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Biological N₂ fixation and N loss as N₂O and N₂ in a white clover-based system of dairy production

A dissertation submitted to Trinity College Dublin for the degree of

Doctor of Philosophy

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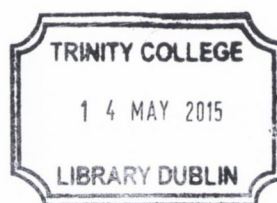
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DECLARATION

I hereby certify that this thesis has not been submitted as an exercise for a degree at this or any other University and is entirely my own work unless otherwise indicated.

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PREFIX

All field measurements (including biological N₂ fixation, nitrous oxide, dinitrogen and N losses to groundwater measurements) were carried out by the author from October 2010 to the end of the data collection phase of this study in March 2013. Data collected before October 2010 was supplied by Teagasc and the relevant studies are referenced where appropriate.

The white clover based dairy production system used in this study was already designed and established prior to the commencement of this study and was managed by Teagasc personnel throughout the duration of the study. This included the management of the pastures and livestock on the system.

Shoot *B*-values for white clover used during the biological N₂ fixation study in this thesis were determined by Dr. Euan James and Dr. Petro Iannetta at the James Hutton Institute, Dundee, Scotland as part of a collaboration between Teagasc and Trinity College Dublin with the James Hutton Institute.

ABSTRACT

Pasture-based livestock production systems have a relatively large requirement for nitrogen (N) input compared to other food production systems. These inputs may consist of organic N, mineral N or biologically-fixed N. Of all N that enters these systems, only a small proportion (15-40%) is converted to saleable agricultural products. The remaining N (surplus N) is largely unaccounted for and can be either lost from the system through a range of pathways including gaseous N losses to the atmosphere as nitrous oxide (N₂O), ammonia (NH₃), and dinitrogen (N₂), N losses to groundwater or N may be immobilised in soil organic matter, all of which have differing environmental consequences. Thus the quantification of N losses is critical for assessing the environmental impacts of ecosystem N dynamics and accordingly for improving our ability of N management. This study carried out detailed N flow measurements on a dairy production system in order to account for all N entering into, and leaving from, the system and to determine the fate of farm surplus N which has remained an area of high uncertainty for the research community. This study also allowed for a much more holistic view of N movements on a farm system compared to most previous studies which have primarily focused on either individual N inputs or losses. Encompassed in this holistic study was a comprehensive comparison of three techniques used to measure biological N₂ fixation under varying fertiliser N inputs over a sustained period of two years and a long term study of N₂O from white clover based pastures to access inter-annual variation in emissions, both of which are lacking in the literature.

The experiments were carried out on a perennial ryegrass (*Lolium perenne* L.)/white clover (*Trifolium repens* L.; 18.2%; grass/clover) based system of dairy production at Solohead Research Farm, Tipperary, Ireland (52° 51' N, 08° 21' W) under moist maritime climatic conditions. Biological N₂ fixation (BNF) associated with white clover was measured using three techniques (¹⁵N natural abundance, ¹⁵N isotope dilution and nitrogen difference) under four fertiliser N inputs (0, 86, 140 and 280 kg N ha⁻¹ yr⁻¹). Nitrous oxide emissions were measured over a four-year period (October 2008 to November 2012) using a static chamber technique. These N flow measurements were combined with measurements of N₂ gas, N losses to water, N exported in animal products, N deposition in rainfall and estimates of NH₃ volatilisation to complete a farm N balance for the dairy production system over two production years: 2011 and 2012.

Biological N₂ fixation in above-ground herbage ranged from 14 to 128 kg N ha⁻¹ yr⁻¹, as measured by both ¹⁵N techniques, across the range of fertiliser N inputs and over the two years of the study. Fertiliser N input reduced BNF and there was also significant inter annual variation in BNF due to differences in clover herbage yield between years. Both ¹⁵N based techniques produced similar estimates of BNF while the nitrogen difference technique gave different results. Nitrous oxide emissions displayed large intra and inter-annual variation with annual estimates ranging from 4.4 to 34.4 kg N ha⁻¹. Weather driven variables including monthly soil temperature (December) and annual rainfall accounted for a large proportion of the variation in annual N₂O emissions. Nitrogen inputs accounted for N outputs (N exported in products and N losses) within experimental error in the farm N balance for the system. Total N losses of 180 kg N ha⁻¹, average for both years, were in the following proportions: 6.5% as losses to groundwater, 38.5% as NH₃, 8.5% as N₂O and 46% as N₂ gas. The heavy textured soils with high soil organic carbon and impeded drainage, and moist maritime climate at this site created conditions conducive to denitrification which may explain the relatively high rates of N losses as N₂ gas and at the same time the relatively low N losses to groundwater.

Although N surpluses on pasture based livestock production systems can be substantial, the results of this study suggest that a high proportion of surplus N may be lost as environmentally benign dinitrogen gas when the system is on heavy textured soils with impeded drainage and under moist maritime climatic conditions. Such soil types are representative of around 23% of Irish soils. This study also highlights where the emphasis should be (NH₃ and N₂O) in terms of minimising environmentally damaging N losses and improving the N use efficiency of such systems of dairy production.

EXECUTIVE SUMMARY OF MAIN FINDINGS

- Annual above ground biological N_2 fixation (BNF) ranged between 14 to 128 kg N ha^{-1} in perennial ryegrass/white clover pastures in Ireland
- The ^{15}N isotope dilution and ^{15}N natural abundance techniques provided similar annual estimates of BNF across a range of fertiliser N inputs on non-grazed perennial ryegrass/white clover pastures over a two year period
- The nitrogen difference technique produced variable estimates of BNF
- Fertiliser N input increased herbage yields but reduced BNF and increased potential N loss to the environment on perennial ryegrass/white clover pastures. The results of this study, suggest that intermediate fertiliser N (86 and 140 kg N ha^{-1} yr^{-1}) inputs achieve a balance between minimising detrimental impact on BNF and potential N loss while producing more herbage DM yield than grass/clover pastures receiving no fertiliser N
- Substantial inter annual variation in annual nitrous oxide (N_2O) emissions (4.4 to 34.4 kg N_2O-N ha^{-1}) was record from perennial ryegrass/white clover based grassland over four years
- Weather and soil conditions contributed to the high level of inter-annual variation in N_2O emissions. Monthly soil temperature (December) and annual rainfall were the dominant factors accounting for abiotic variation in annual N_2O emissions on perennial ryegrass/white clover pastures.
- The complete farm N balance for the dairy production system ranged from -40 to 10 kg N ha^{-1} yr^{-1} over two years and N outputs accounted for N inputs within experimental error in this study. This study, therefore, accounted of all farm N surplus and effectively closed the N balance on a dairy production system at Solohead Research farm, which has been a research question for over a decade.
- Estimated ammonia volatilization was the largest environmentally damaging N loss from the dairy production system in this study

- Nitrogen losses to water were relatively low from the dairy production system in this study. Nitrate-N concentrations (mg l^{-1}) (0.8 to 5.5: mean 2.7) in groundwater were well below the maximum allowable concentration of $11.3 \text{ mg NO}_3\text{-N l}^{-1}$ under the European Nitrates Directive over the two year duration of this study
- Environmentally benign dinitrogen gas was the dominant product of denitrification and accounted for up to 46% of farm surplus N, on average over two years, on the dairy production system in this study
- This study illustrated that, once a pasture based system is based on heavy soil in high rainfall areas, damaging N losses to water can be minimal and a large proportion of surplus N (previously perceived to be almost entirely damaging to the environment) can be lost by environmentally benign N_2 gas

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LIST OF ABBREVIATIONS

%Nd_{fa} = percentage of plant N derived from the atmosphere

°C = degrees Celsius

‰ = parts per thousand

¹⁴N = N isotope with 14 neutrons in their atom

¹⁵N = N isotope with 15 neutrons in their atom

ACP = agricultural catchments programme

ANOVA = analysis of variance

asl = above sea level

Atom% ¹⁵N = abundance of ¹⁵N atoms as a percentage of the total (¹⁵N / (¹⁴N + ¹⁵N)) × 100

Atom% ¹⁵N excess = a measure of a materials ¹⁵N content above that of atmospheric N₂

ATP = adenosine triphosphate

BFN = biological fixed N₂

BNF = biological N₂ fixation

B_s-value = δ¹⁵N of legume shoots fully dependant on N₂ fixation for their plant N

C = carbon

C₂H₂ method = acetylene inhibition method used to measure denitrification

CAN = calcium ammonium nitrate

Clover = white clover

CLRTAP = convention on long range trans-boundary air pollution

cm = centimetre

CO₂ = Carbon dioxide

d = day

DM =dry matter

DON = dissolved organic N

DNRA = dissimilatory nitrate reduction to ammonium

EC = European community

EEC = Economic European community

EF = emission factor

EPA = environmental protection agency

EU = European Union

g = grams

GAP = good agricultural practice for the protection of waters

GC = gas chromatograph

G_{EF} = global emission factor

GHG = greenhouse gas

H₂ = hydrogen

ha = hectare

HNO₃⁻ = nitric acid

hr = hour

ID technique = isotope dilution technique used to measure biological N fixation

IPCC = inter-governmental panel on climate change

KCL = potassium chloride

kg = kilogram

kt = kilo tonnes

m = metre

mg = milligrams

ml = millilitre

mm = millimetre

MSON = mineralisation of soil organic nitrogen

Mt CO₂ eq = mega tonnes CO₂ equivalents

N = nitrogen

N₂ = dinitrogen gas

N₂O = nitrous oxide

NAP = nitrates action programme

NA technique = natural abundance technique used to measure biological N fixation

ND = nitrates directive

NDiff = nitrogen difference technique used to measure biological N fixation

NECD = national emissions ceiling directive

NH₂OH = hydroxylamine

NH₃ = ammonia

NH₄⁺ = ammonium

NO₂⁻ = nitrite

NON-ETS = non-emissions trading sector

NO_x = nitrogen oxides

N_r = reactive nitrogen

NS = not significant

NUE = nitrogen use efficiency

P = phosphorus

Perennial ryegrass/white clover = grass/clover

pH = parts hydrogen

ppm = parts per million

PVC = polyvinylchloride

s.d. = standard deviation

s.e. = standard error

S.E.M. = standard error of the mean

S.I. = statutory implement

SAS = statistical analysis software

SNB = soil nitrogen balance

SOMN = soil organic matter nitrogen

t = tonne

TAN = total ammonical nitrogen

Tg = teragram

TON = total oxidised nitrogen

UK = United Kingdom

UN = United Nations

UNFCCC = United Nations Framework Convention on Climate Change

USA = United States of America

VWC = volumetric soil water content

WFD = water framework directive

WFPS = water filled pore space

yr = year

δ = delta units

μm = micrometre

CHAPTER 1. GENERAL INTRODUCTION

1.1. Overview

Agriculture has been identified as a major source of N loss to water and emissions of ammonia (NH_3) and nitrous oxide (N_2O) to the atmosphere, all of which are detrimental to the environment (Oenema *et al.*, 2011). The global demand for meat and dairy products is predicted to increase by over 30%, driven by increased affluence in the developing world (Alexandratos and Bruinsma, 2012). This is likely to result in an increase in ruminant animal numbers and further acceleration of the N cycle. Therefore, such N losses from agriculture are expected to increase in coming decades (Di and Cameron, 2002; Smith *et al.*, 2007). These losses are also expected to contribute to global climate change, which will put further pressure and stresses on food production (Wheeler and von Braun, 2013).

In Ireland, the abolition of E.U. milk quotas in 2015, coupled with the envisaged increase in dairy production under Food Harvest 2020 targets (DAFF, 2011) is anticipated to increase the scale and intensity of dairy production systems with associated projected increases in gaseous and aqueous N losses. A major challenge facing agriculture in Ireland will be to exploit the opportunity for growth while meeting national and international regulatory targets and ceilings related to N losses. These include: the nitrates directive (ND) (91/676/EEC) and the EU water frame directive (WFD) (OJEC, 2000) concerned with N losses to groundwater, the EU Climate and Energy Package related to greenhouse gases (GHG) including N_2O and the National Emissions Ceiling Directive (NECD) (2001/81/EC) related to NH_3 emissions. Major focus will have to be placed on pasture-based production systems, which dominate Irish agriculture and are known to have relatively high N losses (Schröder *et al.*, 2003; Treacy *et al.*, 2008; Buckley *et al.*, 2013; Mihailescu *et al.*, 2013).

Nitrogen use efficiency (proportion of N imported into farms leaving in agricultural products: NUE) on Irish pasture-based livestock production systems is relatively low ranging between 18% to 42% (Treacy *et al.*, 2008; Buckley *et al.*, 2013; Mihailescu *et al.*, 2013). Low NUE ultimately leads to a surplus of N on farms. Typical farm N surpluses in Ireland range from 69 to 239 kg N ha⁻¹ (Mihailescu *et al.*, 2013). This surplus N is the main substrate for N losses such as N_2O , NH_3 gas and N losses to groundwater, all of which have environmental consequences. This has raised concerns over the environmental impact of pasture-based livestock production systems. On the other hand, other possible N losses/sinks include emissions of dinitrogen (N_2) gas and immobilisation of N in soil

organic matter which are environmentally benign. Despite much research, large uncertainty exists around the relative contribution of individual N losses/sinks in accounting for surplus N on pasture based livestock production systems.

Biological N₂ fixation (BNF) associated with legumes such as white clover has been found to be a substantial N input for pastures-based systems supplying between 55 to 296 kg N ha⁻¹ year⁻¹ on grazed pastures based on white clover (clover) (Ledgard and Steele, 1992). Little information, however, is available on the quantity of N fixed on perennial ryegrass/white clover (grass/clover) based grasslands in Ireland. Many different techniques have been devised to measure BNF. The technique used to quantify BNF may itself affect the estimates of BNF you attain in any individual experiment. Biological N₂ fixation on pasture based production systems is essentially regulated by soil N concentration, N fertiliser use, grazing management, clover variety, animal grazing, temperature and soil moisture. Direct measurement of BNF and investigation into factors regulating BNF is needed if the maximum benefit from BNF is to be achieved for Irish pasture-based production systems.

Nitrous oxide has been found to be a major pathway of N loss from pasture-based production systems. Indeed, grasslands have been identified as a major source of N₂O globally (de Klein *et al.*, 2008). This is important in an Irish context as permanent grassland represents approximate 90% of Ireland's utilisable agricultural area. Nitrous oxide emissions from temperate grassland vary widely from -0.5 to 51.3 kg N₂O-N ha⁻¹ yr⁻¹ and are essentially regulated by management practices, soil type and climate. Estimates of N₂O emissions from Irish grasslands are lacking. Alterations in regulatory drivers of N₂O over time create large temporal variation in emissions and many studies have found emissions to vary inter-annually (Velthof *et al.*, 1996; Hyde *et al.*, 2006; Flechard *et al.*, 2007; Rafique *et al.*, 2011; Rees *et al.*, 2013). However, there is a lack of long term (>2 years) N₂O studies internationally, that have investigated the factors that drive inter-annual variation in emissions.

1.1.1. Thesis structure

This thesis is organised into five chapters: This chapter, introduces the reader to the background literature associated with this thesis including an overview of the N cycle and a more detailed review of the important aspects of this thesis including BNF, N losses as

N₂ and N₂O, and farm N balances. The legislation governing N losses in Irish agriculture is also outlined. This section also outlines the gaps of knowledge addressed in this thesis, the objectives and hypotheses. Chapter 2, following this general introduction, investigates BNF using three techniques on grass/clover pastures receiving four different N fertiliser inputs, Chapter 3 investigates inter-annual variation in N₂O emissions on grazed and N fertilised grass/clover pastures over a four year period, in Chapter 4 the results from Chapter 2 and 3 are combined with other N flow measurements to complete a detailed farm N balance for a grass/clover based system of dairy production and finally Chapter 5 discusses the major findings, implications and conclusions of this study and includes future research recommendations. The publications and various disseminations arising from the work carried out in this study are outlined in the Appendix.

1.1.2. Legume Futures

This study was carried out as part of Teagasc and Trinity College Dublin's contribution to the EU Framework Programme 7 project 'Legume Futures' (www.legumefutures.de). This four year project has twenty partners in thirteen different European countries. The full title of the project is 'Legume-supported cropping systems for Europe'. The overall aim of the project is to 'develop the use of legumes in cropping systems to improve the economic and environmental performance of European agriculture'. The results of this study primarily contributed to Work Package 3, which is concerned with the environmental impact of legume supported production systems.

1.2. Nitrogen cycle

1.2.1. Overview of reactive N and the N cycle

Nitrogen is an essential nutrient for the growth and functioning of plants, animals and humans. It is an essential part of all proteins and enzymes and thus, N drives the key metabolic processes involved in growth and energy transfer (Erisman *et al.*, 2013). The largest sink of N, globally, is atmospheric N₂ gas which accounts for almost 80% of our atmosphere. Dinitrogen is a stable form of N in which two atoms of N are held by a strong triple bond and is of no biological use in this form. A process termed 'N fixation', however, can break this triple bond and reduce N₂ to reactive N (N_r) which is 'biologically available'. Following fixation, N_r can undergo a number of microbial, chemical and

physical processes (immobilisation, mineralisation, ammonification, nitrification, plant assimilation) before being returned to atmospheric N_2 by denitrification to complete the N cycle (Figure 1.1). During these processes, N_r exists as N compounds including both inorganic (ammonia (NH_3), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), nitric acid (HNO_3), nitrous acid (NO), nitrogen oxide (NO_x) and nitrous oxide (N_2O)) and organic forms (urea, amines, nucleic acids and proteins). The N cycle is known to be inherently leaky from the initial fixation step to the final return of N_r to atmospheric N_2 . Reactive N can be volatilised as NH_3 gas, emitted as NO_x and N_2O gas to the atmosphere and leached to groundwater. This has negative implications for drinking water, air quality, freshwater eutrophication, biodiversity loss, stratospheric ozone depletion and climate change (Erisman *et al.*, 2013; Fowler *et al.*, 2013). Human modification of the N cycle has seen a doubling of the global N cycling in the last century (Erisman *et al.*, 2008; Fowler *et al.*, 2013). The increase in N_r in the global N cycle has its benefits in terms of food production, nonetheless, it also has potential disadvantages for the environment and human health.

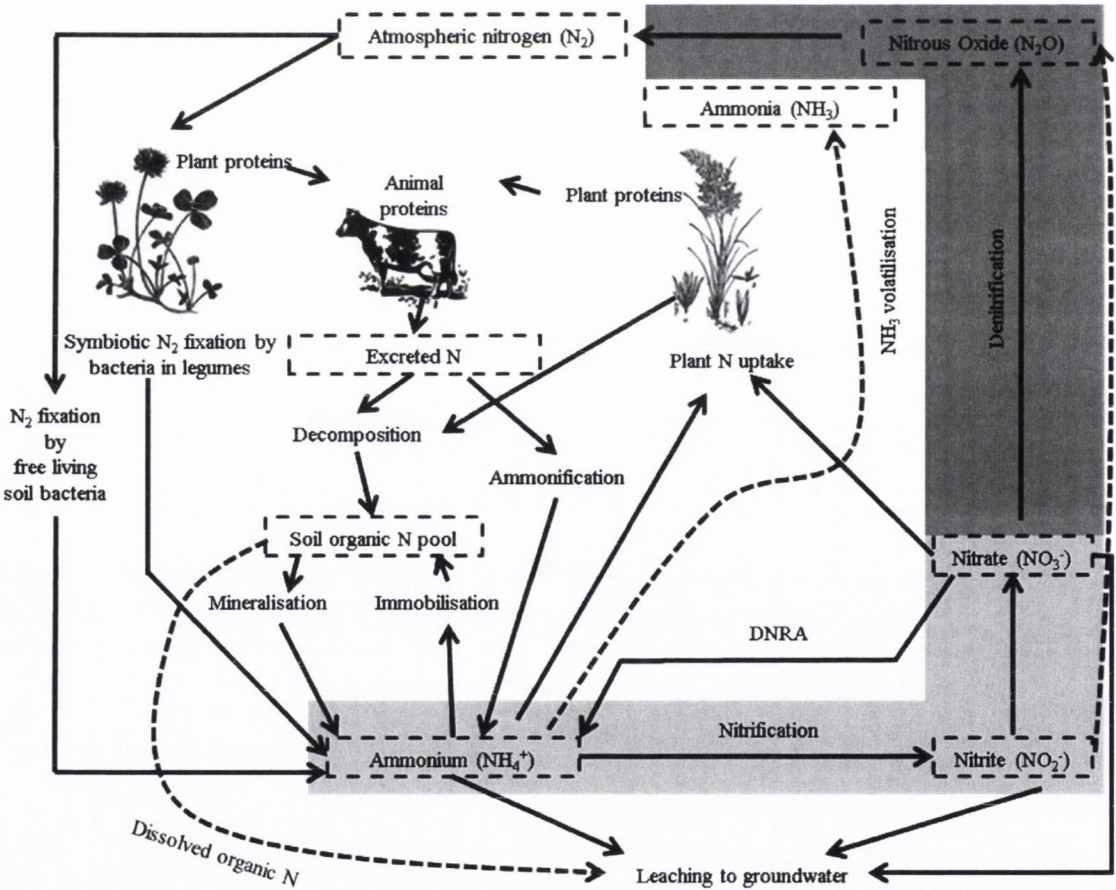


Figure 1.1. Schematic of the soil nitrogen cycle. DNRA: dissimilatory nitrate reduction to ammonium. Source: (Jarvis, 1993; Bussink and Oenema, 1998; Stevens and Laughlin, 1998; McNeill and Unkovich, 2007; Jackson *et al.*, 2008; Unkovich *et al.*, 2008; Baggs, 2011; Jarvis *et al.*, 2011)

1.2.2. Overview of farm N cycle

The farm N cycle is dynamic with multiple inputs, outputs, losses and internal movements and transformations of N (Figure 1.2). (1) Typical livestock farms import N in the form of animal feed and livestock purchases. (2) Nitrogen can be added to both crop and livestock farms through N inputs from synthetic fertiliser, manures, seed, atmospheric deposition of N, bedding material (straw) and N_2 fixation by legumes. The primary output of N is through the sale of (3) animal products such as milk and (4) livestock or (5) as arable produce such as cereals and straw. (6) Nitrogen can also be removed from the farm in the form of exported manures. Environmentally damaging losses of N are a reality on the majority of farming systems. These can include losses to, (7) groundwater in the form of NO_3^- , NH_4^+ and dissolved organic N (DON) and (8) to the atmosphere as N_2O , NH_3 gas and NO_x . (9) Dinitrogen gas is also a major loss of N from farms although it is environmentally benign (Jarvis *et al.*, 2011).

Nitrogen is also cycled internally within farms. (10) Nitrogen can be up taken by crops or (11, 12) by forages which are directly consumed by livestock. This consumed N is often far in excess of the requirement for the production of milk and meat protein (Tamminga, 1992), and (13) is returned to fields either directly as dung and urine by grazing livestock or (14) indirectly through the application of animal manure to fields after collection and (15) storage in livestock housing and manure storage facilities.

(16) Another important part of N cycling on farms is the movement and transformation of N within soil. Added N can be sequestered into relatively immobile forms of organic N in soil. Likewise, N can be released from this organic soil N pool through mineralisation to forms of N that can be subject to plant uptake or other forms of N transformation and losses (Jarvis *et al.*, 2011).

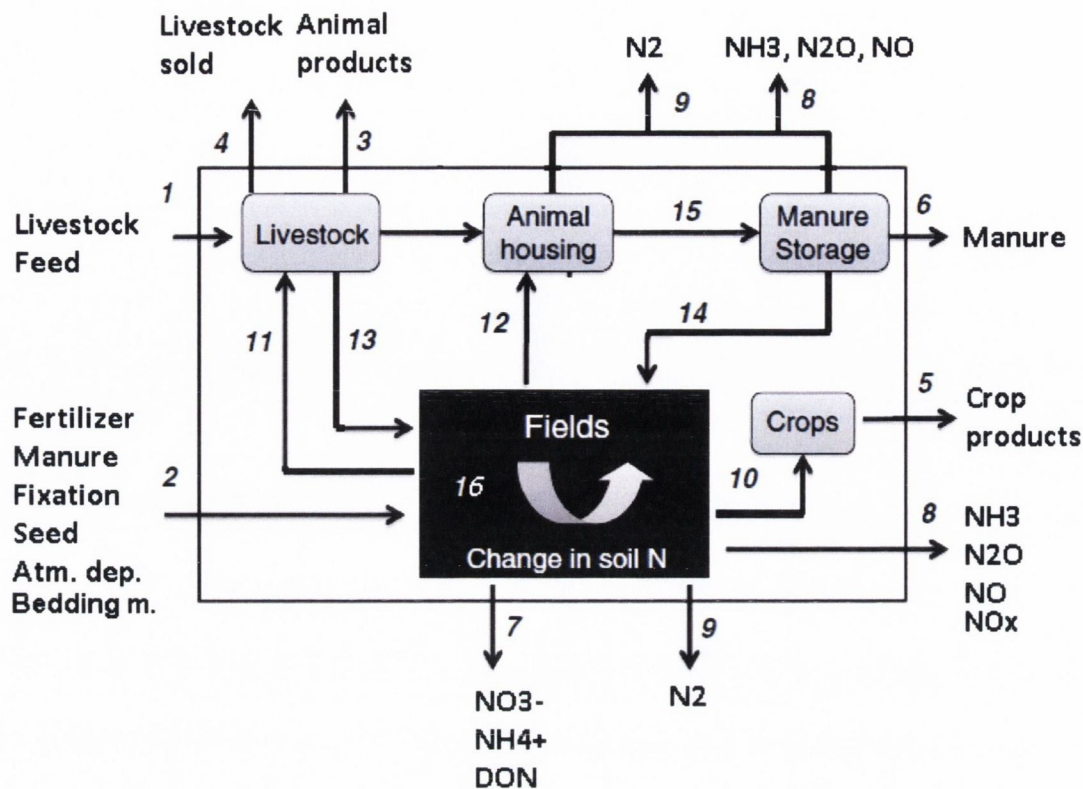


Figure 1.2. Nitrogen flows and transformations on farms. The numbers refer to the flows or transformation processes described in the text. Adapted from Jarvis *et al.*, (2011)

1.2.3. Nitrogen inputs and transformations

1.2.3.1. Mineralization and immobilization

Soil organic matter N (SOMN) is the largest pool of N in terrestrial ecosystems with between 5 to 15 t N ha⁻¹ present in the soil layers exploited by plant roots (Jarvis *et al.*, 2011). In the upper 15 cm of soils in the United Kingdom (UK), there can be between 3 and 10 t of organic N ha⁻¹ under grassland (Humphreys *et al.*, 2003). This N is locked up in organic compounds which are not available for biological processes. Nitrogen mineralisation, however can convert organic N to inorganic N (Myrold and Bottomley, 2008). This process in soil is balanced by the conversion of inorganic soil N to organic N through the process of N immobilization (Myrold and Bottomley, 2008). One to 2% of SOMN may in any given year be converted to inorganic N (NH₄⁺) (Jarvis *et al.*, 2011). This mineralised N is an important source of N for plant uptake. The extent of this soil N supply can be measured as that taken up by the plant in the absence of other N inputs such as fertiliser and manure-N, legume fixation and atmospheric deposition. Reported ranges of SOMN mineralisation, measured in this manner, have been reported to range from 60 to 250 kg N ha⁻¹ yr⁻¹ in Irish grasslands and between 43 to 233 N ha⁻¹ yr⁻¹ in grasslands in the

UK and the Netherlands (Humphreys *et al.*, 2003). However, this measurement is not a true reflection of total SOMN mineralisation as not all mineralised N is taken up by plants and may be lost through various N loss pathways.

The decomposer and liable organic soil N pools dominate short term N cycling. These N pools are more responsive to organic N inputs in animal manures and plant residues and to changes in soil temperature, moisture and structure and hydrology due to soil disturbance such as tillage or drainage (Jarvis *et al.*, 2011). Mineralization of SOMN is regulated by soil moisture, soil temperature, C/N ratio of the organic matter, soil pH and soil texture (Konrad, 2008). Under certain management or weather conditions net N mineralization may take place which leads to a large release of inorganic N. For example, in arable cropping soil disturbance from tillage promotes net mineralisation (Jarvis *et al.*, 2011).

1.2.3.2. *Nitrification and denitrification*

Nitrification is the microbial oxidation of NH_3 to NO_3^- . Nitrate is the substrate for denitrification which is responsible for the stepwise reduction of soil NO_3^- to gaseous N_2 . Both processes are dealt with in further detail in section 1.4.4.

1.2.3.3. *Plant N assimilation (uptake)*

Plants predominantly take up soil inorganic N, preferably NH_4^+ as opposed to NO_3^- , to meet their N requirements for growth. Plants uptake N via transport systems in the plasma membrane of root cells, which is regulated by the N requirement of the plants as well as root system architecture and mechanisms that regulate the activity of N transport systems within the plant. Nitrogen uptake by individual plants depends on species and respective growth potentials. External factors also regulate plant N uptake such as soil NH_4^+ , NO_3^- , organic compounds, soil pH, light and temperature (Jackson *et al.*, 2008). The N uptake of any plant over a given period is generally a function of N supply and demand. Grass, for example, has a relatively large demand for N compared to arable crops. This is partly due to the C3 metabolism characteristic of grass species and partly because of the relatively high-N ($>30 \text{ g N kg}^{-1}$ dry matter (DM)) photosynthetic foliage rather than low-N (14 to 20 g N kg^{-1} DM) storage organs or seeds as is the case for most arable crops (Humphreys *et al.*, 2003). Pastures producing close to maximum yields, in Ireland, of between 14 and 16 t DM ha^{-1} annually needs to take up around 450 $\text{kg N ha}^{-1} \text{ yr}^{-1}$ (Humphreys *et al.*, 2003). Plant N uptake requirements can vary temporally over time and tends to be highest during

vegetative growth up until flowering (Jarvis *et al.*, 2011). Concurrently, the timing of N fertiliser applications should be in line with plant demand, to optimise N fertiliser use efficiency. The rate of N input should also be adjusted to match plant requirements. Nitrogen that is surplus to immediate plant demand can be lost by various N loss pathways. Figure 1.3 depicts the typically daily N requirements of grasslands based on five years of grass growth data from Moorepark, Co. Cork, Ireland. Herbage yield ranged from 14.5 to 16.3 t DM ha⁻¹ yr⁻¹ (mean 15.5t DM ha⁻¹ yr⁻¹) over the period (Humphreys *et al.*, 2003).

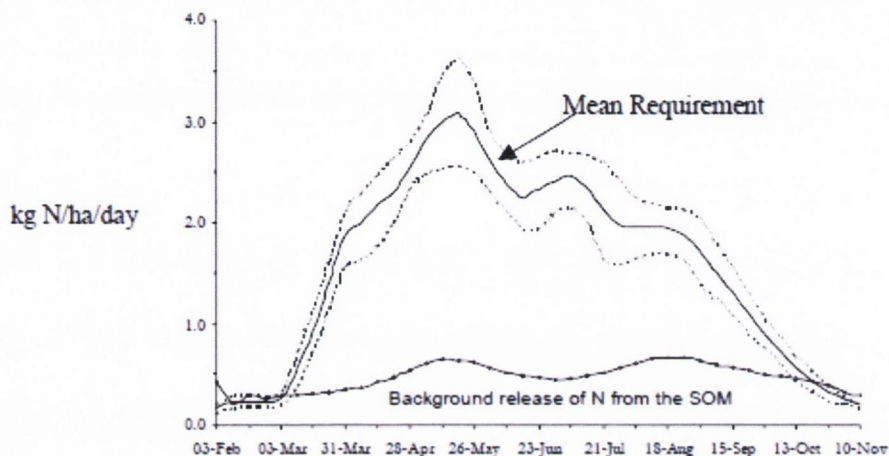


Figure 1.3. Generalised requirement for N fertiliser (kg N ha d⁻¹) by grassland during the growing season assuming a constant 30 g N kg dry matter compared to background release of N from SOM. Mean requirement is the mean of five years of data. Year to year variation in weekly N requirement is indicated using the $\pm$ standard deviation (s.d.) of the mean requirement (dashed lines). Source: (Humphreys *et al.*, 2003).

1.2.3.4. Nitrogen cycling in excreta

Livestock play a major part in nitrogen cycling in pasture-based production systems through their excretion of ingested N in urine and dung (Haynes and Williams, 1993). Up to 75 to 95% of the N ingested from herbage is excreted and either directly or indirectly return to pasture (Whitehead, 1995; Nielsen *et al.*, 2003; Spek *et al.*, 2013). Urine-N generally accounts for up to 75% of excreted N (Lantinga *et al.*, 1987). While grazing, this N is deposited unevenly in localised areas and in amounts equivalents of 1000 kg N ha⁻¹ in a single cow urination patch (area of 0.16 to 0.49 m²) and 130 and 2000 kg N ha⁻¹ for sheep (area 0.008 to 0.05 m²) and cattle dung patches (area 0.05 to 0.09), respectively (Lantinga *et al.*, 1987; Haynes and Williams, 1993). These N loads are often in excess of immediate plant demand (see Figure 1.3) and therefore only 1 to 45% of this N is up taken by pasture with the remainder removed through various N loss pathways (Wachendorf *et al.*, 2008; Selbie *et al.*, 2012). Nitrogen excreted while indoors is stored and applied to land

in a more uniform fashion as slurry or farmyard manure, however, it is also subject to N losses during storage and land application (Stevens and Laughlin, 2001a; Misselbrook *et al.*, 2005; Lampe *et al.*, 2006; Jones *et al.*, 2007; Gilhespy *et al.*, 2009; Sanz *et al.*, 2010; Schröder *et al.*, 2010; Pereira *et al.*, 2011; Bourdin *et al.*, 2014).

1.2.3.5. *Nitrogen fixation and nitrogen fertiliser*

Dinitrogen can be fixed chemically using the Haber-Bosch process by reacting atmospheric N₂ with hydrogen in the presence of iron at high pressure and temperature. Dinitrogen fixation is also carried out by free-living bacteria and by bacteria in symbiosis with leguminous plants (see next section). However, the fixation of N₂ through Haber-Bosch (120 Tg N yr⁻¹) in 2010 was double that of natural terrestrial sources of N_r (63 Tg N yr⁻¹). It has been estimated that almost half of the human population at the beginning of the twenty first century depends on industrially produced N fertiliser for their food (Erisman *et al.*, 2013; Fowler *et al.*, 2013).

As already outlined, grassland pastures have a large demand for N to reach maximum yields (section 1.2.3.3). The N requirement of pastures can be met by the mineralisation of SOMN, N recycled in excreta and by biological fixed N, when leguminous plants are present in pasture. Additional inorganic N fertiliser can also be applied to increase N supply and thus pasture production (Humphreys *et al.*, 2003). Nitrogen fertiliser has been reported to account for between 80 and 86%, on average, of the N imported onto Irish farms (Treacy *et al.*, 2008; Buckley *et al.*, 2013; Mihailescu *et al.*, 2013). Inorganic N fertiliser use was reported to be (mean ± s.d.) 155 ± 61 kg N ha⁻¹ yr⁻¹, on average over 2009 and 2010, on 195 Irish dairy farms (Buckley *et al.*, 2013). This mean figure is somewhat lower than the mean figure of 239 kg N ha⁻¹ yr⁻¹ reported by Treacy *et al.* (2008) for 21 Irish dairy farms in the period 2003 to 2006. This decrease in N fertiliser use coincides with the introduction of the EU Nitrates Directive regulations in 2006 (section 1.6.1), which set limits on input of N in manures excreted by ruminant livestock of 250 kg ha⁻¹ yr⁻¹ and on input of total N (livestock manures plus manufactured fertiliser) of 490 kg ha⁻¹ yr⁻¹ on Irish farms (Humphreys, 2008).

The next section reviews symbiotic biological N₂ fixation, which is an important N input to legume based agricultural systems.

1.3. Biological N₂ fixation

Dinitrogen gas represents almost 80% of the earth's atmosphere but is of no biological use to plants in this form. Biological N₂ fixation is the process whereby a number of species of bacteria (primarily Rhizobia) use the enzyme nitrogenase to convert atmospheric N₂ gas to NH₃. Biological N₂ fixation has been calculated to contribute up to 50 million tonnes of N to agricultural ecosystems annually (Herridge *et al.*, 2008). Many forms of biological N₂ fixation exist. Heterotrophic free-living N₂ fixers have been found to contribute small amounts of N to agricultural systems (mostly <5 kg N ha⁻¹ yr⁻¹) (Unkovich *et al.*, 2008). Cyanobacteria form symbioses with plants from the Gunnera and Azolla plant genus, and with Cyads seed plants and lichens, all of which can fix N₂. Significant amounts of N₂ can be fixed by bacteria associated with non-legumes such as sugar cane, in the order of 10 to 65 kg N ha⁻¹ yr⁻¹ (Boddey *et al.*, 1995; Urquiaga *et al.*, 2012). Actinorhizal plants (mainly trees e.g. Alder: *Alnus*) form bacteria symbioses which have been found to fix between 0.2 and 40 kg N ha⁻¹ yr⁻¹ (Andrews *et al.*, 2011). The most important form of biological N₂ fixation, however, is the symbiosis between legumes and Rhizobia. These symbiotic relationships have been reported to contribute in the range of 1 to 680 kg N ha⁻¹ yr⁻¹ to agricultural ecosystems (Unkovich *et al.*, 2008). More commonly observed ranges of 50 to 250 kg N ha⁻¹ yr⁻¹ have been associated with pasture and forage legumes (Unkovich *et al.*, 2008) and 55 to 296 kg N ha⁻¹ yr⁻¹ for grazed pastures based on white clover (*Trifolium repens* L.) (Ledgard and Steele, 1992), which will be the focus of this review. Pastoral systems in many temperate regions of the world use clover, because of its benefits to animal feed quality, in terms of higher crude protein and DM digestibility relative to other pastoral grasses such as perennial ryegrass (Dewhurst *et al.*, 2009), persistence under grazing (Frame and Newbould, 1986) and inputs of N *via* fixation of atmospheric N₂ (Ledgard and Steele, 1992) and subsequent economic benefit (Humphreys *et al.*, 2012).

1.3.1. Nodulation and mechanism of biological N₂ fixation

Biological N₂ fixation is dependent on the establishment of a symbiotic relationship between legume plants and an effective *Rhizobium* strain. White clover is generally nodulated by a saprophytic soil bacterium called *Rhizobium leguminosarum* biovar *trifolii*, which exists naturally in Irish and UK soils (Sprent, 2009; Oldroyd *et al.*, 2011). This relationship begins with the nodulation of the legume plant. There are a number of steps in the nodulation process. Following an initial 'chemical cross-talk' (for further information

see the extensive review of Oldroyd *et al.* (2011)) rhizobia bacteria colonise growing root hairs of the plant and subsequently invade the (usually) curled root hairs of the host plant via a plant cell wall-enclosed tube called an infection thread (Figure 1.4) (Ashihara *et al.*, 2011; Sprent *et al.*, 2013). The infection thread, conveying the rhizobia, grows into the cortex of the root and branches repeatedly (Sprent *et al.*, 2013).

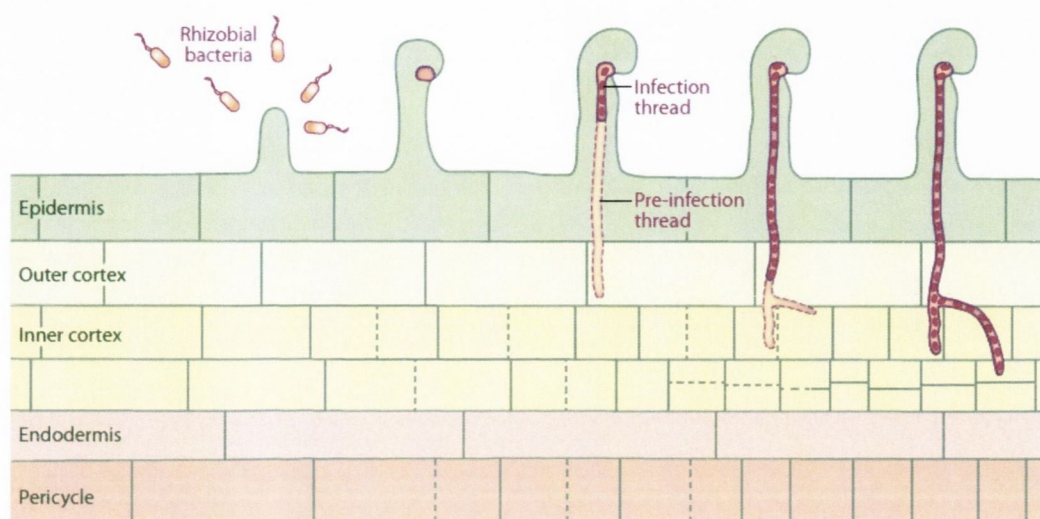


Figure 1.4. Early stages of nodulation and Rhizobia bacterial infection of a legume root hair showing; the curled root hairs, development of the infection thread and initiation of cell division (indicated by dotted lines) to form nodules. Source: (Oldroyd *et al.*, 2011)

Concurrently, cell division is induced in the root cortex (Figure 1.4) to form root nodules (Figure 1.5 a, b, c). The rhizobia enter developing root nodules via their release from the tips of infection threads. Within the cells of the nodules the bacteria differentiate into their symbiotic form, the bacteroid, which is essentially an organelle devoted to fixing N_2 (Figure 1.5 d) (Ashihara *et al.*, 2011; Sprent *et al.*, 2013). Free-living rhizobia are not usually capable of N_2 fixation and it is only in this symbiotic form that they can fix N_2 . Rhizobia can also infect legumes where (usually) no infection threads are formed. These include infection where rhizobia enter at the 'crack' where lateral or adventitious roots emerge or by epidermal infection where bacteria enter between epidermal cells (Sprent *et al.*, 2013).

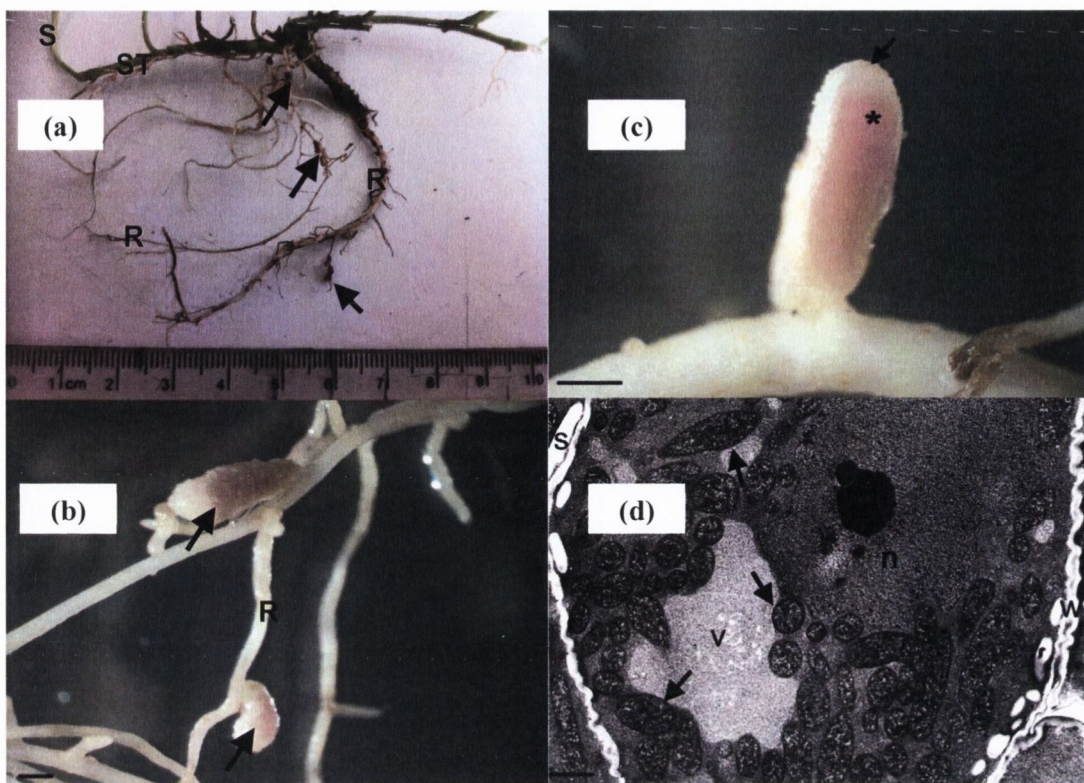


Figure 1.5. White clover plant showing stem (S), stolon (ST) and roots (R) with nodules (arrows) (a); close up of white clover roots (R) with nodules (arrows) (b); sliced open nodule on white clover root revealing the presence of the red pigmented protein leghaemoglobin (*) and the growing tip of the nodule (meristem : arrow) (c) transmission electron micrograph of a white clover nodule cell showing the N_2 -fixing Rhizobium bacteroids (arrows), nucleus (n), cell wall (W), vacuole (V) and starch (S). Scale bars (black line) located on the bottom left corner of (b), (c) and (d) are 2mm, 2mm and 2 μ m long, respectively. Images for this figure were produced from white clover during the authors experimental work in conjunction with experimental work carried out at the James Hutton Institute, Dundee.

The chemical reaction in the N_2 fixation process in these bacteroids can be illustrated as:



This reaction is catalysed by an enzyme known as nitrogenase which is expressed by the bacteroids. Fixed N is initially in the form of NH_4^+ which is then converted by the plant to less toxic amides for transport to the shoots. In return for this fixed N the plant supplies the bacteroids with carbohydrates from photosynthesis, water and an environment that allows the bacteroids to regulate their nitrogenase expression and activity including the oxygen carrying protein leghaemoglobin, which is essential for active N_2 fixation (Figure 1.5 c) (Andrews *et al.*, 2009; Oldroyd *et al.*, 2011).

Dinitrogen fixation is an energy demanding process which is represented by the 16 molecules of adenosine triphosphate (ATP). For example, Ryle *et al.* (1989) found that nitrogenase-linked respiration accounted for 13.4 to 16% of the clover's net shoot photosynthesis over a 24 h period. The overall, energy and water costs per unit N assimilated are, respectively, likely to be around 5 and 7% greater for N₂ fixing plants than for non-N₂ fixing plants relying on assimilation of soil NH₄⁺ and NO₃⁻ via the roots (Andrews *et al.*, 2009). Due to the higher energy demand of N₂ fixation clover plants will preferentially assimilate NO₃⁻ and or NH₄⁺ through the roots when available in soil. However, clover does not rely solely on N uptake from the soil and tends to retain at least 15% of its N from N₂ fixation even at optimal soil N levels (Davidson and Robson, 1986). White clover, has often been found to acquire 70 to 99% of its annual N supply from N₂ fixation (Ledgard *et al.*, 2001; Carlsson and Huss-Danell, 2003; Rasmussen *et al.*, 2012; Oberson *et al.*, 2013). This dependence on N₂ fixation may be enhanced due to more efficient uptake of soil N by the companion grasses with which clover is usually grown (Hogh-Jensen and Schjoerring, 1994, 1997; Carlsson and Huss-Danell, 2003).

1.3.2. Factors affecting biological N₂ fixation

The factors which affect clover growth/production and the proportion of plant N derived from the atmosphere (%Ndfa) regulates N₂ fixation associated with clover. Both management and climatic factors can have an effect (Ledgard and Steele, 1992; Hogh-Jensen and Schjoerring, 1994; Menneer *et al.*, 2003, 2005). The main factors regulating N₂ fixation on managed grass/clover pastures include: soil N content, N fertiliser use, grazing management, clover variety, animal grazing, temperature and soil moisture.

1.3.2.1. Soil N concentration and N fertiliser use

In low N input grass/clover pastures (no N fertiliser applied), temporal variation in clover growth and N₂ fixation may be intrinsic, due to dynamic interactions between grass and clover that are mediated by changes in soil N availability. For example, increased soil N availability following large inputs from N₂ fixation favour greater grass growth compared to clover, which leads to leaf shading of clover by grasses (Frame and Newbould, 1986; Ledgard, 2001). This is due to the grasses superior growth response to N compared to clover. On the other hand, when available soil N declines following the reduction in N₂ fixation due to competition from grasses, the relative growth rate of clover becomes greater than the grass with which it coexists because of its N₂ fixing ability (Schwinning and

Parsons, 1996; Hogh-Jensen and Schjoerring, 2010). This cycling of species dominance may take place over a period of months or years. This feedback mechanism between soil N and N₂ fixation acts as a limit on the level of N inputs to legume/grass pastures. Consequently, it also regulates the potential for N losses to the environment (Ledgard, 2001).

In moderate to intensive N input grass/clover pastures the use of N fertiliser at various levels has been shown to reduce the %Ndfa in clover plants due to the substitution of atmospheric N₂ for soil N and to reductions in nitrogenase activity (Kaiser *et al.*, 1997; Crush and Caradus, 2001; Ledgard *et al.*, 2001). This, however, generally leads to only relatively short-term reductions in N₂ fixation. More substantial long term reductions in N₂ fixation can be caused by the decrease in the density of clover herbage in pasture, which can also be associated with N fertiliser use (Hogh-Jensen and Schjoerring, 1994; Ledgard *et al.*, 1996; Elgersma *et al.*, 2000; Ledgard *et al.*, 2001).

Ledgard *et al.*, (2001) found a reduction in %Ndfa from 77% to 45% on grazed grass/clover pastures receiving annual fertiliser N inputs of 400 kg ha⁻¹ compared to those receiving no N fertiliser. The corresponding decrease in annual N₂ fixation was from 154 to 39-53 kg N ha⁻¹. The reduction in %Ndfa was most likely caused by an increase in soil NH₄⁺ and NO₃⁻ concentrations when N fertiliser was applied. Furthermore, Hansen and Vinter (2001) found negative relationships between both soil NH₄⁺ ($y = -0.07 + 0.97$; s.e. slope = 0.01; $R^2 = 0.07$; $P < 0.001$) and NO₃⁻ concentrations ($y = -0.09 + 0.91$; s.e. slope = 0.003; $R^2 = 0.41$; $P < 0.001$) with %Ndfa.

Reductions in N₂ fixation, pasture clover content, stolon biomass and nodule biomass in grazed dairy pastures receiving 200 or 400 kg N ha⁻¹ yr⁻¹ compared to pastures receiving no N fertiliser have also been reported (Harris and Clark, 1996). Conclusions from this and other studies (Hoglund and Frankow-Lindberg, 1998) suggest that the reduction in clover DM production caused by N fertiliser use, and hence a subsequent reduction in N₂ fixation, is a result of increased competition from companion grasses. Grasses have a greater ability to uptake N from soil compared to clover which, gives them a competitive advantage over clover where fertiliser N is applied (Schwinning and Parsons, 1996). Hogh-Jensen and Schjoerring, (2010) provided further evidence on this in a plot study in Denmark where clover and perennial ryegrass were grown in monoculture and in mixture at two N fertiliser rates (Figure 1.6). The input of N fertiliser significantly increased annual grass

DM production when grown in monoculture and in mixture with clover. In contrast, annual clover DM production remained static when grown in monoculture and was significantly reduced when grown in mixture with grass following fertiliser N application.

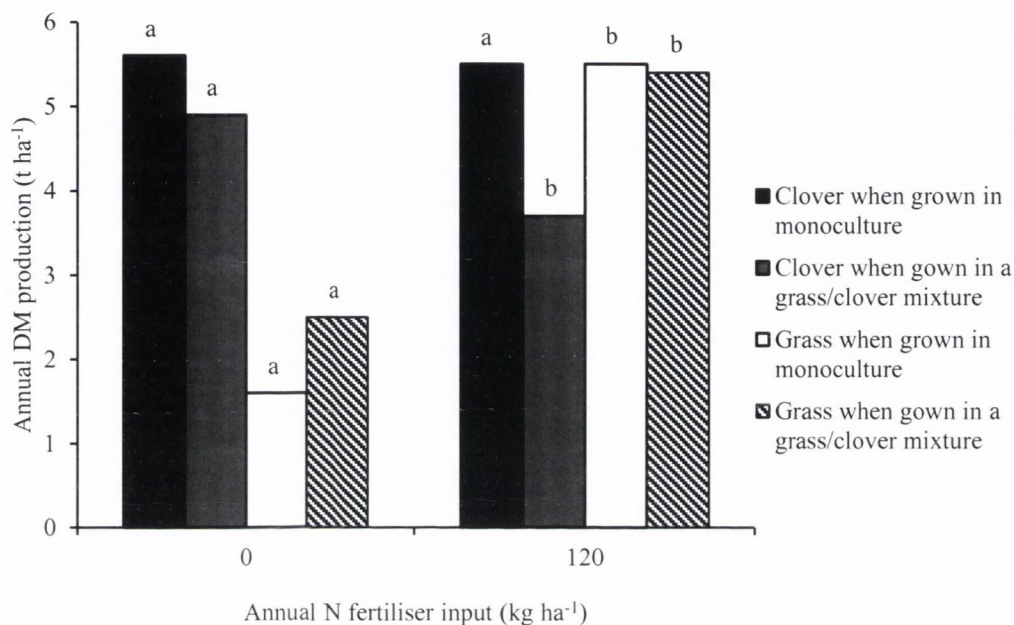


Figure 1.6. Individual annual DM production of white clover and perennial ryegrass when grown as a monoculture or in a mixture (white clover and perennial ryegrass), as affected by annual fertiliser N input. Different letters indicate significant differences ($P < 0.05$) within each ley type. Adapted from Høgh-Jensen and Schjoerring (2010).

Long term grazing studies in New Zealand and Europe have found reductions in sward clover content with N fertiliser use (Harris and Clark, 1996; Ledgard *et al.*, 2001; Trott *et al.*, 2004). For example, Trott *et al.*, (2004) found large reductions in pasture clover content with increasing N fertiliser input under a range of grazing and cutting managements during a five year study (Figure 1.7).

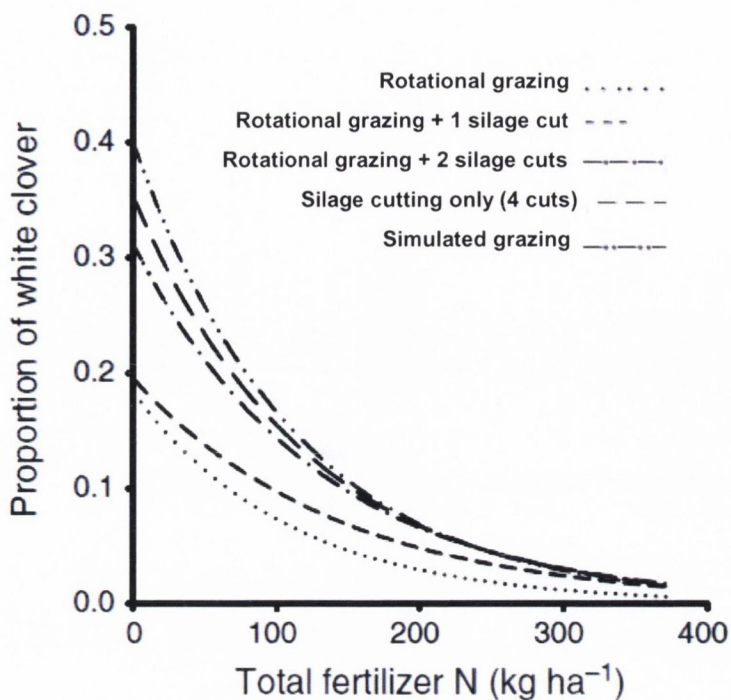


Figure 1.7. Relationship between pasture white clover content and annual N fertiliser input across a range of grazing and cutting managements (Trott *et al.*, 2004).

1.3.2.2. Animal grazing

Nitrogen excreted at pasture can reduce N_2 fixation similar to the use of N fertiliser. For example, Menneer *et al.* (2003) found that application of cow urine ($746 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) reduced annual N_2 fixation on a grass/clover pasture from 232 to 145 kg N ha^{-1} . No significant difference was found in clover DM production between urine affected and non-urine affected pastures in this New Zealand based study. The main contributor to the decrease in annual N_2 fixation was the 38% decrease in annual %Ndfa in clover on pastures receiving urine compared to no urine. Similarly, Vinther (1998) found a reduction in N_2 fixation in grass/clover pastures of between 42% and 63% over a four month period depending on the timing of urine application. In the same study, reductions in N_2 fixation from 89 to 69 kg N ha^{-1} over a four month period were found up to a distance of 10 cm from dung patches. Taking into consideration stocking density (4 to 6 cows ha^{-1} in this case), residence time in the pasture, length of the grazing season, urine and dung excretion *per day* and the area affected by individual patches, Vinther, (1998) estimated that cattle excreta can reduce N_2 fixation in grass/clover pastures by 10 to 15% annually, at the field scale, compared to grass/clover pastures receiving no excreta. Using similar calculations, Menneer *et al.*, (2003) found that between 25% and 46% of grazed pasture could be

affected by urine deposition annually in New Zealand leading to reductions in clover N₂ fixation of between 10% and 40%, respectively, at the field scale.

Animal treading/poaching during grazing can have an adverse effect on N₂ fixation. Phelan *et al.*, (2013b) found significant reductions in annual clover DM production on areas of a pasture subjected to animal treading (2.6 t DM ha⁻¹) compared to non-grazed areas (3.1 t DM ha⁻¹). Significant reductions in N₂ fixation of 165 to 143 kg N ha⁻¹ yr⁻¹, respectively, were subsequently found in treaded compared to non-grazed areas in the same study. Likewise, Menneer *et al.* (2005) found a tendency for reduced N₂ fixation following a moderate and severe single treading event created by walking dairy cows through plots at a stocking intensity of 4.5 cows 100 m⁻¹ for 1.5 and 2.5 hours, respectively (Table 1.1).

Table 1.1. Effects of a different single treading (“pugged”) events on selected components of total herbage yield, pasture composition, N yield, N₂ fixation in white clover and the proportion of white clover N derived from the atmosphere (%Ndfa) on a grass/clover pasture in New Zealand (Menneer *et al.*, 2005).

Pasture parameter	Non-pugged	Moderately pugged	Severely pugged	SED
Total DM yield (kg ha ⁻¹ yr ⁻¹)	10149	8562	6683	417***
Grass DM yield (kg ha ⁻¹ yr ⁻¹)	6817	5497	4264	558***
Grass N yield (kg ha ⁻¹ yr ⁻¹)	197	154	116	16***
Clover DM yield (kg ha ⁻¹ yr ⁻¹)	2046	1854	989	479 [†]
Clover proportion (%) ^a	20	22	15	4NS
Clover N yield (kg ha ⁻¹ yr ⁻¹)	89	78	40	21 [†]
Clover fixed N yield (kg ha ⁻¹ yr ⁻¹)	76	66	36	18 [†]
%Ndfa ^b	85	85	90	NS

^aCalculated using total clover DM yield

^bCalculated using the ¹⁵N isotope dilution method.

***, [†] and NS: significant at $P < 0.001$, $P < 0.1$ and not significant, respectively.

1.3.2.3. Grazing management

Pasture management has a large impact on the interaction between clover and the companion grasses with which it is usually grown. White clover has lower growth rates and is generally less competitive for light than grasses, particularly over the autumn to spring period. Management practices that reduce this competition from companion grasses will promote clover growth and hence N₂ fixation. Lowering defoliation height and increasing defoliation frequency on grass/clover pastures are two such strategies which can reduce competition from grasses (Phelan, 2013). The umbrella-like leaf structure of clover exposes it to higher leaf loss at high defoliation heights (<5 cm above ground level) compared to the blade-like leaf structure of grass leading to reduced clover DM production. Carrying grass/clover pastures with high herbage mass for extended periods, particularly over winter, can result in increased shading of clover leaves and stolon

growing points, which leads to lower pasture clover content (Phelan *et al.*, 2014). For example, Phelan *et al.* (2014) found a 1.46 t DM ha⁻¹ increase in annual clover DM production under simulated grazing when defoliation height was kept to 2.7 cm compared to 6 cm (above ground level) during the summer-to-winter period (2nd July to 16th December). Corresponding annual N₂ fixation increased by 40 kg N ha⁻¹. Reducing the defoliation frequency from 84 to 42 days in the same study also increased annual clover DM production by 1.7 t DM ha⁻¹ and N₂ fixation by 50 kg N ha⁻¹. Similarly, Phelan *et al.*, (2013a) found that annual clover DM production increased from 1.7 to 2.3 t DM ha⁻¹ when experimental pastures were grazed to 4 cm as opposed to 6 cm post-grazing height on a dairy systems-scale grazing trial. There was no difference in milk production *per* cow between the two post grazing height treatments. Furthermore, Ledgard *et al.*, (2001) found in a grazing trial in New Zealand receiving annual N fertiliser inputs of 400 kg ha⁻¹ that clover DM production and N₂ fixation increased where stocking rates were increased from 3.3 to 4.4 dairy cows ha⁻¹ (33% increase). This was due to reduced post-grazing height under the higher stocking rate, which reduced competition from grasses.

1.3.2.4. White clover variety

Ledgard *et al.*, (1996) evaluated nine clover varieties from Ireland, New Zealand, Belgium and the UK in mixtures with perennial ryegrass under grazing by sheep in New Zealand. The study found significant differences in annual clover DM production, %Ndfa and N₂ fixation between the nine varieties (Figure 1.8). The three large-leaved varieties fixed the most N₂ under no N fertiliser input, but N₂ fixation was reduced with N fertiliser input in all varieties to similar levels with the exception of the large-leaved varieties Aran and Kopu (Figure 1.8). Similarly, Elgersma *et al.*, (1998) found significantly higher annual clover DM production, N yield, clover content and N₂ fixation from a large-leaved variety (Alice) compared to medium (Retor) and small-leaved (Gwenda) varieties under no N fertiliser input in year four and five of a long-term plot experiment in the Netherlands.

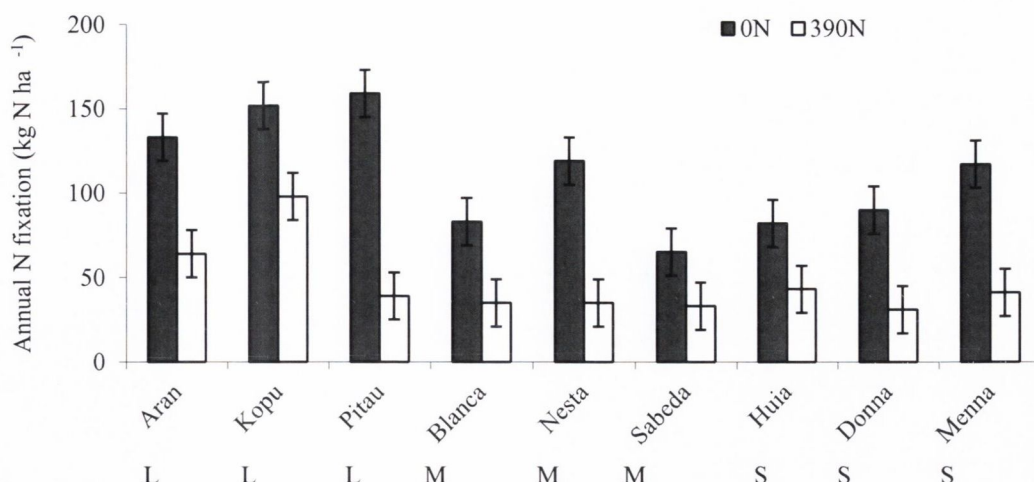


Figure 1.8. Effect of N fertilization ($0 \text{ kg N ha}^{-1} \text{ yr}^{-1}$: 0N; $390 \text{ kg N ha}^{-1} \text{ yr}^{-1}$: 390N) and white clover variety on annual N_2 fixation in a mixed grass/clover pasture rotationally grazed by sheep in New Zealand. Error bars represent least significant differences ($P < 0.05$). L = large, M = medium and S = small leaf size classification, respectively. Adapted from Ledgard *et al.* (1996).

1.3.2.5. Temperature

Temperature affects clover root, stolon, and shoot growth, and N_2 fixation activity (Frame and Newbould, 1986; Ryle *et al.*, 1989; Murray *et al.*, 2000). The minimum air temperature for clover growth is considered to be 6°C with the optimum around 25°C , while active N_2 fixation requires a temperature of 9°C (Frame and Newbould, 1986). Murray *et al.* (2000) found significantly higher leaf (286% increase), stolon (133% increase), root DM yield (87% increase) and growing points (88% increase) on two clover varieties grown in a controlled temperature room with a $24/18^\circ\text{C}$ day/night temperature regime compared to plants growth in a similar room with a $12/6^\circ\text{C}$ day/night temperature regime.

Ryle *et al.* (1989) measured ethylene production (as indicator of nitrogenase activity using the acetylene reduction assay), nitrogenase-linked respiration and basal root + nodule respiration of clover subjected to temperatures ranging from 4 to 33°C . The measured variables increased linearly from 5 to 23°C . Temperatures above 23 to 25°C did not further enhance the rate of nitrogenase activity, which remained essentially the same up to the maximum temperature of 33°C .

Temperature also affects the interactions between clover and grasses. Perennial ryegrass ($5\text{--}6^\circ\text{C}$) has a lower soil temperature growth threshold than clover ($9\text{--}10^\circ\text{C}$) (Davies, 2001)

which, gives perennial ryegrass a competitive advantage over clover, particularly in the early and late stage of the growing season (Hoglund and Frankow-Lindberg, 1998). Furthermore, Kleter (1968) found clover content in permanent grassland in the Netherlands to be strongly related to spring temperatures, particularly in April.

1.3.2.6. Soil moisture

White clover growth is sensitive to extremes in soil moisture, which can occur under drought and flooding conditions. Drought conditions have often been reported to reduce clover growth and thus N₂ fixation (Lucero *et al.*, 1999; Goh and Bruce, 2005; Garcia *et al.*, 2010). Goh and Bruce (2005) reported clover yield to be significantly higher on irrigated (1.5 t DM ha⁻¹ yr⁻¹) compared to non-irrigated (1 t DM ha⁻¹ yr⁻¹) grass/clover pastures subject to summer drought conditions in Canterbury, New Zealand. Corresponding annual N₂ fixation were 52 and 27.4 kg N ha⁻¹ in the irrigated and non-irrigated pastures, respectively. Perennial ryegrass is also reported to have a competitive advantage over clover under drought conditions due to its larger root system and its ability to control transpirational losses more efficiently, thus prolonging its growth period compared to clover under such conditions (Lucero *et al.*, 1999).

Flooding leads to low soil oxygen levels and suppresses plant growth. Huber *et al.* (2009) reported lower total plant production from clover under simulated flooding conditions compared to clover maintained at a soil moisture percentage of 25%. Flooding also affects N₂ fixation activity. A reduction in N₂ fixation activity of clover was reported immediately after a flood irrigation event on a New Zealand pasture (Mundy *et al.*, 1988). Dinitrogen fixation activity subsequently increased after the flood irrigation event. The lack of oxygen supply for nitrogenase activity within the root nodules following the flood irrigation event was proposed as the main cause of reduced N₂ fixation activity.

1.3.3. Methods of measuring biological N₂ fixation

Only when accurate estimates of N₂ fixation can be measured can we look to identify and understand the factors that affect N₂ fixation (Unkovich *et al.*, 2008). Many different methods have been devised to measure biological N₂ fixation, each having advantages and limitations. Estimates of N₂ fixation may be influenced, in part, by the method of measurement used (Ledgard and Steel, 1992). Some studies have found variation in estimates of N₂ fixation using different methods across different legumes (Hogh-Jensen

and Schjoerring, 1994; Carranca *et al.*, 1999). Biological N₂ fixation and nitrogenase activity have been quantified using the acetylene reduction method (nitrogenase activity), ureide (N solutes) method and N mass balance method in the past but are generally out of use owing to inaccuracy in their estimates (Unkovich *et al.*, 2008). The three main methods currently used in the field are the ¹⁵N natural abundance (NA), ¹⁵N isotope dilution (ID), and the nitrogen difference (N Diff) method which have been reviewed extensive elsewhere (Witty, 1983; Danso *et al.*, 1993; Chalk and Ladha, 1999; Unkovich and Pate, 2000; Carlsson and Huss-Danell, 2003; Unkovich *et al.*, 2008). This section gives an overview of these techniques.

1.3.3.1. ¹⁵N Natural abundance method

Stable isotopes are elements that have variations in the number of neutrons in their atoms. For example, N has two stable isotopes, with the lighter isotope (¹⁴N) being much more abundant in the natural environment than the heavier ¹⁵N isotope. The natural abundance of atmospheric ¹⁵N (0.3663 atom %) is very stable globally and is taken as zero (0.0‰) for N₂ fixation calculations. Deviation from atmospheric ¹⁵N abundance is usually expressed in terms of $\delta^{15}\text{N}$ units (‰), commonly known as “delta” units. One $\delta^{15}\text{N}$ unit is 1‰ deviation from the ¹⁵N atmospheric abundance (Carlsson and Huss-Danell, 2003).

The application of the ¹⁵N NA method relies on the slightly higher natural abundance of ¹⁵N (4 to 12‰) in most soils compared to atmospheric ¹⁵N abundance. The higher ¹⁵N levels in soil is brought about by isotopic discrimination of the heavier ¹⁵N isotope compared to the lighter ¹⁴N isotope during biological, chemical and physical processes in soil (Bremer and van Kessel, 1990). When the $\delta^{15}\text{N}$ of atmospheric N and soil N are different the $\delta^{15}\text{N}$ of N₂-fixing legume plants deriving their N from both the atmosphere, through N₂ fixation, and from the soil (Figure 1.9 B) will be different to that of non-N₂ fixing plants who acquire their N solely from the soil (Figure 1.9 C), and from N₂-fixing legume plants that acquire their N solely from the atmosphere (Figure 1.9 A). This difference in plant $\delta^{15}\text{N}$ can be measured using an isotope ratio mass spectrometer, and subsequently the proportion of legume N derived from the atmosphere can be calculated. The N₂ fixation (kg N ha⁻¹) over a given time period can be calculated by multiplying the %Ndfa by the total legume N yield over the given time period (Ledgard and Steele, 1992; Unkovich and Pate, 2000; Carlsson and Huss-Danell, 2003).

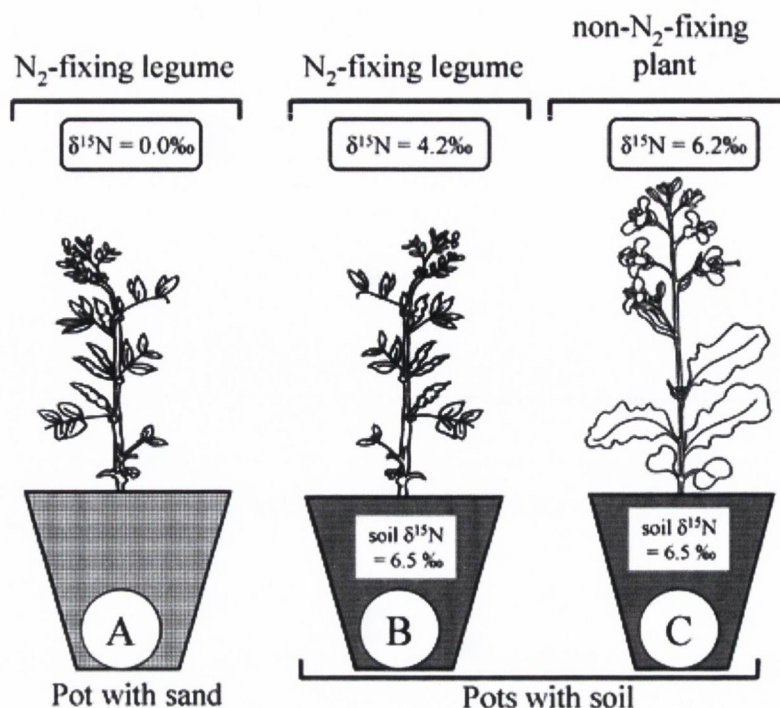


Figure 1.9. Illustration of the $\delta^{15}\text{N}$ natural abundance method for estimating N_2 fixation, highlighting that the $\delta^{15}\text{N}$ of a legume completely dependent on atmospheric N_2 (A) should have a $\delta^{15}\text{N}$ in shoots very close to that of its N source, atmospheric N_2 (0‰); that a plant totally dependent on soil N (C) will have $\delta^{15}\text{N}$ close to that of soil N; and that a plant able to utilise both soil and atmospheric N_2 (B) will have a $\delta^{15}\text{N}$ which reflects the relative contributions from these two N sources (Unkovich *et al.*, 2008).

The major advantage of this method over other methods is that it can be applied in most situations where a fixing and non-fixing plant is present in the same location. It also does not require the addition of expensive ^{15}N -labelled fertilisers and the setup of experimental measurement plots, which is the case with the ID technique. The method can, therefore, be applied at farm level (Unkovich and Pate, 2000; Unkovich *et al.*, 2008). Although the ID method requires the fixing and non-fixing plant to have similar N uptake characteristics, this is of relatively little importance with the NA method, as it uses the small natural ^{15}N enrichment of the soil which is generally relatively stable over time and depth (Ledgard and Steele, 1992).

The potential problem that exists for the NA method is the isotopic fractionation between ^{14}N and ^{15}N by nitrogenase during N_2 fixation. Recent debate suggests that there is little fractionation between ^{14}N and ^{15}N by nitrogenase during the actual N_2 fixation process and that the subsequent fractionations during deamination of NH_3 in bacteroids maybe a more important source of fractionation (Unkovich, 2013). Nevertheless, a *B*-value must be determined to account for this fractionation, whichever it is, when calculating the %Ndfa by the legume. The *B*-value is essentially the $\delta^{15}\text{N}$ of shoots of legumes that are fully

dependent upon N_2 fixation for their growth (Figure 1.9A) (Ledgard and Steele, 1992; Unkovich and Pate, 2000; López-Bellido *et al.*, 2010). The $\delta^{15}N$ of whole legume plants fully dependent upon N_2 fixation is usually close to 0‰, while the $\delta^{15}N$ of legume shoots is commonly less than 0‰ (Unkovich, 2013). As most estimates of N_2 fixation rely on the analysis of plant shoots rather than the whole plant, a B -value must be used to correct for the fractionation of ^{14}N and ^{15}N between legume shoots and roots (Unkovich *et al.*, 2008). Failing to use a B -value will lead to an over estimation of %Nd_{fa} when estimating N_2 fixation using the analysis of legume shoots and non-legume reference plants (Unkovich and Pate, 2000). B -values are usually determined by growing the legume in question in a glasshouse in N-free sand culture as described by Unkovich *et al.* (2008), but B -values should ideally be determined under conditions relevant to the field, as they can be influenced by several factors, such as nutrition, moisture and rhizobial strain (Ledgard and Steele, 1992). Doughton *et al.*, (1992) developed a method of calculating a B -value under field conditions where the NA and ID method are used simultaneously to estimate N_2 fixation. The technique is a process of adjusting values of $\delta^{15}N$ for fixed N until %Nd_{fa} calculated by the NA best matches the calculated %Nd_{fa} from the ID method (Doughton *et al.*, 1992). A limited number of studies using both of these ^{15}N methods have used this technique to calculate a B -value (Menneer *et al.*, 2003). Okito *et al.*, (2004) also used a simplified version of this technique to calculate B -values for four tropical legumes. On the other hand these indirect techniques assume that the ID method is completely accurate at measuring fixation which may not be the case (Chalk and Ladha, 1999) and recent comments suggest that they should not be relied upon (Unkovich, 2013).

The other potential limitation of the NA method is the need to have an appropriate non- N -fixing reference plant to provide a measure of plant available soil N. An assumption of the method is that the $\delta^{15}N$ of non- N_2 -fixing reference plants is identical to the $\delta^{15}N$ of soil N utilised by the legume, but this may not always be the case, and so it is strongly advised to use more than one reference plant (i.e. two or more species) to reduce the errors associated with this limitation (Unkovich *et al.*, 2008).

Low soil $\delta^{15}N$ values may also be a limitation to this method in certain locations. It is recommended that the $\delta^{15}N$ of plant available soil N be above 6‰ when using the NA method, as below this value the accuracy in estimating N_2 fixation decreases markedly (Ledgard and Peoples, 1988). Low $\delta^{15}N$ values have previously been found in pasture soils (Riffkin *et al.*, 1999; Menneer *et al.*, 2003). Large spatial variation in $\delta^{15}N$ of plant-

available soil N immediately after urine deposition and under grazing conditions have also been found (Eriksen and Høgh-Jensen, 1998; Menneer *et al.*, 2003), which may be due to the lower values of $\delta^{15}\text{N}$ of urine deposited during grazing (Steele and Daniel, 1978). Furthermore, Menneer *et al.*, (2003) in a study comparing ^{15}N techniques to measure the impact of urine deposition on N_2 fixation from clover suggested that the NA method should be used with caution in intensively grazed dairy pastures where frequent inputs of urine affect large areas.

1.3.3.2. ^{15}N isotope dilution method

The ID method has been the most widely used method for providing time integrated measurements of N_2 fixation in field settings (Unkovich and Pate, 2000). The method is essentially the same as the ^{15}N NA method, except that the soil used to grow the N_2 fixing and non-fixing reference plant is artificially enriched in ^{15}N , using ^{15}N -labelled compounds, above the background soil ^{15}N natural abundance (Unkovich *et al.*, 2008). The ^{15}N -labelled fertiliser is added to increase the difference between soil and atmospheric ^{15}N abundance. The difference in ^{15}N between N_2 fixing and non-fixing reference plants will be increased, thus allowing for more precise estimations of %Ndffa compared to the NA method (Carlsson and Huss-Danell, 2003). The legume and reference plant materials are analyzed for their ^{15}N content and the dilution of the ^{15}N content of the legume plant material by the ^{14}N derived from atmospheric N_2 (99.6337 at. % ^{14}N) relative to that of the reference plant is then used to calculate %Ndffa (Unkovich and Pate, 2000).

Isotope fractionation (i.e. *B*-value) does not have to be considered when using this method since the enrichments used greatly exceed natural variations in ^{15}N (Unkovich *et al.*, 2008), and this gives it an advantage over the NA method. The addition of ^{15}N -labelled material enriches the soil in ^{15}N above background ^{15}N abundance and eliminates the sources of errors caused by low soil $\delta^{15}\text{N}$ values, which can be a problem with the NA method (Unkovich *et al.*, 2008). Moreover, Hansen and Vinther (2001) concluded that the ID method was better than the NA method for measuring N_2 fixation in intensively grazed pastures due particularly to the sources of errors caused by the ^{15}N depletion in urine depositions and by the high spatial variation of soil $\delta^{15}\text{N}$. On the other hand, ^{15}N -labelled fertiliser is very expensive, which may limit its use in certain studies. It is recommended to allow time for the stabilisation of soil ^{15}N levels following application of ^{15}N labelled fertilisers. This is required to reduce the errors associated with changes in the ^{15}N

enrichment of the N taken up from the soil by the N₂-fixing and non-fixing plants, particularly if their N uptake patterns differ. This stabilisation period may take anything from 1 to 12 months depending on soil type (Unkovich *et al.*, 2008).

1.3.3.3. *Nitrogen difference method*

The NDiff method is based on the difference in N yields between a N₂ fixing and non-fixing reference plant grown in the same soil. The total N yield in the non-fixing plant is subtracted from the total N yield of the N₂-fixing plant under study, and the difference is then ascribed to N₂ fixation (Ledgard and Steele, 1992; Unkovich and Pate, 2000). The NDiff method can also be applied in intercropping or mixtures of legume and non-legume plants. The amount of N₂ fixed is then calculated as the difference in total N yield of the mixture (legume and non-legume control) minus the total N yield of the non-legume monocrop (Unkovich *et al.*, 2008).

Legumes and non-legumes are usually grown in adjacent plots to measure N₂ fixation using the NDiff method. The plots must be big enough to account for spatial variation in soil N fertility and to facilitate normal growth. The herbage on each plot is harvested after a specific growing period. Herbage yield and N content are determined to calculate total N yield (Unkovich *et al.*, 2008). The principal assumption is that the legume and non-legume use exactly the same amount of soil N, and that the total N yield of the legume is always higher than the non-legume. To minimise differences in soil N uptake by legume and non-legumes, the non-legumes needs to be as close as possible in phenology (development rate) and stature (particularly rooting intensity and depth) to that of the legume. The technique is most effective at low soil N fertility where differences in soil N uptake will be small and dependence on N₂ fixation is likely to be large (Unkovich *et al.*, 2008).

The main advantage of the NDiff method over other methods is that it is cheap and easy to establish (Carlsson and Huss-Danell, 2003; Unkovich *et al.*, 2008). It also provides a time integrated measure of N₂ fixation. However, the major assumption behind this method, that legume and non-legume plants remove equal amounts of N from soil, is not robust due to potential differences in rooting depth, growth rates and soil N demand between different plant species (Unkovich *et al.*, 2008). The NDiff method generally underestimates N₂ fixation, as legumes utilise less soil N compared to non-legumes (Ledgard and Steele, 1992; Carranca *et al.*, 1999; Carlsson and Huss-Danell, 2003). Therefore, the selection of an appropriate non-legume plant is vital for this method. The NDiff method has been found

to give similar estimates of N_2 fixation compared to other methods, such as the ID and NA methods (Carranca *et al.*, 1999; Unkovich *et al.*, 2008), but in the absence of complementary approaches, many authors have concluded that the NDiff method cannot be solely relied on to measure N_2 fixation (Unkovich and Pate, 2000; Unkovich *et al.*, 2008). Reports in the literature have even found that the NDiff method can give negative values of N_2 fixation (Ledgard and Steele, 1992; Unkovich and Pate, 2000).

The next section discusses important N losses pathways from agricultural systems, with a specific focus on pasture-based systems.

1.4. Nitrogen losses

1.4.1. Nitrogen losses to groundwater

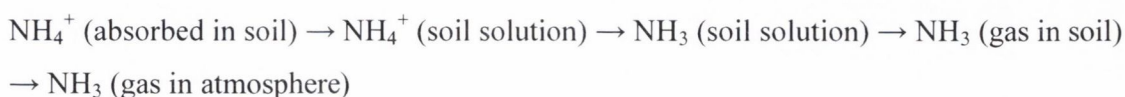
Nitrogen losses to groundwater generally occur when soil N is surplus to plant requirement in periods when excess water percolates through the soil profile (effective drainage) by matrix or macro pore flow, which transports N compounds below the rooting zone (Stark and Richards, 2008). The highest risk periods in pasture-based systems are in early spring, late autumn and winter, when grass N uptake is low and rainfall exceeds transpiration leading to effective drainage. Soil NO_3^- is a negatively charged ion and is not held by soil cation exchange sites in the way that NH_4^+ is, and is therefore the primary soil N compound lost to water (Di and Cameron, 2002). Nitrogen leaching losses have been reported to range from 6 to 204 kg N ha⁻¹ yr⁻¹ under pasture-based systems and 4 to 107 kg N ha⁻¹ yr⁻¹ under arable systems (Ledgard *et al.*, 1999; Webb *et al.*, 2000; Di and Cameron, 2002; Sieling and Kage, 2006; Necpálová *et al.*, 2012).

Irrespective of farming system N leaching is regulated by soil type, weather conditions and management practices. Fine textured soils are generally found to have lower leaching losses than coarse textured soils due to the slower drainage and greater denitrification capacity of these soils. Weather conditions such as rainfall will have a direct impact on effective drainage and thus N leaching, particularly following N applications on free draining soils. Management practices such as N application rate and timing may affect N leaching if they lead to soil N surplus to plant requirement (Di and Cameron, 2002). Grazing management can also have an impact on N leaching. For example, increasing the grazing period by grazing earlier in the spring and later in autumn will increase the amount of urine N deposited in high risk periods as N leaching under urine patches (197 to 674 kg

N ha⁻¹ yr⁻¹) can be substantial (Wachendorf *et al.*, 2008; Selbie *et al.*, 2012; Zaman and Nguyen, 2012; Welten *et al.*, 2013). There are a number of mitigation strategies to reduce N leaching which are discussed in detail by Di and Cameron (2002).

1.4.2. Ammonia volatilisation

Ammonia volatilisation is a chemical reaction responsible for the exchange of NH₄⁺ from soil to NH₃ gas in the atmosphere. The key reactions involved in the process can be represented as follows (McNeill and Unkovich, 2007):



Ammonium ions in soil are the substrate of NH₃ volatilisation. The major source of NH₄⁺ in agriculture is the ammonification of urea in animal urine and N fertilisers. This process is also known as urea hydrolysis and is carried out by the enzyme urease, which is produced by practically all microbial and plant species. Its activity is regulated by urea concentration, pH, temperature and water content (Kissel *et al.*, 2008). The hydrolysis of urea also produces hydroxyl ions and, hence, soil can increase to pH 8. Ammonium ions subject to elevated pH levels are prone to NH₃ volatilisation (Saggar *et al.*, 2004).

Ammonia can be emitted from many different sources on farms including N fertiliser application (primarily urea based fertiliser), livestock N excreted indoors (housing and milking yard) and outdoors, and during storage and land spreading of animal manures. It is, therefore, a difficult N loss to quantify for an entire farm system. Many studies have quantified NH₃ from individual farm sources (Lockyer and Whitehead, 1990; He *et al.*, 1999; Misselbrook *et al.*, 2006; Todd *et al.*, 2008; Sanz *et al.*, 2010; Pereira *et al.*, 2011). The number of reported emissions from entire farm systems, in contrast, is small and reported emissions range from 25 to 78 kg ha⁻¹ on continuously (no animal housing) grazed pasture based systems (Ledgard *et al.*, 1999; Eckard *et al.*, 2007) and from 36 to 105 kg ha⁻¹ on intensive dairy production systems (with animal housing) in northwest Europe (Bussink and Oenema, 1998; Jarvis and Ledgard, 2002).

There are a number of climatic and management factors that influence the rate of NH₃ volatilisation. These factors affect (i) the rate of conversion of NH₄⁺ to NH₃ gas (ii) the transfer of NH₃ gas between the soil solution and the atmosphere and (iii) the supply of

substrate for NH_3 volatilisation (van der Weerden and Jarvis, 1997; Saggar *et al.*, 2004). These factors include solar radiation, pH, wind speed, air temperature (Hatch *et al.*, 1990), slurry application technique and timing (Huijsmans *et al.*, 2001), and N fertiliser type and rate of application (van der Weerden and Jarvis, 1997). Extensive reviews of NH_3 mitigation strategies have been discussed elsewhere (Saggar *et al.*, 2004; Webb *et al.*, 2005; Ndegwa *et al.*, 2008; Webb *et al.*, 2010).

1.4.3. Nitrous oxide and dinitrogen emissions

The production of N_2 in soils is fundamentally important in the global N cycle as it is the mechanism by which the global N cycle is balanced by returning inorganic forms of N to the atmosphere (Knowles, 1982). Nitrous oxide is a potent GHG with a global warming potential 310 times greater than carbon dioxide (CO_2) (IPCC, 2007). Moreover, it is a major contributor to stratospheric ozone depletion (Ravishankara *et al.*, 2009). Emissions of N_2O and N_2 are also an important economic loss of N from agricultural soils. Annual N_2O -N emissions from temperate grassland generally range from -1.6 to 51.3 kg ha⁻¹ (Hyde *et al.*, 2006; Cardenas *et al.*, 2010; Rafique *et al.*, 2011; Rees *et al.*, 2013). Reports of annual N_2 emissions in the literature are scarce. Those of which exist are generally reported as 'total denitrification' ($\text{N}_2 + \text{N}_2\text{O}$). Total denitrification has been reported to range from 0 to 239 kg N ha⁻¹ yr⁻¹ in agricultural and forest soils (Barton *et al.*, 1999). A recent review of total denitrification in temperate grasslands found daily emissions to range from 0.0001 to 6.84 kg N ha⁻¹ d⁻¹ (median: 0.0171 kg N ha⁻¹ d⁻¹, mean $\pm$ s.e: 0.258 $\pm$ 0.050 kg N ha⁻¹ d⁻¹) (Saggar *et al.*, 2013). Nitrogen balance studies suggest that total denitrification may range from 30 to 300 kg N ha⁻¹ yr⁻¹ (Kroeze *et al.*, 2002).

1.4.4. Processes producing nitrous oxide and dinitrogen

Nitrous oxide is produced, consumed and influenced by an array of processes in soil (Figure 1.10) (Bremner, 1997; Stevens and Laughlin, 1998; Wrage *et al.*, 2001; Baggs, 2008). Nitrification and particularly denitrification are considered the most important of these processes (Stevens *et al.*, 1998; Müller *et al.*, 2004; Saggar *et al.*, 2013) and for this reason are the focus of this review. The reader is referred to Butterbach-Bahl *et al.* (2013) and Baggs (2011) for detailed information on all processes producing N_2O and N_2 . All N_2O produced from different processes may mix to form one pool before being reduced to N_2 by denitrification. Both N_2 and N_2O can be produced by different processes simultaneously in the same soil profile due to the existence of aerobic and anaerobic soil

micro-sites (Stevens and Laughlin, 1998). Hybrid N_2O or N_2 can also be produced directly via chemo- or co-denitrification. This process occurs during the sequential denitrification reaction pathway, with either a NO or NO_2^- enzyme complex binding to nucleophilic N species, such as amines, azides, hydrazine or amino compounds (Spott *et al.*, 2011).

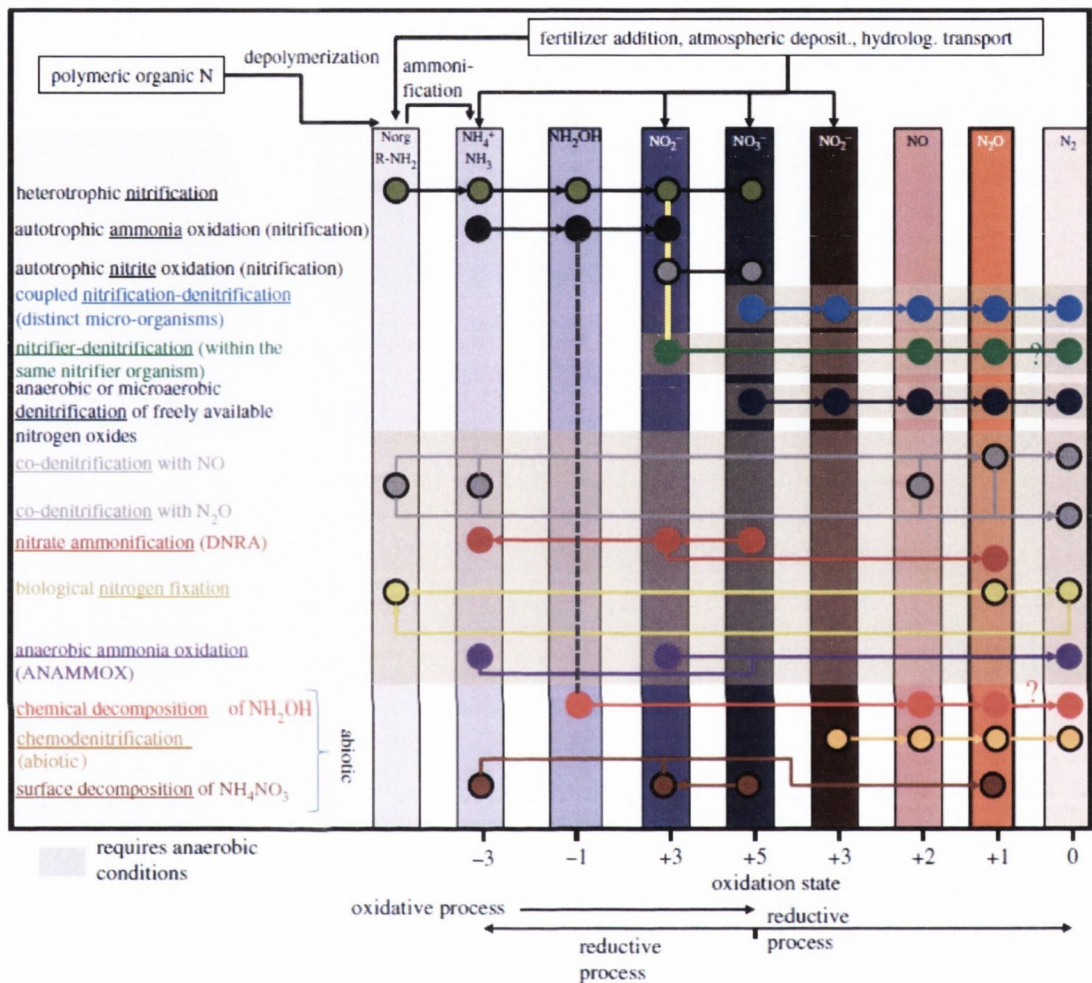


Figure 1.10. Soil N transformations producing and influencing nitrous oxide and dinitrogen production in soil. According to current knowledge, anaerobic ammonia oxidation does not contribute to N_2O formation or consumption. By contrast, N_2O may at least serve as a substrate for biological dinitrogen fixation. Processes predominantly requiring anaerobic (or micro-aerobic) conditions are underlined by grey illuminated segments. Norg/R-NH₂, monomeric organically bound N forms; NH_4^+ , ammonium; NH_3 , ammonia; NH_2OH , hydroxylamine; NO_2^- , nitrite; NO_3^- , nitrate; NO , nitric oxide; N_2O , nitrous oxide; N_2 , molecular dinitrogen. DNRA, Dissimilatory Nitrate Reduction to Ammonium. Source: (Butterbach-Bahl *et al.*, 2013)

1.4.4.1. Nitrification

Nitrification can be autotrophic or heterotrophic. Autotrophic nitrification is a two-step process whereby NH_4^+ is oxidised to NO_3^- under generally aerobic conditions. During the first step, NH_4^+ is reduced to hydroxylamine (NH_2OH), which act as an intermediate between NH_4^+ and NO_2^- (Figure 1.11). Hydroxylamine is subsequently reduced to NO_2^- in

this first step. The first step in the process (NH_4^+ to NO_2^-) is carried out by ammonium oxidizing bacteria and in some instances by ammonium oxidizing archaea (Baggs, 2011). The second step (NO_2^- to NO_3^-) is carried out by NO_2^- oxidizers (Wrage *et al.*, 2001). Nitrous oxide can be produced as a by-product during autotrophic nitrification (Stevens and Laughlin, 1998). In general, heterotrophic nitrification has the same substrates, intermediates and products as autotrophic nitrification but has been found to oxidise organic forms of N such as urea as well as NH_3 . Heterotrophic nitrification is generally regarded to be only a minor source of N_2O compared to autotrophic nitrification. Nevertheless it may produce significant amounts of N_2O under certain soil conditions such as low pH, high oxygen contents and high availability of organic material (Wrage *et al.*, 2001).

The enzymes and the functional genes involved in each step of nitrification are illustrated in Figure 1.11 (Groffman *et al.*, 2006; Baggs, 2008; Firestone *et al.*, 2012; Müller and Clough, 2012; Wakelin *et al.*, 2013). These enzymes are very important for the regulation of nitrification. For instance, nitrification inhibitors such as dicyandiamide (DCD) have been found to reduce the rate of conversion of NH_4^+ to NO_2^- . This inhibitor deactivates the ammonia mono-oxygenase enzyme of soil ammonia oxidizers, making the enzyme ineffective for the first step of the nitrification process (Amberger and Amberger, 1989; Skiba *et al.*, 2011). The use of such nitrification inhibitors is an effective mitigation strategy for N_2O emissions (Di and Cameron, 2012; Dai *et al.*, 2013; Wakelin *et al.*, 2013). Nitrification is an important prerequisite for denitrification as it produces its NO_3^- substrate.

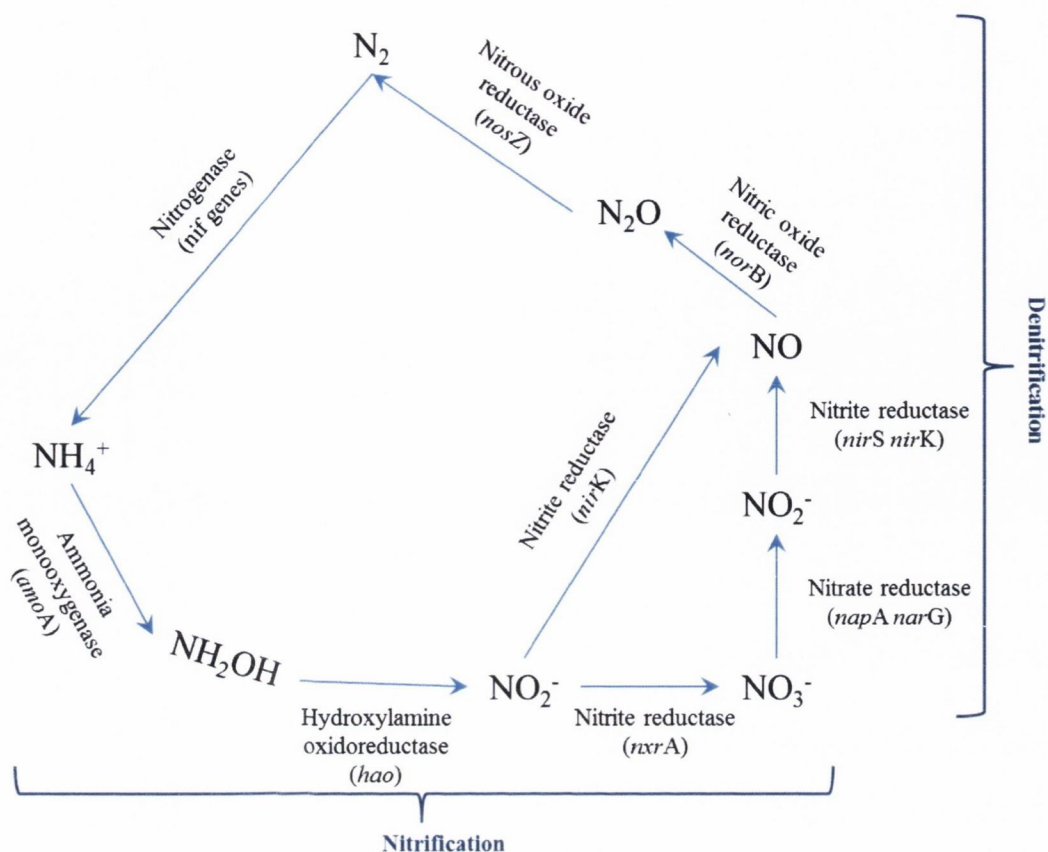


Figure 1.11. Enzymes and the functional genes (in parenthesis) which, encode these enzymes, involved in the nitrification and denitrification process. Source; (Baggs, 2011; Müller and Clough, 2012)

1.4.4.2. Denitrification

Denitrification is a reductive process whereby NO_3^- or NO_2^- is reduced to NO , N_2O or N_2 (Figure 1.10 and 1.11) by an array of denitrifying micro-organisms (bacteria, archaea, fungi and other eukaryotes) (Baggs, 2011). Fungi have been found to be important contributors to N_2O , in particular, in permanent grassland soil (Laughlin and Stevens, 2002). However, bacteria are considered to be the most important denitrifiers. During denitrification bacteria generally couple N oxide reduction with oxidation of organic matter (mostly organic C) to produce energy (Firestone, 1982). During denitrification NO_x act as terminal electron acceptors and organic C as electron donors (Stevenson and Cole, 1999; Morley and Baggs, 2010). Denitrifying bacteria can use either NO_3^- or oxygen as an electron acceptor, but are generally only considered to reduce NO_3^- when oxygen is unavailable (i.e. under suboxic conditions). However, Bateman and Baggs (2005) found denitrification to be the dominant process producing N_2O under aerobic soil conditions (20% WFPS) while Patureau *et al.* (2000) isolated several bacteria which were capable of

aerobic denitrification. Despite these findings, denitrification, in general, is promoted by anaerobic conditions (Baggs, 2011).

Enzymes are required in each step of the denitrification process (Figure 1.11) and therefore play a major part in the regulation of N_2O and N_2 emissions. In fact, fungi and some species of bacteria do not possess N_2O reductase and therefore are unable to reduce N_2O to N_2 (Richardson *et al.*, 2009). The genes which encode the enzymes involved in denitrification have been used as molecular markers to investigate denitrifier community size and structure. These markers have been linked to denitrification process rates. The key enzyme recognised as a precursor to gaseous N emissions is NO_2^- reductase, which is encoded by the *nirS* or *nirK* gene (Müller and Clough, 2012). The ratio between *nirS* and N_2O reductase (*nosZ*) has also been related to the $\text{N}_2\text{O}:\text{N}_2$ ratio (Regan *et al.*, 2011).

1.4.5. Factors affecting N_2O and N_2 emissions

The main factors regulating soil N_2O and N_2 emissions are soil NO_3^- and NH_4^+ concentrations, soil moisture (particularly water-filled pore space; WFPS), organic C, soil pH, and soil temperature (Luo *et al.*, 1999; van Spanning *et al.*, 2007; Cuhel *et al.*, 2010). Therefore management (application of N and grazing intensity) and climatic factors (rainfall and temperature) have a large influence on both N_2 and N_2O emissions from agricultural soils.

1.4.5.1. Application of nitrogen

The application of N (fertiliser N and animal slurries) has been shown to increase N_2O and N_2 emissions in numerous studies (McTaggart *et al.*, 1997; Ellis *et al.*, 1998; Smith *et al.*, 1998; Rudaz *et al.*, 1999; Horgan *et al.*, 2002; Dobbie and Smith, 2003a; Jones *et al.*, 2007; Luo *et al.*, 2007; Abdalla *et al.*, 2010a). This is primarily due to increased concentrations of NO_3^- and NH_4^+ in the soil for nitrification and denitrification. For instance the application of inorganic fertiliser N results in an increase in NO_3^- concentrations in the upper soil layer shortly after N application, while application of organic N fertilisers results in a slow increase in soil NO_3^- over a longer period of time (Jones *et al.*, 2007). A meta-analysis conducted by the author of some of the available literature reveals a general increase in annual N_2O emissions with increasing annual N

input (Figure 1.12). The relatively large scatter in the data in Figure 1.12 suggests that N input is only one of many factors which influence N₂O emissions.

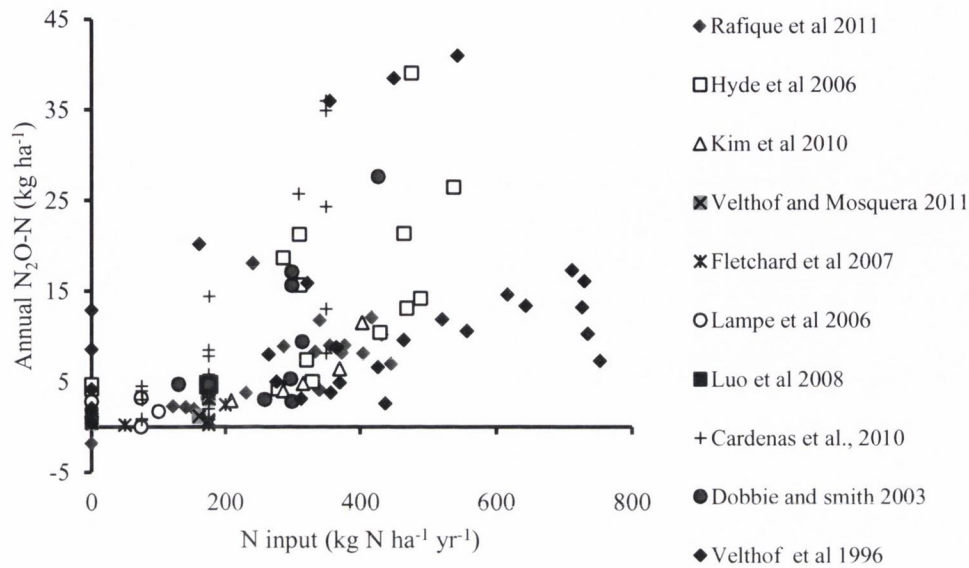


Figure 1.12. Meta-analysis of the relationship between annual N₂O-N emissions and reported N input across a broad range of international studies at various grassland sites.

The form of fertiliser N applied can have a large impact on emissions. Nitrate based inorganic N fertiliser are found to produce higher rates of N₂ and N₂O emissions as opposed to urea based fertilisers (Horgan *et al.*, 2002; Dobbie and Smith, 2003a). This may be due to losses of N through immobilisation of NH₄⁺ following the hydrolysis of urea (Horgan *et al.*, 2002), also urea based fertilisers are prone to N losses in the form of NH₃ volatilisation (van der Weerden and Jarvis, 1997). These losses lead to a reduction in available substrate for nitrification and denitrification.

Slurry and other organic manures are also found to induce substantial emissions (Ellis *et al.*, 1998; Lampe *et al.*, 2006; Jones *et al.*, 2007; Schils *et al.*, 2008; Velthof and Mosquera, 2011). The application of organic manures to grassland is found to stimulate emissions of N₂O over a longer period compared to inorganic fertiliser and greater emissions in some cases. These prolonged and sometime greater emissions maybe due to (i) higher total N inputs, (ii) the slower accumulation of NO₃⁻ in the soil from the mineralisation and subsequent nitrification of organic N, (iii) the presence of easily available C contained in organic manures, (iv) changes in soil pH and soil aeration following increased respiration and (v) changes in soil moisture associated with the application of organic manures (McTaggart *et al.*, 1997; Ellis *et al.*, 1998; Barton and Schipper, 2001; Jones *et al.*, 2007). The technique use to apply organic manure to soil is also found to affect denitrification and N₂O production. Soils which receive organic manure by injection (organic manure injected

to 10 cm depth in the soil) are found to have greater emissions of N_2O and increased denitrification rates compared to soil receiving organic manure by surface application (splash plate) (Ellis *et al.*, 1998).

The combined application of organic manures and inorganic N fertiliser has been reported to substantially increase emissions of N_2 and N_2O compared to singular applications of each N source. Increased emissions have been attributed to the coupled supply of C in organic manures and readily available N in inorganic fertilisers. The ^{15}N enrichment of evolved N_2O in such studies indicates that the N added in inorganic N fertiliser is much more susceptible to be emitted as N_2O compared to the N added in organic manures under these combined applications (Stevens and Laughlin, 2001a; Dittert *et al.*, 2005).

The excessive N loads, additions of organic C and specific amino acids associated with dung and urine deposition on grazed pastures have been reported to stimulate considerable daily and cumulative emissions of N_2O and N_2 from grassland soils (Monaghan and Barraclough, 1993; Allen *et al.*, 1996; Bhandral *et al.*, 2003; Clough *et al.*, 2009; Zaman and Blennerhassett, 2010; Wakelin *et al.*, 2013). Clough *et al.* (2009) reported significantly higher daily N_2O emissions (13 to 1,414 g $\text{N}_2\text{O-N ha}^{-1}$) on urine amended pasture compared to soil receiving no urine (0.2 to 93 g $\text{N}_2\text{O-N ha}^{-1}$) over a 78 day period in New Zealand. Bhandral *et al.*, (2003) also reported higher N_2O emissions from urine amended pasture relative to pasture amended with water.

1.4.5.2. Soil moisture and aeration status

Soil water filled pore space is a commonly used measure of soil moisture and an indicator of soil aeration status. Soil bulk density is taken into account during the calculation of WFPS, which allows soil moisture to be compared across different soil types. Therefore, WFPS is considered to be a better parameter for soil moisture measurements compared to soil volumetric moisture content. Many studies have found WFPS (i.e. aeration status) to be a major factor affecting N_2O and N_2 production with emissions generally increasing under elevated WFPS or low aeration status (Figure 1.13) (Luo *et al.*, 1999; Rudaz *et al.*, 1999; Dobbie and Smith, 2003a; Bateman and Baggs, 2005; Liu *et al.*, 2007; Luo *et al.*, 2007). Increases in WFPS as a result of rainfall or irrigation often restricts soil aeration (Barton *et al.*, 1999), leading to anaerobic soil conditions conducive to denitrification and hence increased N_2O and N_2 emissions.

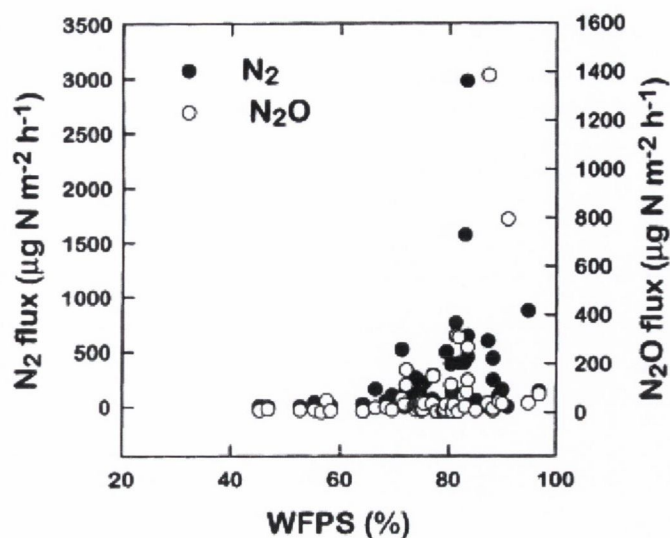


Figure 1.13. Relationship between fluxes of N_2 and N_2O with soil water filled pore space (WFPS) (Rudaz *et al.*, 1999).

Nitrification is the dominant process producing N_2O at 30-60% WFPS under relative aerobic soil conditions and little N_2 is normally produced (Figure 1.14). Nitrous oxide and particularly N_2 production increases as WFPS increases to between 60-85% and N_2O is produced primarily by denitrification. At >85% WFPS most of the soil profile is under anaerobic conditions and denitrification is the dominant process producing N_2O . Many studies have also found N_2O emissions to reduce when WFPS reaches 85-90% WFPS (Figure 1.14). This can be attributed to the reduction of N_2O to N_2 under such anaerobic conditions (Smith *et al.*, 1998) due to the stimulation of N_2O reductase, which generally requires anaerobic conditions (Stevens *et al.*, 1997; Rudaz *et al.*, 1999; Bateman and Baggs, 2005; Liu *et al.*, 2007; Richardson *et al.*, 2009; Cuhel *et al.*, 2010).

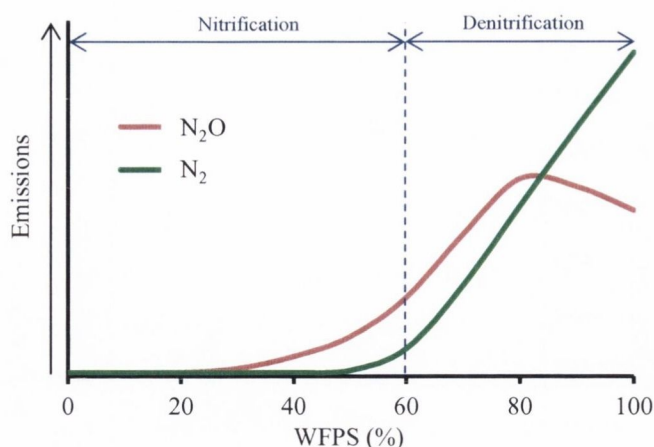


Figure 1.14. Conceptual illustration of the effects of soil water filled pore space (WFPS) on emissions of N_2O and N_2 and the dominance of processes producing N_2O . This illustration was based on the findings of Bateman and Baggs (2005), Smith *et al.* (1998) and Liu *et al.* (2007)

Rudaz *et al.*, (1999) and Luo *et al.*, (2007) among many studies have found N_2O and N_2 emissions to increase when soil moisture content reaches and increases above field capacity or a certain threshold of WFPS. These threshold values differ according to soil type, and are generally greater for coarse-textured than fine textured soils. For example, reported threshold soil water contents range from 74% to 83% in sandy and sandy loam soils, from 62% to 83% in loam soils and from 50% to 74% in clay loam soils (Barton *et al.*, 1999).

Animal treading has been reported to trigger denitrification, due to soil compaction which increases WFPS and therefore increases soil anaerobic conditions (Saggar *et al.*, 2004). For example, Bhandral *et al.*, (2003) found compaction to create a seven fold increase in N_2O emissions from various N sources on pasture soil (fine sandy loam) in New Zealand (Table 1.2). Soil compaction was also found to significantly increase N_2O emission factors (EF; % of N applied emitted as N_2O) from 1.30% to 2.92% on treatments receiving high ($373.2 \text{ kg N ha}^{-1}$) and low ($186.4 \text{ kg N ha}^{-1}$) application of urine N on grassland in the Netherlands (van Groenigen *et al.*, 2005).

Table 1.2. Total N₂O-N emitted (kg N ha⁻¹) over 12 weeks from different forms of N applied at a rate of 600 kg N ha⁻¹ to compacted and uncompacted pasture (Bhandral *et al.*, 2003).

Treatments	Main plots		Mean
	Compacted	Uncompacted	
Urine	9.17	2.94	6.05
Nitrate-N ^a	61.74	4.37	33.05
Ammonium-N ^a	9.17	2.64	5.90
Urea	9.11	2.13	5.62
Water	2.62	1.12	1.87
Mean	18.35	2.64	
LSD (0.05) Compacted versus uncompacted (<i>n</i> = 24) 3.43			
LSD (0.05) Compacted + uncompacted mean (<i>n</i> = 8) 3.93			
LSD (0.05) Treatments (<i>n</i> = 4) 5.94			

^aNitrate-N was applied as potassium nitrate and ammonium-N as ammonium sulphate.

Water in soil is also thought to control denitrification by influencing substrate movement. Luo *et al.*, (1999) suggested, in a study investigating factors controlling denitrification, that observed effects of soil water content on denitrification may have been due to the easier movement of NO₃⁻ or soluble-C to denitrifying micro-sites in soil.

1.4.5.3. Organic carbon

Soil C is required by denitrifiers as an electron donor, and also indirectly affects the oxygen status of aerobic soils (Groffman *et al.*, 1987). Available C increases heterotrophic respiration in aerobic soils, decreasing soil oxygen concentrations and creating anaerobic microsites for denitrifiers. Unlike soil water content and nitrate, the amount of C required for denitrification has not been regularly cited in the literature (Barton *et al.*, 1999). The rate of denitrification is most often related to the amount of dissolved organic C in porewater or groundwater, or the amount of soluble organic C rather than the total amount of solid organic C present. Solid-phase organic C contents of soil or geological deposits may also give some indication of the potential for denitrification (Buss *et al.*, 2005). Brettar *et al.*, (2002) observed a positive correlation between denitrification rate and total soil organic C. Furthermore, in a study carried out by Myrold and Tiedje (1985) aiming to determine the factors which most influence the denitrification capacity of a soil found that the addition of plant C was the largest factor contributing to the denitrification capacity of a soil through the mechanism of microbial growth.

The amount of C available in the soil surface horizon is generally sufficient to sustain denitrifying bacteria but not in the subsurface soil horizon (Stevenson and Cole, 1999). Murray *et al.*, (2004) and Clough *et al.*, (1998a) found that the addition of C to subsurface soil stimulates denitrification activity in these soils. Murray *et al.*, (2004) also reported that the form of C administered to soil can influence denitrification potential. The study used three forms of C; glucose, starch and cellulose. The simplest carbohydrate (glucose) was found to stimulate the most denitrification activity in subsoil (70-90cm) and topsoil (0-20cm). Denitrification activity in this study appeared to be limited by the relative recalcitrance of the C substrate supplied, since the availability of C substrate is determined by their decomposability and their ability to support microbial growth (Murray *et al.*, 2004).

1.4.5.4. Soil temperature

Soil temperature influences nitrifying and denitrifying bacteria activity in the soil and, thus, emissions of N₂ and N₂O. An increase in temperature also leads to increased activity of other microbial communities. This increases soil respiration and hence CO₂ production leading to an increase in soil anaerobic micro-sites. For example denitrification rates from soil incubated in a laboratory study at 25 °C were found to be significantly higher than those of soil incubated at 10, 15 or 20 °C (Abdalla *et al.*, 2009a).

Many studies have found total denitrification and N₂O emission to be very sensitive to temperature. One measure of temperature sensitivity is the Q₁₀ value (the rate of increase in a biological reaction when temperature increases by 10°C). Values differ across different soil types and temperature ranges. Abdalla *et al.* (2009a) reported Q₁₀ values ranging from 4 to 6 for temperatures between 10 to 25°C, whilst Dobbie and Smith, (2001) reported values of 3.7 and 2.3 for temperatures between 5 to 12°C and 12 to 18°C respectively for N₂O emissions.

Increased N₂O emissions have been reported during soil freeze thaw periods which have been reported to account for up to 25 to 78% of annual emissions (Kaiser *et al.*, 1998; Virkajärvi *et al.*, 2010). The increase in emission may be due to; (i) the release of easily degradable N and C through the lysis of micro-organisms and disrupted soil aggregates, (ii) the reduction in N₂O reductase activity, (iii) the physical release of accumulated N₂O during soil thawing which was produced by active micro-organisms in unfrozen soil water and (iv) by a reduction in the diffusion resistance to gases that occurs during thawing

(Christensen and Tiedje, 1990; Christensen and Christensen, 1991; van Bochove *et al.*, 2000; Teepe *et al.*, 2001; Holtan-Hartwig *et al.*, 2002; Müller *et al.*, 2002; Mørkved *et al.*, 2006; de Bruijn *et al.*, 2009). Denitrification is found to be the primary process producing N_2O during these periods (Müller *et al.*, 2002; Mørkved *et al.*, 2006). This may be due to changes in nitrifying and denitrifying communities between frozen and unfrozen soil conditions (Wertz *et al.*, 2013). An important characteristic of the soil which increases the potential for N_2O emission during freeze thaw periods is the capacity of soil for NH_4^+ fixation by clay minerals and NO_2^- and NH_4^+ immobilisation by soil microbial biomass. Soils with a high content of active microbial and/or fungal biomass and clay-dominated aggregates may therefore be particularly prone to high N_2O emissions during freeze thaw events (Müller *et al.*, 2002).

1.4.6. Methods of measuring N_2O and N_2 emissions

Nitrous oxide emissions can be measured with relative ease in the field using the static chamber method (Hutchinson and Moision, 1981) due to its low atmospheric concentration and the availability of sensitive detectors for this gas (Groffman *et al.*, 2006). Despite some limitations (Rochette and Eriksen-Hamel, 2007; Kroon *et al.*, 2008), this method is generally recognized to give reliable estimates of N_2O fluxes. On the other hand the measurement of N_2 fluxes and therefore total denitrification ($\text{N}_2\text{O} + \text{N}_2$) is far more difficult. This is primarily due to the high background concentration of N_2 in the atmosphere and the high spatial and temporal variation in the emissions of N_2 (Groffman *et al.*, 2006). Current methodologies for measuring N_2 have a number of limitations; (i) they disturb the physical setting of the process, (ii) disturb natural processes, (iii) alter substrate concentrations, (iv) lack sensitivity and (v) are expensive and time consuming (Khalil and Richards; Groffman *et al.*, 2006). Nitrogen balances (van der Salm *et al.*, 2007) and modelling (Henault and Germon, 2000; Heinen, 2006) have been used to estimate annual N losses through denitrification. The acetylene inhibition (C_2H_2) and ^{15}N gas-flux method, however, are the only methods suitable for direct measurement of total denitrification ($\text{N}_2\text{O}+\text{N}_2$) in the field (Stevens and Laughlin, 1998).

1.4.6.1. Acetylene inhibition method

The acetylene inhibition method has been the most commonly applied method to measure total denitrification ($\text{N}_2\text{O}+\text{N}_2$) and is based on the ability of C_2H_2 to inhibit the reduction of

N_2O to N_2 . In the presence of C_2H_2 , N_2O becomes the terminal product of denitrification and is considered the product of total denitrification. Acetylene (10% v/v) is generally added to the headspace of a sealed soil core and N_2O concentration is measured over an incubation period ranging from 1 to 24 hours. The comparison of N_2O emissions from soil with and without the addition of C_2H_2 is often used to determine the $\text{N}_2:\text{N}_2\text{O}$ ratio. Therefore the method does not measure N_2 directly *per se*. The method is easy to carry out and relatively inexpensive compared to the ^{15}N gas-flux method. This allows for a large numbers of samples to be taken, which is important due to the high spatial and temporal variation of denitrification rates (Groffman *et al.*, 2006). In spite of this, its accuracy and future application has been seriously questioned due to a number of major limitations associated with the method (Groffman *et al.*, 2006; Stevens and Laughlin, 1998). Acetylene inhibits nitrification (Groffman *et al.*, 2006) which is known to produce N_2O and affects the supply of soil NO_3^- for denitrification (Stevens and Laughlin, 1998). Bollmann and Conrad, (1997) found that high concentrations of C_2H_2 enhance the decomposition of NO_x to NO_2 which leads to an underestimation of denitrification rate when the C_2H_2 inhibition method is used. Many other problems associated with the C_2H_2 inhibition method are reported by Groffman *et al.*, (2006).

1.4.6.2. ^{15}N gas-flux method

The ^{15}N gas-flux method involves the application of highly enriched (20-99 atom % ^{15}N) ^{15}N fertiliser to a designated plot of soil, enclosing it with a chamber and measuring the evolution of N_2 and N_2O in the chamber headspace by quantifying the increase in ^{15}N labelled gases (Stevens and Laughlin, 1998). This method along with gas-phase mass spectrometry allows separate fluxes of N_2 and N_2O to be quantified simultaneously in the field. The flux of N_2 is quantified from the measurement of the differences in isotopic composition between N_2 in normal atmosphere and the enriched atmosphere in the chamber headspace above the ^{15}N fertilised soil. The flux of N_2O is quantified by the change in N_2O concentration in the headspace as in the static chamber method. The enrichment and distribution of ^{15}N atoms in the N_2O molecules of the enriched headspace can also be used to determine the origin of N_2O fluxes i.e. nitrification, denitrification or chemo-denitrification (Arah, 1997; Stevens and Laughlin, 1998, 2001b). This method allows all N transformation to proceed naturally, which is not the case with the C_2H_2 inhibition method. Therefore the ^{15}N gas-flux method may give more reliable estimates of

N₂O and N₂ fluxes. Nonetheless this method alters N substrate supply and requires the addition of expensive ¹⁵N fertiliser and isotope analysis (Stevens and Laughlin, 1998).

Following this overview of N losses the next section describes N balances which can be used to estimate a production systems potential for N loss and its' NUE.

1.5. Farm N balances

1.5.1. Farm N balances

The N movements within agriculture/farm N system can be measured and quantified to create N balances. Nitrogen balances measure the difference between the N inputs into, and outputs from, an agricultural system (Parris, 1998; Ledgard *et al.*, 1999; Treacy *et al.*, 2008; Jarvis *et al.*, 2011). The farm-gate N balance is the main N balance used due to its relative ease of completion. This type of balance is a useful tool that can be used to estimate the N pollution potential and NUE of individual farms or farming sectors across many countries.

A farm-gate balance takes into account the N entering and leaving a farm via the farm-gate. Inputs include synthetic fertiliser, feeds, seeds, manure, atmospheric deposition and purchases of livestock. Nitrogen outputs include the export of agricultural products such as milk, cereals, livestock, and manures (Schröder *et al.*, 2003). The difference between the N imported onto and exported from farms will create a farm-gate N deficit, balance or surplus. The farm-gate import of N generally exceeds the export of N in livestock based farms creating a farm-gate N surplus (Treacy *et al.*, 2008; Buckley *et al.*, 2013; Mihailescu *et al.*, 2013). This surplus N can be lost from the farm, therefore, these balances are useful in comparison studies to predict the potential for N loss from different production systems (Schröder *et al.*, 2003).

Farm-gate N balance studies have reported large farm-gate N surpluses for example (mean $\pm$ s.d.) 238 ± 74 kg N ha⁻¹ on 120 specialised dairy farms in Belgium (Neuens *et al.*, 2006), (mean $\pm$ s.d.) 175 ± 39 kg N ha⁻¹ on 63 conventional pilot dairy farms in Denmark (Nielsen and Kristensen, 2005), 47 to 601 kg N ha⁻¹ on 41 contrasting dairy farms in Australia (Gourley *et al.*, 2012) and 87 to 389 kg N ha⁻¹ across 21 intensively managed dairy farms in Ireland (Treacy *et al.*, 2008).

Comparison of farm-gate N balances across 9 regions of the Atlantic Area of Europe was undertaken in the ‘Green Dairy’ project between 2004 and 2006 (Table 1.3). A total of 139 commercial pilot farms, specialised primarily in dairy production, were included in the study. Irish dairy farms were the highest users of fertiliser N in the study, which accounted for up to 90% of total N imports. Despite this, the N surpluses on the Irish farms were within the range of those found across other EU countries (Raison *et al.*, 2006). More recent N balance studies carried out in Ireland by Buckley *et al.*, (2013), using data from 195 specialist dairy farms contributing to the National Farm Survey (www.teagasc.ie/nfs/) in 2009 and 2010 reported N surpluses (mean $\pm$ s.d.) of $138.9 \text{ kg ha}^{-1} \pm 58.5$. This is much lower than previous N surpluses found in Ireland between 2003 and 2006. Raison *et al.* (2006) reported a value of 240 kg N ha^{-1} and Treacy *et al.* (2008) reported a value of 232 kg N ha^{-1} . This large decrease in reported N surpluses coincides with the introduction of EU Nitrates Directive regulations in 2006 (section 1.6.1).

Table 1.3. Farm-gate N balances in the ‘Green Dairy’ dairy farms (average of 3 years of monitoring between 2004 and 2006; Raison *et al.*, 2006)

	Scotland ^a	Southern Ireland	Southern England	Brittany ^b	Pays de la Loire ^b	Aquitaine	Basque country	Galicia	North Portugal
	kg N ha ⁻¹ yr ⁻¹								
Fertiliser N	114	269	234	57	66	147	28	136	212
Fixation	1	0	1	22	14	0	7	0	0
Animal	0	0	0	21	12	0	0	0	0
Feeds	63	32	82	85	59	81	315	319	524
Others	3	0	13	8	3	10	4	2	18
Total imports	181	301	330	193	154	238	352	457	754
Milk	43	47	54	31	27	30	86	98	180
Meat	4	14	6	25	7	5	7	10	22
Crops	0	0	4	16	27	48	0	0	10
Animal	0	0	0	4	0	0	2	0	40
Total exports	47	61	64	76	61	83	95	108	252
N Surplus	134	240	266	117	93	155	257	349	502

^athe N balance is only carried out on the dairy cow unit brought to the total agricultural area. It does not include the other herbivores (beef cattle).

^bnot counting the milk + off-ground mixed farms, the surplus = 75 kg N/ha in Brittany and 69 in Pays de Loire.

Nitrogen surpluses are largely governed by N inputs and are generally higher on dairy production systems compared to arable systems (Figure 1.15) due to higher N inputs and lower NUE (discussed in the next section) associated with dairy production systems.

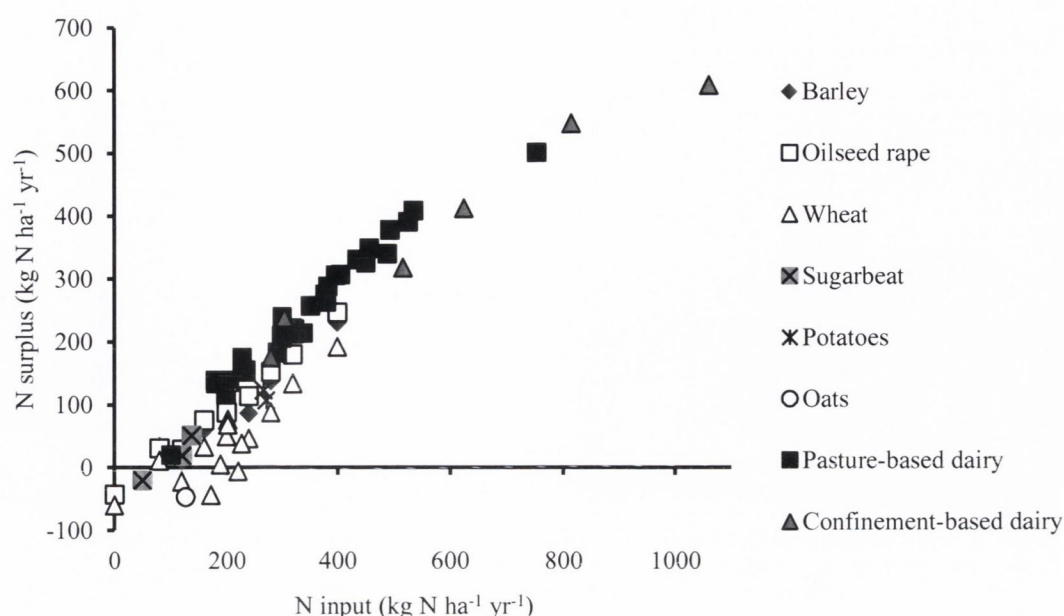


Figure 1.15. Relationship between N surplus and N input on various arable crops and two types of dairy production systems. Source: (Ledgard *et al.*, 1999; Webb *et al.*, 2000; Nielsen and Kristensen, 2005; Nevens *et al.*, 2006; Raison *et al.*, 2006; Sieling and Kage, 2006; Bassanino *et al.*, 2007; Fangueiro *et al.*, 2008; Humphreys *et al.*, 2008b; Ryan *et al.*, 2011; Buckley *et al.*, 2013; Mihailescu *et al.*, 2013).

1.5.2. Nitrogen use efficiency on farms

The N surpluses reported in the literature and in comparison studies such as the ‘Green Dairy’ project illustrate that NUE (i.e. the percentage of total N input that is exported from the farm in agricultural products: $\text{total N exports} \div \text{total N imports} \times 100$) on livestock farms is generally poor. Efficiencies at farm level have been found to range from 5.7 to 68.1% (Buckley *et al.*, 2013) but more commonly range from 15 to 45% (Nielsen and Kristensen, 2005; Nevens *et al.*, 2006; Bassanino *et al.*, 2007; Fangueiro *et al.*, 2008; Treacy *et al.*, 2008).

Low NUE is primarily due to losses of N from the farm system (see section 1.4). The extent of these losses varies during different transformation/movements of N through the farm system. The recovery of N input ($\text{N removed in pasture or crops} \div \text{N input to fields} \times 100$) in the field is relatively efficient in both pasture (63 to 89%; Necpálová *et al.*, 2013b) and arable systems (63 to 81.9%; on average; Webb *et al.*, 2000; Sieling and Kage, 2006). This is the main movement of N in arable systems, in contrast livestock systems require many more movements and transformations of N which ultimately leads to higher N losses and lower NUE.

1.5.3. Accounting for nitrogen surplus

Many studies have completed farm-gate N balances or have measured individual N losses as outlined earlier. However, there is a lack of studies which have combined these measurements in an attempt to account for N surplus. In general, studies attempting to close the N balance have been unable to account for between 45 to 90% of N surplus (Kroeze *et al.*, 2002). Fangueiro *et al.*, (2008) found that NH₃ emissions account for between 36 and 59 % of the N surplus in indoor milk production systems in Portugal. In Australia, Eckard *et al.* (2007) accounted for up to 76% of N surpluses, on average, on non- N fertilised grass/clover based system but only accounted for 51 %, on average, of N surpluses on N fertilised grass/clover based systems. Humphreys *et al.*, (2008a) found that NH₃ volatilisation and NO₃⁻ losses to ground water accounted for only 23 and 4% respectively, of the N surpluses on experimental pasture-based milk production systems in Ireland. On-going research at Solohead Research Farm in Ireland has been investigating this research question for over 10 years (Humphreys *et al.*, 2008a; Li *et al.*, 2011; Yan *et al.*, 2013). Accumulated results indicate that up to 65% of N surplus is still unaccounted for following measurements/estimates of NH₃, N leaching and N₂O emissions (Table 1.4).

Table 1.4. Farm-gate N balance, measured losses of N as nitrous oxide, ammonia volatilization and leached N and resulting unaccounted for N surplus on the Solohead Research Farm.

	Range	Mean	Reference
	kg N ha ⁻¹ yr ⁻¹		
N inputs			
Fertiliser N	80	80	(Humphreys <i>et al.</i> , 2008b)
Feed	24	24	(Humphreys <i>et al.</i> , 2008b)
Atmospheric deposition	10	10	(Humphreys <i>et al.</i> , 2008b)
Biological N fixation	85-160	123	(Phelan <i>et al.</i> , 2014)
Total N imports	199-274	237	
N exports			
Milk sales	61-65	63	(Humphreys <i>et al.</i> , 2008b)
Livestock sales	2	2	(Humphreys <i>et al.</i> , 2008b)
Live-weight change	2	2	(Humphreys <i>et al.</i> , 2008b)
Total N exports	65-69	67	
N surplus	130-209	170	
N losses			
N leaching	18-23	21	(Necpálová <i>et al.</i> , 2012)
Nitrous oxide	6-8	7	(Li <i>et al.</i> , 2011)
Ammonia volatilisation	31-32	32	(Humphreys <i>et al.</i> , 2008b)
Total N losses	55-63	59	
Unaccounted for N		111	
Unaccounted for N (%)		65	

Major uncertainties exist around farm N balance studies and thus the fate of surplus N. Studies have relied heavily on modelling estimates for the calculation of N inputs, such as BNF associated with legumes. Losses such as NH_3 and N_2O have also relied on modelling or default emissions factors (Watson and Atkinson, 1999). There is also a lack of N balance studies which have measured N_2 to account for N surplus.

The next section outlines the legislation and policy concerned with water quality, greenhouse gas emissions and pollutant gases in Ireland all of which are important in the context of N surpluses and losses from farming systems.

1.6. Legislation and policy governing N losses

1.6.1. Losses to water

The EU have introduced directives and legislation to reduce nutrient loss, in particular NO_3^- loss, from agriculture to water bodies in order to reduce water quality in Europe. The two most important of these are the nitrates directive (ND) (91/676/EEC) and the E.U. water framework directive (WFD) (OJEC, 2000). The ND was adopted in 1991 and has the objective of reducing water pollution caused by nitrates from agricultural sources and preventing further such pollution, with the primary emphasis on the management of livestock manures and other fertilisers. The EU Water Framework Directive is a multi-part and multi-phase piece of legislation with the primary aim of achieving at least 'good ecological and chemical' status in all water bodies in Europe by 2015 and maintaining them into the future (OJEC, 2000).

Under the ND each member is required to complete a Nitrates Action Programme (NAP). This NAP is subject to review and amendment, if necessary, every four years. Ireland's first NAP began in 2006 and was reviewed in 2010 and early 2014. A revised version of the ND (S.I. 31 of 2014), known as the European Union's Good Agricultural Practice for the Protection of Waters (GAP) Regulations 2014 gives legal effect to Ireland's third NAP under the ND. Under the adopted GAP regulations farmers are not permitted to apply over $170 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ in the form of organic N which includes livestock deposition and mechanical application. So called 'closed periods' have also been introduced under GAP where both organic and inorganic N and phosphorus (P) application to land are prohibited in winter due to the high risk of losses to water in this period. Closed periods for inorganic fertilisers are from 15th September to 12th January in southern counties and from 15th September to

31st January for northern counties. Closed periods for organic fertilisers is from 15th October to 12th January in southern counties and from 15th October to 31st January for northern counties. In line with these closed periods, minimum slurry storage requirements for the winter period, being 16 weeks in southern counties and 22 weeks in northern counties, are mandatory for livestock farms (Humphreys, 2008).

Ireland has been granted so called 'derogation' under the GAP regulations which allows intensive grassland farmers to apply up to 250 kg organic N ha⁻¹ yr⁻¹ under certain conditions. At present less than 10% of Irish farmers apply for derogation (Teagasc, 2013c). Ireland's original application for derogation in 2005 was based on the assumption that there is generally large denitrification potentials in Irish soils given the moist maritime climate (high net precipitation), the heavy textured nature of the soils and long growing period (Teagasc, 2013c). This will result in the removal of NO₃⁻ from soil thus reducing the risk of losses to groundwater.

The ND requires member states to (i) monitor water quality and identify waters which are polluted, (ii) evaluate the effectiveness of the NAPs' and (iii) report progress to the EU Commission. The agricultural catchments programme (ACP) was, thus, established to monitor NO₃⁻ and P levels in six catchments across Ireland with the ND requirements in mind. The ACP also evaluates the efficacy of Ireland's derogation status under the ND. The most recent report from the ACP in 2012 indicted that NO₃⁻ concentrations in surface water in all six catchments were below the EU maximum acceptable concentration for drinking water of 11.3 mg l⁻¹ NO₃⁻-N (Teagasc, 2012).

1.6.2. Greenhouse gas emissions

Climate change policy in Ireland was developed in the context of national and EU commitment to the 1992 United Nations Framework Convention on Climate Change (UNFCCC). The principal aim of the convention was to stabilise GHG concentration in the atmosphere to prevent interference with the global climate. Ireland has adhered to its first GHG commitment under the adopted Kyoto Protocol, which was to reduce GHG emission to 13% above 1990 levels (i.e. 62.6 Mt CO₂ eq yr⁻¹) over the period 2008-2012 (EPA, 2014b). Further stringent GHG emission reductions have been drawn up under the EC Climate and Energy Package adopted in 2008. This package requires Ireland to reduce GHG in the non- EU Emissions Trading Sectors (ETS: agriculture, transport, residential

and waste sectors) by 20% compared to 2005 levels (i.e. 37.5 Mt CO₂ eq yr⁻¹) over the period 2013 to 2020 (EPA, 2014b).

The agricultural sector in Ireland is the largest contributor to total national GHG emissions (Figure 1.16). Enteric fermentation (45%), manure management (27%), N application to agricultural soils (22%) and fossil fuel burning within agriculture (5%) are the sources of Irelands agricultural GHG emissions. Nitrous oxide currently accounts for 38% of agricultural GHG emissions (EPA, 2013). Pasture-based livestock production is the main contributor to this, as 90% of Irelands agricultural area is under permanent pasture.

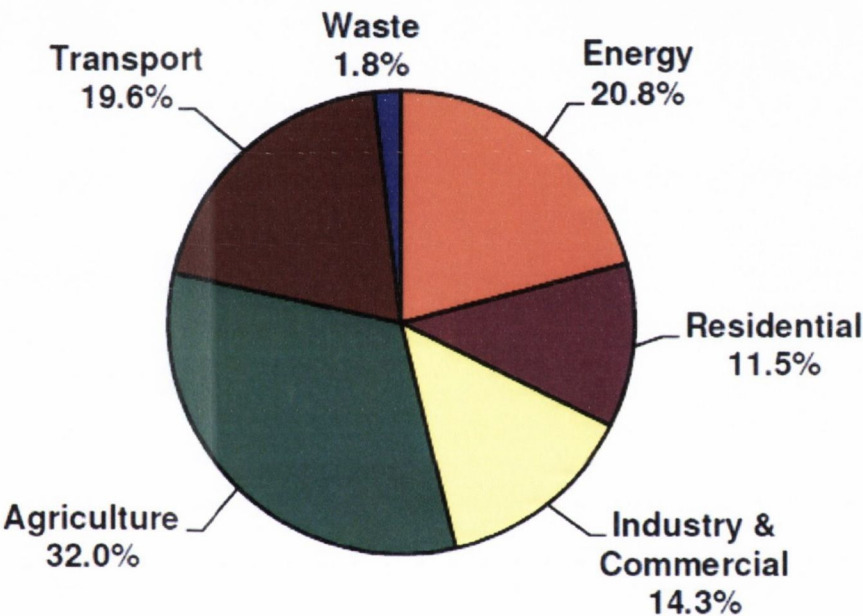


Figure 1.16. Irelands national greenhouse gas emissions by sector in 2011 (EPA, 2013).

1.6.3. Ammonia volatilisation

The convention on long range trans-boundary air pollution (CLRTAP) was established in 1979 following the recognition at international level that co-operation was required to reduce the emissions of pollutant gases which contribute to acidification of, and eutrophication in natural ecosystem, and ground-level ozone pollution. The CLRTAP works under a number of protocols. Following the UN Gothenburg protocol of 1999, the European Communities NECD 2004 set a limit on annual national emissions of sulphur dioxide, NO_x, volatile organic compounds and NH₃ for each EU country (EPA, 2014a). Of these pollutants, NH₃ is the greatest concern to the Irish agricultural sector. Agriculture accounts for virtually all (>98%) NH₃ emissions in Ireland. Management of animal manures and excretion at pasture produced 92.8% of NH₃ emissions in agriculture and chemical fertilisers accounted for the remaining 7.2% in 2012 (EPA, 2014a). Under the

NECD targets Ireland was required to reduce emissions to $\leq 116 \text{ kt NH}_3 \text{ yr}^{-1}$ by 2010. National emissions were under this limit in 2010 (106.2 kt), mostly due to a reduction in livestock numbers and N fertiliser use in this period, and currently stand at 104.62 kt. However, a lower target of 108.9 kt yr^{-1} has been set for 2020 following a review by the NECD.

1.6.4. Future projections and implications

The recently developed Food Harvest 2020 (DAFF, 2011) is an industry led strategy, supported by the government, for green growth in the Irish agri-food sector up to 2020. Ambitious targets have been set for each commodity mostly based on value with the exception of the dairy industry, where a 50% increase in volume is envisaged by 2020 following the abolition of EU milk quotas in 2015. Total bovine numbers and N fertiliser use are projected to increase by 1% and 11%, respectively by 2020 under Food Harvest 2020 targets (Teagasc, 2013c). Agricultural GHG emissions (12%) and NH_3 emissions are projected to increase in this period and contribute to a breach in national non-ETS GHG emission and national NH_3 emission limits, respectively (Figure 1.17) (EPA, 2014a, b).

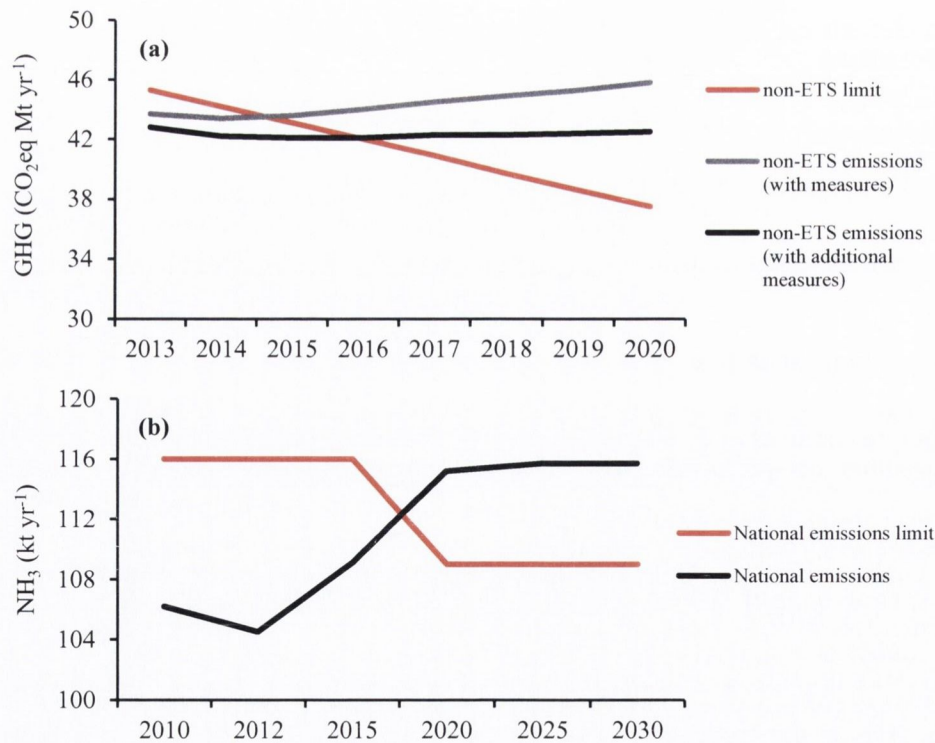


Figure 1.17. Projected non-ETS emissions with measures (existing policy and measures) and with additional measures (existing policy and measures with planned additional policy and measures) in comparison with linear reduction pathway required between 2013 and 2020 in non-ETS emissions under the EU Climate and Energy Change Package (a) and projected national NH_3 emissions in comparison with the NECD national NH_3 emission limits (b). Source: (EPA, 2014a, b).

It is possible that further strict regulations could be put in place to reduce GHG emissions and N losses from agriculture if national limits are not adhered to. This will have the effect of reducing productivity or at least increasing production costs on Irish farms in some circumstances (Dillon and Delaby, 2009). Therefore, identification and evaluation of cost effective mitigation strategies will have to be a key focus for Irish agricultural research. The determination of the most prominent N loss pathways from farms will be important part of this.

1.7. Gap of knowledge, objectives and hypotheses

Many studies have completed farm-gate N balances or have measured individual N losses as outlined earlier. However, there is a lack of studies which have combined these measurements in an attempt to account for farm surplus N. Major uncertainties, therefore, exist around the fate of surplus N. Previous studies have relied heavily on modelling estimates for the calculation of N inputs, such as BNF associated with legumes. There is also a lack of N balance studies which have measured gases emissions such as N₂O and N₂, in particular, to account for farm surplus N. Previous studies at Solohead Research farm have been unable to account for up to 65% of farm surplus N and suggest that surplus N may be lost as N₂, however, this remains to be proven.

Biological N₂ fixation has been quantified on a range of agricultural systems outside of Ireland, however, there is a lack of information on the quantity of N₂ fixed on perennial ryegrass/white clover based pastures in Ireland. Techniques used to measure BNF have previously been compared. Few studies, however, have compared three techniques over a long-term period (2 years) and under varying fertiliser N inputs in order to have a robust comparison and assessment of these techniques.

While N₂O emissions have been quantified from pasture-based systems on many occasions there is a lack of long term (>2 years) N₂O studies internationally, that have investigated the factors that drive inter-annual variation in emissions.

This study was conducted on a white clover-based system of dairy production at Solohead Research farm under moist maritime climatic conditions on a clay loam soil with impeded drainage. Detailed N flow measurements were conducted over two years, which included BNF (using three techniques: nitrogen difference, ¹⁵N natural abundance and ¹⁵N isotope dilution), N₂O emissions (over four years), N₂ emissions and N losses to groundwater. The

overall objectives of this thesis were to (i) account for all N inputs into, and outputs from, a pasture-based dairy production system and (ii) determine the relative importance of individual N losses to the fate of farm surplus N. The hypothesis of this thesis is that measurement of nitrous oxide, dinitrogen and N losses to groundwater and estimates of ammonia volatilisation will account for farm surplus N on a white clover based system of dairy production in Ireland based on a clay loam soil under moist maritime climatic conditions.

Specific hypotheses within individual experimental chapters of this thesis include:

- (i) Techniques used to quantify BNF (^{15}N isotope dilution, ^{15}N natural abundance and nitrogen difference) differ in their estimates of BNF
- (ii) Addition of fertiliser N, particularly high ($280 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) fertiliser N regimes, create discrepancies between the ^{15}N isotope dilution and ^{15}N natural abundance techniques used to estimate BNF
- (iii) Increasing fertiliser N input on grass/clover pastures reduces BNF and increases potential of N losses on such pastures
- (iv) Nitrous oxide emissions from a N fertilised and grazed grass/clover pasture vary inter-annually due to changes in weather conditions over time
- (v) Environmentally benign N_2 gas accounts for between 40 to 65% of the previously unaccounted for farm surplus N on a dairy production system in Ireland under moist maritime climatic conditions on a clay loam soil with impeded drainage

The next three chapters are experimental chapters, which addresses the objectives and specific hypothesis of the thesis outlined in this section.

CHAPTER 2. COMPARISONS OF BIOLOGICAL NITROGEN FIXATION IN ASSOCIATION WITH WHITE CLOVER (*TRIFOLIUM REPENS* L.) UNDER FOUR FERTILISER NITROGEN INPUTS AS MEASURED USING TWO ¹⁵N TECHNIQUES

Based on: Burchill, W., James, E., Lanigan, G., Li, D., Williams, M., Iannetta, P. and Humphreys, J. 2014. Comparisons of biological N fixation of white clover under four fertiliser nitrogen inputs measured using two ¹⁵N techniques. Plant and Soil 385, 287-302 DOI 10.1007/s11104-014-2199

2.1. Abstract

The objectives of this study were to compare techniques for measuring biological nitrogen fixation (BNF) and to assess how fertiliser nitrogen (N) input affects the balance between BNF and sustainable herbage production on perennial ryegrass (*Lolium perenne* L.)/white clover (*Trifolium repens* L.; grass/clover) grassland. Biological N₂ fixation and herbage production by white clover based grassland was measured in 2011 and 2012 under four nominal annual fertiliser N inputs: 0 (0N), 86 (86N), 140 (140N) and 280 kg ha⁻¹ (280N). Biological N₂ fixation was measured using the ¹⁵N isotope dilution and ¹⁵N natural abundance techniques under all fertiliser N inputs and also using the nitrogen difference technique under 0N. The two ¹⁵N techniques produced similar annual estimates of above-ground BNF across the range of fertiliser N inputs. Fertiliser N input resulted in higher herbage dry matter yield, but reduced annual BNF which averaged 80, 64, 66 and 47 kg ha⁻¹ on 0N, 86N, 140N and 280N, respectively, across both techniques and years. The two ¹⁵N techniques were comparable in estimating BNF. Intermediate fertiliser N inputs achieved a balance between minimising detrimental impact on BNF and potential N loss while producing more herbage DM yield than clover pastures receiving no fertiliser N.

2.2. Introduction

White clover (*Trifolium repens* L.; clover) forms a symbiotic relationship with soil bacteria, collectively termed rhizobia. White clover has a particular affinity for *Rhizobium leguminosarum* bv. *trifolii*, and their nitrogen (N)-fixing symbiosis can produce over 200 kg ha⁻¹ yr⁻¹ of plant available N (Carlsson and Huss-Danell 2003; Ledgard 2001). This has the potential to reduce the need for synthetic fertiliser N and, therefore, lessen both the environmental impact and production costs associated with pasture based livestock production (Ledgard *et al.*, 2009; Li *et al.*, 2011; Humphreys *et al.*, 2012; Yan *et al.*, 2013).

Accurate and reliable techniques for quantifying biological N₂ fixation (BNF) are essential to assess the effects of management and climate. The nitrogen difference (NDiff), ¹⁵N natural abundance (NA) and ¹⁵N isotope dilution (ID) techniques have been widely used to measure BNF across a range of legumes and have been found to differ in their estimates (Evans *et al.*, 1987; Cadisch *et al.*, 1989; Hogh-Jensen and Schjoerring, 1994; Carranca *et al.*, 1999; Unkovich and Pate, 2000; Carlsson and Huss-Danell, 2003; Menneer *et al.*, 2004). Pairwise comparisons of these techniques have been made in many studies, but all

three techniques have only been assessed in a single study (Carranca *et al.*, 1999). Many of the available comparisons of the techniques are also based over a relatively short time period (≤ 1 year) and over a limited number of harvests/sampling occasions. There is also a lack of studies comparing these techniques across a range of fertiliser N inputs.

Previous studies have found that the addition of fertiliser N reduces BNF (Ledgard *et al.*, 2001; Trott *et al.*, 2004) and increases environmental N losses in grass/clover pastures (Bouman *et al.*, 2010; Li *et al.*, 2011). Nonetheless, the use of fertiliser N on such pastures is a very cost effective way of increasing herbage production from an economic perspective. There is, however, uncertainty as to how fertiliser N input affects the balance between BNF and sustainable herbage production in terms of economical production and minimising potential N losses. The objectives of this study were to (i) compare techniques of measuring BNF in white clover based grassland across a range of fertiliser N inputs over two years and (ii) assess how fertiliser N affects the balance between BNF and sustainable herbage production on perennial ryegrass (*Lolium perenne* L.)/white clover pastures. This study hypothesises that; (i) techniques used to quantify BNF (^{15}N isotope dilution, ^{15}N natural abundance and nitrogen difference) differ in their estimates of BNF, (ii) addition of fertiliser N input, particularly high ($280 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) fertiliser N regimes, create discrepancies between the ^{15}N isotope dilution and ^{15}N natural abundance techniques used to estimate BNF, and (iii) increasing fertiliser N input on grass/clover pastures reduces BNF and increases potential of N losses on such pastures.

2.3. Materials and techniques

2.3.1. Experimental site

This study was carried out at the Teagasc Solohead Research Farm, Tipperary, Ireland ($52^{\circ}51'\text{N}$, $08^{\circ}21'\text{W}$; 95 m asl) (Figure 2.1). The predominant soils are poorly drained Gleys (90%) and Grey Brown Podzolics (10%) with a clay loam texture (29% clay, 36% silt and 34% sand) and low permeability. The soils are seasonally wet, waterlogged or flooded due to impeded drainage and a shallow water table depth (0 to 2.2 m below ground level) at the site. Mean soil organic carbon, total N content, bulk density and pH at 0 to 10 cm depth were 5.12%, 0.54%, 0.87 g cm^{-3} and 6.2, respectively.



Figure 2.1. Selection of the permanent pastures at the Solohead Research Farm used in this study.

The site (52 ha) has been under permanent grassland (predominantly perennial ryegrass) for at least 60 years, with the pastures ranging in age from 20 to 30 years. White clover was established and maintained in these pastures since 2000 by surface broadcasting 5 kg ha⁻¹ of clover seed onto silage stubble in each individual paddock at intervals of approximately five years. The mixture of clover cultivars used between 2004 and the present experiment were Crusader, Chieftain and Barblanca, which are classified as medium, medium and large leaf sized cultivars, respectively. The pastures received annual fertiliser N inputs of between 0 and 90 kg N ha⁻¹ and were grazed by Holstein-Friesian dairy cows and occasionally cut for silage for up to 10 years prior to the commencement of the current study.

Mean annual rainfall at the site (1997-2010) was 1036.3 mm. Mean monthly soil temperature (1997-2010) in the top 10 cm ranged between 2.5 and 11°C during winter (November-January) to 10.4 and 18.2°C in summer (May-July).

2.3.2. Experiment layout and design

The experiment was laid down in a randomised design with three replicates. There were four nominal fertiliser N treatments: 0 (0N), 86 (86N), 140 (140N) and 280 (280N) kg N ha⁻¹ yr⁻¹. Two ¹⁵N techniques, NA and ID were used to determine the proportion of N derived from the atmosphere (%Nd_{fa}) as subplot treatments within each fertiliser N treatment. The NDiff technique was used to determine BNF under 0N.

Fenced plots were established in October 2010 in paddocks across Solohead Research Farm receiving the four nominal fertiliser N inputs. These plots were not grazed during the study. Each plot was 14 m × 2 m except for that receiving 0N which was 14 m × 4.5 m. Each plot contained a cutting plot (10×1.2 m) for herbage yield determination and two micro-plots (1 m²), where %Nd_{fa} was measured using the two ¹⁵N techniques.

A grass monoculture plot (0N_G) (10×1.2 m) was laid down in each replicate of 0N for the determination of BNF using the ND_{diff} technique. White clover was eliminated from these plots using a herbicide (Duplosan®, Nufarm, Wyke, Yorkshire, UK. Active ingredient = mecoprop-P (48% w/w), application rate = 2 L ha⁻¹).

All plots were cut to 4 cm above ground level following the initial laying down of the experiment in October 2010 and the herbage was removed and discarded. Fertiliser N was applied in split applications in spring (February to May) on the 86N and 140N plots and throughout the main growing season (February to September) on the 280N plots (Table 2.1).

Table 2.1. Date, type and rate of fertiliser N application on treatments receiving nominal annual fertiliser N inputs of 86 (86N), 140 (140N) and 280 (280N) kg ha⁻¹ in 2011 and 2012

Date	Fertiliser N type	Total N (kg ha ⁻¹)		
		86N	140N	280N
2011				
24 th February	Urea	28.8	28.8	28.8
22 nd March	Urea	28.8	28.8	28.8
20 th April	Urea	28.8	84	84
14 th June	CAN ^a			76
25 th July	CAN			34
5 th September	CAN			34
Annual		86	141	286
2012				
15 th February	Urea	28.8	28.8	28.8
13 th March	Urea	28.8		
3 rd April	Urea		95	86
2 nd May	Urea	28.8		
13 th June	CAN			102
24 th July	CAN			34
4 th September	Urea			28.8
Annual		86	124	280

^aCalcium ammonium nitrate

2.3.3. Application of ^{15}N fertiliser

The soil ^{15}N content of the ID micro-plots was enriched above natural abundance levels by applying a solution (two litres) of ^{15}N -enriched (98 atom% ^{15}N) ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$ at a rate of 0.5 kg N ha^{-1} using a spray bottle. The ^{15}N fertiliser was applied to the 0N, 86N and 140N ID micro-plots on the 16th March 2011 and to the 280N plots on the 27th April 2011. Any remaining ^{15}N fertiliser on the herbage was washed into the soil with two litres of deionised water using a watering can with a rosette attachment.

2.3.4. Sampling and analysis

Daily weather data including rainfall (mm), air temperature ($^{\circ}\text{C}$) and soil temperature at 10 cm depth ($^{\circ}\text{C}$) were recorded at the experimental site as described by Fitzgerald and Fitzgerald (2004). Volumetric soil water content (VWC) was recorded at 0-5 cm depth using a HH2 moisture meter in conjunction with a Theta Probe sensor (Delta-T Devices, Burwell, Cambridge, England), at weekly intervals at the site.

Plots were harvested on six occasions in 2011 (27th April, 7th June, 12th July, 9th August, 6th September, 10th October) and on seven occasions in 2012 (14th February, 9th April, 21st May, 22nd June, 23rd July, 4th September, 9th October). At each harvest, plots were harvested to 4 cm above ground level using a mower (Etesia UK Ltd., Warwick, UK). The fresh herbage was weighed in the field using electric weighing scales. A 100 g sub-sample of the fresh herbage was dried for 24 h in an oven with forced-air circulation at 100°C to determine DM content. A second sub-sample of the herbage from the grass monoculture plots with 0N was taken and stored at -20°C before being freeze dried and milled through a 0.2 mm sieve prior to %N analysis. The N concentration in the herbage DM was determined using a Leco N analyser (LECO FP-2000; Leco Corporation, St. Joseph, MI, USA) at the laboratories of the Animal and Grassland Research and Innovation Centre, Teagasc Moorepark, Ireland.

On the same day, a 50 cm^2 area in the centre of each NA and ID micro-plot was cut to 4 cm above ground level using a hand shears (Figure 2.2). The herbage was separated into grass/weeds and clover, dried at 65°C for 48 hours, milled through a 0.2 mm sieve and stored in sealed plastic containers until analysed. Sub-samples of the dried plant material were analysed for their atom% ^{15}N content and %N content using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer

(Sercon Ltd., Cheshire, UK) at the Stable Isotope Facility of the University of California, Davis, USA.



Figure 2.2. Harvesting of white clover and perennial ryegrass samples for atom% ^{15}N determination from one of the ^{15}N isotope dilution micro-plots during the study.

Ten soil cores (0-10 cm depth) were taken the day after plant sampling from the ID and NA micro-plots and bulked. The field moist soil was sieved through a 2 mm sieve to remove any stones or roots. The soil DM was determined by drying 50 g of soil in an oven with forced air circulation at 100°C for 24 hours. Another 50 g of soil was shaken continuously for 2 hours in a solution of 200 ml of 2M KCL to extract inorganic N. Following filtration (Whatman no.2 filter paper, d=0.125 m, 0.45 μm) the solution was stored at -20°C until analysed. The extractions were analysed for ammonium N ($\text{NH}_4^+\text{-N}$) and total oxidised N (TON) on a Thermo Konelab analyser (Technical Lab Services, Ajax, Ontario, Canada) at the water laboratory of Teagasc, Johnstown Castle Environmental Research Centre, Wexford, Ireland.

The clover content (%) of each plot was determined in April, June, August and October each year. Twenty five random subsamples (100 $\times$ 150 mm) were cut at a height of 4 cm above ground level using a hand shears in each plot. All sub-samples at each sampling date were bulked for each plot. The clover in each bulked sample was separated from the remaining herbage. A 100 g sub-sample of each of the clover and the remaining herbage was dried at 100°C for 24 hours and weighed to determine clover content of herbage DM.

2.3.5. Calculations

The %Ndfa in clover for both ^{15}N techniques was calculated according to Unkovich *et al.* (2008) see <http://www.aciar.gov.au/publication/MN236>. Reference plant (perennial ryegrass and dicot weeds) and clover shoot ^{15}N contents were expressed in delta units ($\delta^{15}\text{N}$), which is a measure of the ^{15}N content of the sample in parts per thousand (‰) relative to atmospheric N_2 : (sample atom% ^{15}N minus 0.3663) / (0.3663) $\times$ 1000. These “delta units” were then used to calculate %Ndfa using equation 2.1:

$$\text{Ndfa (\%)} = \frac{\delta^{15}\text{N reference plant} - \delta^{15}\text{N clover}}{\delta^{15}\text{N reference plant} - B_s} \quad [2.1]$$

where $\delta^{15}\text{N}$ are of the shoots of the non-N-fixing reference plant and clover and B_s is the $\delta^{15}\text{N}$ of clover shoots fully dependant on N_2 fixation for their plant N (see section 2.3.6).

For the ID technique, reference plant and clover shoot ^{15}N contents were expressed as atom% ^{15}N excess, which is a measure of the ^{15}N content of the sample minus the ^{15}N content of atmospheric N_2 (i.e. sample atom% ^{15}N minus 0.3663). Equation 2.2 was then used to calculate %Ndfa for the ID technique:

$$\text{Ndfa (\%)} = \left(1 - \frac{\text{atom \% } ^{15}\text{N excess reference plant}}{\text{atom \% } ^{15}\text{N excess clover}} \right) \times 100 \quad [2.2]$$

Perennial ryegrass was the dominant species in the pasture. Other dicot species, such as *Taraxacum officinale* (dandelion), *Ranunculus repens* L. (creeping buttercup), *Bellis perennis* (daisy) and *Rumex obtusifolius* L. (broad-leaf dock) were occasionally present in small quantities on some of the plots. Separation and ^{15}N analysis of grass only and a combination of these dicot species from the June 2012 harvest revealed no difference in $\delta^{15}\text{N}$ values and calculated %Ndfa under each fertiliser N treatment between the two reference plant materials (Table 2.2). Therefore, a combination of the non-legume species was used as reference plant material for both techniques.

Table 2.2. Shoot $\delta^{15}\text{N}$ values of perennial ryegrass and a combination of weed species in the June 2012 harvest and their effect on the proportion of N derived from the atmosphere (%Ndfa) in white clover when used as reference plants using the ^{15}N natural abundance technique in treatments receiving annual fertiliser N inputs of 0 (0N), 86 (86N), 140 (140N) and 280 (280N) kg ha⁻¹.

	Reference plant material		S.E.M. ^b		
	Grass	Weeds ^a	Species	Fertiliser N	Species × Fertiliser N
	Shoot $\delta^{15}\text{N}$ (‰)				
0N	4.9	4.8			
86N	4.7	5.0	0.24 ^{NS}	0.33*	0.46 ^{NS}
140N	4.7	4.1			
280N	2.9	3.3			
	%Ndfa				
0N	98.8	99.2			
86N	87.6	87.1	1.82 ^{NS}	2.49***	3.52 ^{NS}
140N	83.5	84.1			
280N	76.5	78.6			

^a Combination of dicot species, such as *Taraxacum officinale* (dandelion), *Ranunculus repens* (creeping buttercup), *Bellis perennis* (daisy) and *Rumex obtusifolius* (broad-leaf dock)
^b standard error of the mean
 P* < 0.05; * *P* < 0.001; ^{NS} *P* > 0.05

The amount of above-ground N₂ fixation (kg ha⁻¹) in the clover was calculated as the %Ndfa, determined by both the ID and NA techniques, multiplied by the clover N yield. Estimates of %Ndfa from the 140N plots were used to calculate BNF for the first two harvests of 2011 on the 280N plots; these treatments received the same fertiliser N management during this period (Table 2.1). The difference in above-ground herbage N yield between the grass/clover mixture and the grass monoculture in the 0N plots was attributed to above-ground N₂ fixation for the NDiff technique. All techniques provided estimates of BNF partitioned to the above-ground biomass in this study, and that allocated to below ground biomass or transferred to companion grasses was not assessed (Ledgard, 1991; Pirhofer-Walzl *et al.*, 2012; Lesuffleur *et al.*, 2013; Carlsson and Huss-Danell, 2014).

2.3.6. B_s-value determination

As only shoot material was used in the NA technique, a shoot-derived B_s-value was required in order to account for any fractionation of ^{14}N and ^{15}N that may occur during the N fixation process and subsequently during translocation between the nodulated roots and shoots within the legume (Unkovich *et al.*, 2008). B_s-values were thus determined, at the James Hutton Institute Dundee, for the three clover cultivars (Crusader, Chieftain and Barblanca) that were previously surface broadcast at the experimental site, and the mean

B_s -value of the three cultivars was used in the calculation of %Ndfa using the NA technique in this study. Surface-sterilised seeds of each cultivar were grown in a controlled greenhouse environment in plastic pots and were fed a N-free nutrient solution (James *et al.*, 1991) (Figure 2.3). The pots were filled with a mixture (1:1) of sand and perlite and there were nine replicate plots *per* cultivar in a randomised block design. One day after sowing (31th August 2012), the seeds were inoculated with *R. leguminosarum* strains originally isolated and identified according to the technique of Bontemps *et al.* (2010) from clover (cv. Chieftain) root nodules formed in a sterile medium (as outlined above) in response to inoculation with a crushed nodule suspension from clover plants taken from the experimental site. There were four plants per pot following germination, establishment and thinning. Herbage was harvested on the 14th November 2012, allowed to regenerate, and then harvested again on the 14th January 2013. Plants were >9 cm in height prior to each harvest. Nodulation was confirmed by visual inspection following the final harvest. The shoot $\delta^{15}\text{N}$ of the white clover plants was determined as described above.



Figure 2.3. Selection of the white clover cultivars grown in a controlled greenhouse environment in plastic pots for the determination of shoot derived B_s -values for each cultivar

2.3.7. Statistical analysis

All data were statically analysed using SAS 9.3 (SAS 2011, Institute Inc., Cary, NC, USA). Data were checked for normality by using residual normality and variance analysis. All variables (DM yield, N yield, soil N content, clover content, shoot $\delta^{15}\text{N}$, shoot ^{15}N excess, %Ndfa, B_s -values and BNF) were analysed using ANOVA. Fixed effects included

fertiliser N treatment, technique, species, variety, year and harvest, where appropriate, with year or harvest included as repeated measures in the model. All possible interactions between fixed effects were tested in each ANOVA. Post hoc Holm-Tukey and Tukey-Kramer multiple comparisons tests were carried out to determine differences between means. Soil NH_4^+ and soil NO_3^- content required a natural log transformation. The relationships between BNF with annual herbage and soil N variables were tested using Pearson correlation analysis (using replicate values; $n=24$). A statistical probability of $P<0.05$ was considered significant for all statistical tests.

2.4. Results

2.4.1. Weather and soil conditions

There was a large contrast between 2011 and 2012 in terms of seasonal precipitation, air temperatures and soil moisture conditions (Figure 2.4). Rainfall was relatively low during the growing season of 2011.

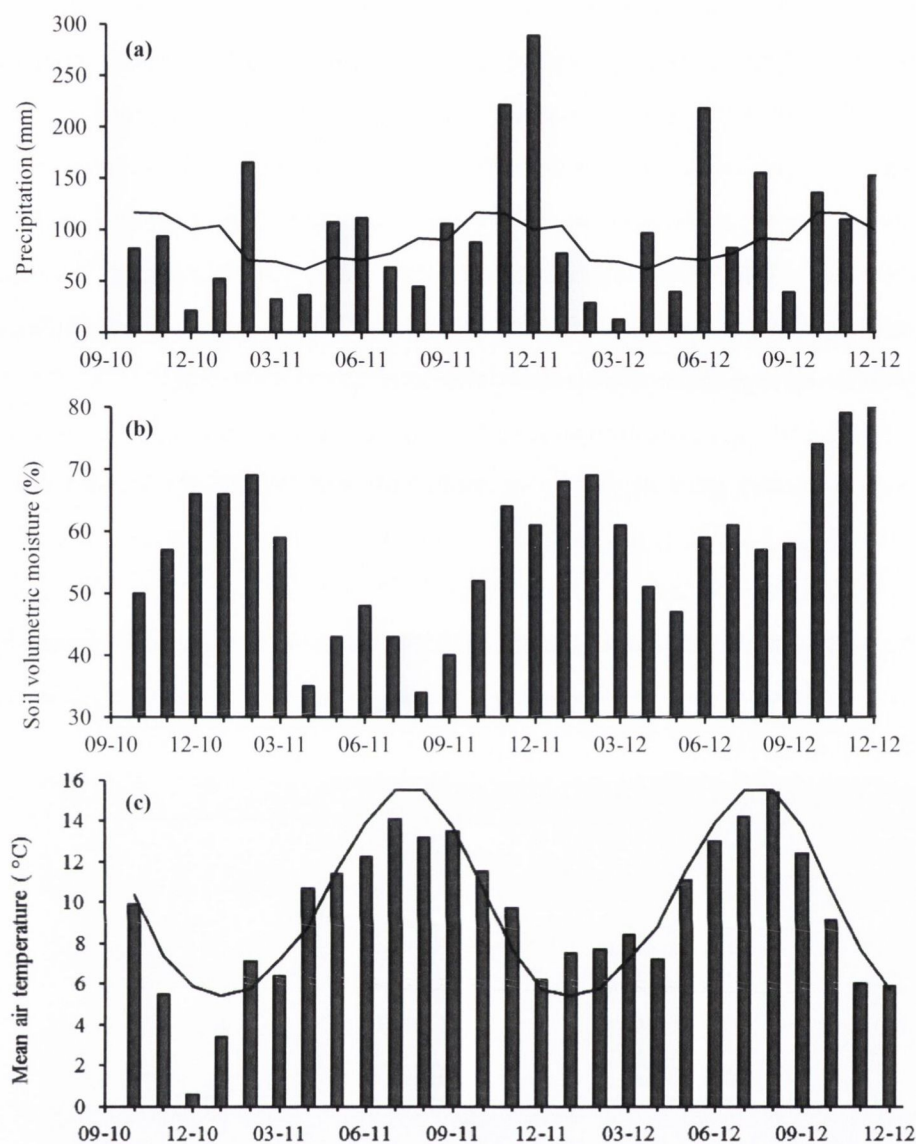


Figure 2.4. Monthly cumulative precipitation (a), mean monthly soil volumetric moisture at 5 cm depth (b) and mean monthly air temperature (c) at Solohead Research Farm during the experimental period (mm-yy). Solid lines indicate long-term mean monthly precipitation and air temperature at the site.

The winter of 2011/2012 had very high rainfall particularly in November and December 2011 (Figure 2.4a). There was also above average rainfall in April, June and August 2012 leading to saturated soil conditions for much of the growing season of 2012 (Figure 2.4b). Monthly mean air temperatures were well below average over the winter of 2010/2011 and remained low until April 2011 (Figure 2.4c). In contrast, air temperatures were above average for much of the winter of 2011/2012 and early spring of 2012.

2.4.2. Herbage dry matter yield and N yield

Grass DM yield generally increased ($P<0.01$) with fertiliser N input and was similar between years (Table 2.3). White clover DM yield was not significantly affected by fertiliser N treatment but was higher ($P<0.001$) in 2011 than 2012 under all fertiliser N treatments (Table 2.3). Total herbage yield generally increased ($P<0.001$) with increasing fertiliser N input. Total herbage yields were significantly higher in 2011 than 2012 (Table 2.3). The grass, clover and herbage N yields had similar fixed and interaction effects as the herbage DM yields (Table 2.3).

Table 2.3. Annual grass, clover and total herbage dry matter (DM) and N yields in treatments receiving annual fertiliser N inputs of 0 (0N), 86 (86N), 140 (140N) and 280 (280N) kg ha⁻¹ in 2011 and 2012.

	2011					2012					S.E.M ^a		
	0N _G	0N	86N	140N	280N	0N _G	0N	86N	140N	280N	Fertiliser N	Year	Fertiliser N × Year
Herbage yields (t DM ha ⁻¹)													
Grass	9.8	8.0	9.2	11.1	11.6	7.2	8.4	10.8	10.0	11.9	0.46**	0.27 ^{NS}	0.61*
Clover	-	3.5	3.2	3.6	4.2	-	1.4	1.5	1.1	0.9	0.21 ^{NS}	0.13***	0.25*
Total	9.8	11.5	12.5	14.7	15.8	7.2	9.8	12.3	11.2	12.8	0.44***	0.25***	0.55*
Botanical composition (%)													
Clover	-	31	26	25	26	-	15	12	10	7	1.56 ^{NS}	1.02***	2.04 ^{NS}
Nitrogen yields (kg ha ⁻¹)													
Grass	265	203	282	321	351	197	199	297	269	331	14.7***	8.8 ^{NS}	19.7*
Clover	-	135	121	135	153	-	52	53	41	30	8.3 ^{NS}	4.9***	9.8*
Total	265	338	403	456	504	197	251	351	310	360	15.5***	8.5***	19.1*

_G Grass monoculture plot

^astandard error of the mean. Differences between means in the table can be deciphered using the presented S.E.M's.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ^{NS} $P > 0.05$

2.4.3. Natural abundance technique: Shoot $\delta^{15}\text{N}$

Grass shoot $\delta^{15}\text{N}$ ranged from +1.6‰ to +8.7‰ and were affected by an interaction ($P<0.001$) between fertiliser N treatment and harvest date (Figure 2.5). Grass shoot $\delta^{15}\text{N}$ was always higher than that of clover shoots under all fertiliser N treatments apart from the first three harvests under 280N in 2011 (Figure 2.5).

Clover shoot $\delta^{15}\text{N}$ values (-1.79‰ to +4.24‰) were negative or similar to the $\delta^{15}\text{N}$ of atmospheric N_2 (0‰) for much of the study, indicating a high reliance on biologically fixed N. Clover shoot $\delta^{15}\text{N}$ values were significantly affected by fertiliser N treatment ($P<0.01$), harvest date ($P<0.001$) and an interaction ($P<0.001$) between fertiliser N and harvest date (Figure 2.5).

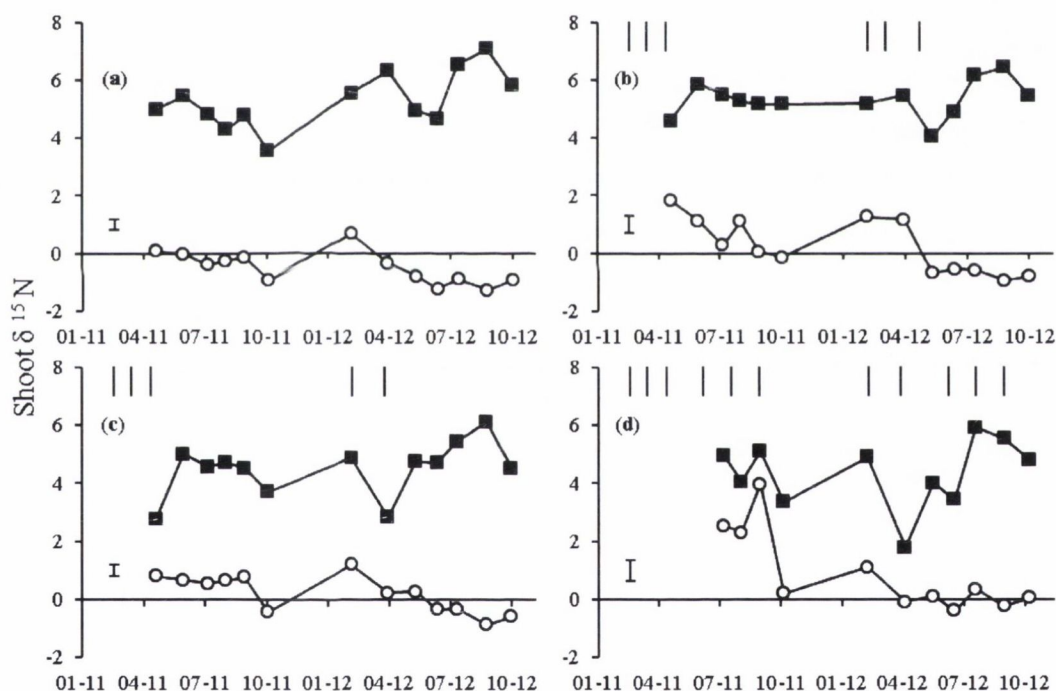


Figure 2.5. Shoot $\delta^{15}\text{N}$ of perennial ryegrass (black box) and white clover (white circle) at each harvest date (mm-yy), on treatments receiving annual fertiliser N inputs of (a) 0, (b) 86, (c) 140 and (d) 280 kg ha^{-1} . Vertical lines indicate dates of fertiliser N application. Error bars represent the interaction S.E.M. of species $\times$ harvest date.

2.4.4. Isotope dilution technique: Shoot atom% ^{15}N excess

The ^{15}N atom% excess of grass ranged from +0.0065 to +0.136 and declined with time ($P<0.001$) following ^{15}N fertiliser application and reached initial stabilisation in August 2011, where it remained relatively stable until February 2012 (Figure 2.6a).

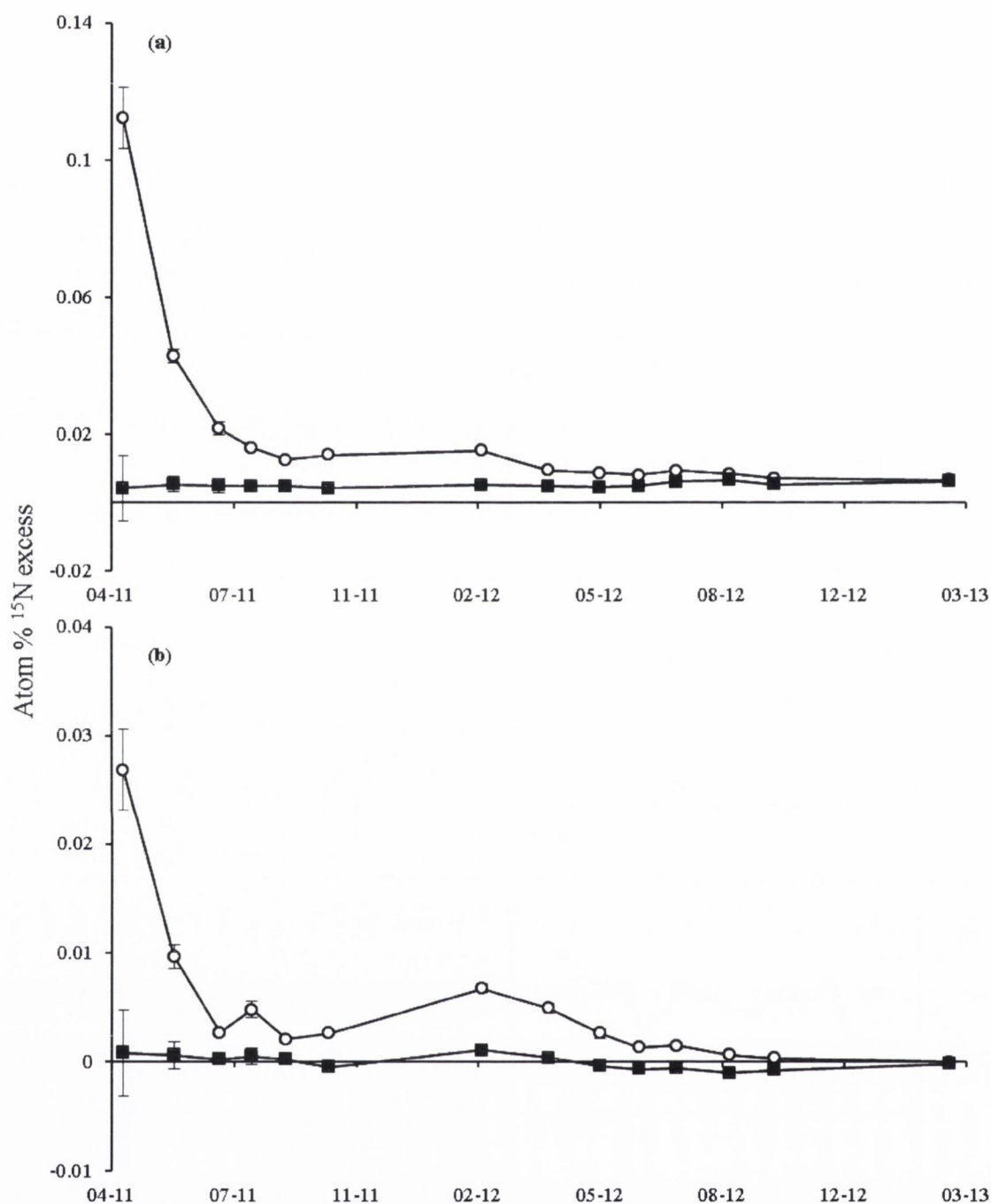


Figure 2.6. Shoot ^{15}N excess of perennial ryegrass (a) and white clover (b) in ^{15}N isotope dilution (white circle) and ^{15}N natural abundance (black box) plots on each harvest date (mm-yy). Data are the mean of plots receiving annual fertiliser N inputs of 0, 86 and 140 kg ha $^{-1}$. The mean of these treatments is illustrated as the isotope dilution plots received ^{15}N -enriched (98 atom% ^{15}N) ammonium sulphate at a rate of 0.5 kg N ha $^{-1}$, on the same date (16th March 2011). Error bars represents the interaction S.E.M of technique $\times$ harvest date. Data points are larger than error bars on some occasions.

Thereafter, it declined slowly to a minimum of +0.0065 in the March 2013, which was similar to natural abundance levels (+0.0062). The atom% ^{15}N excess of clover ranged

from -0.0002 to +0.084 and was much lower than that of grass throughout the experiment (Figure 2.6b). Clover atom% ^{15}N excess declined with time ($P<0.001$) following ^{15}N -labelled fertiliser application to a minimum of -0.0002 in the final harvest in March 2013, which was similar to natural abundance levels (-0.0002). The atom% ^{15}N excess of both the grass and clover under 280N following the later ^{15}N -labelled fertiliser application had a similar trend to 0N, 86N and 140N, reaching initial stabilization in September 2011 and declined to natural abundance levels in March 2013 (data not shown).

2.4.5. Soil N concentrations

Soil NH_4^+ ranged from 1.7 to 32.4 kg N ha $^{-1}$ and was affected by an interaction ($P<0.001$) between fertiliser N treatment and harvest date. There were peaks in soil NH_4^+ following fertiliser N applications under 140N in April 2011 and under 280N in April 2012 (Figure 2.7a).

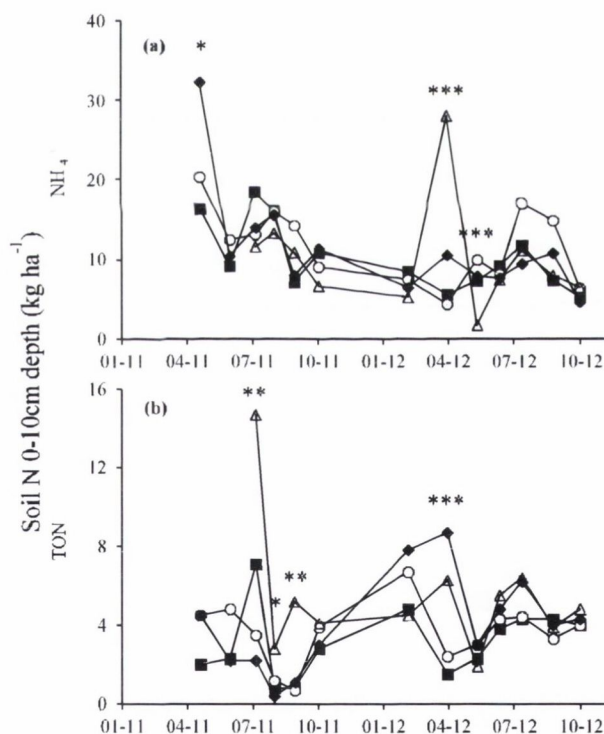


Figure 2.7. Soil ammonium (NH_4^+) (a) and total oxidised nitrogen (TON) (b) on each harvest date (mm-yy) in treatments receiving annual fertiliser N input of 0 (black box), 86 (white circle), 140 (black diamond) and 280 (white triangle) kg ha $^{-1}$. *, **, *** indicate harvest dates where fertiliser N treatment was significant at $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

Soil TON ranged between 0.4 and 14.7 kg N ha $^{-1}$ on individual harvest dates and was affected by an interaction ($P<0.01$) between fertiliser N treatment and harvest date. Soil TON was higher under 280N than all other fertiliser N treatments on most harvests in 2011

and was also higher under 140N and 280N than 0N and 86N in the April 2012 harvest (Figure 2.7b).

2.4.6. Proportion of N derived from the atmosphere (%Ndfa) in white clover

The %Ndfa ranged from 22% to 99%. The highest estimates occurred under 0N while the lowest estimates were recorded following fertiliser N applications under 140N and 280N (Figure 2.8). Estimates of %Ndfa between the two techniques were similar at most harvests, but there were discrepancies between the two techniques particularly following some of the fertiliser N applications (Figure 2.8). The ID technique produced estimates over 100% on some replicates in each fertiliser N treatment on the final harvest in October 2012. For this reason, the October 2012 estimates were excluded from the annual average %Ndfa estimates and from calculations of annual BNF using the ID technique.

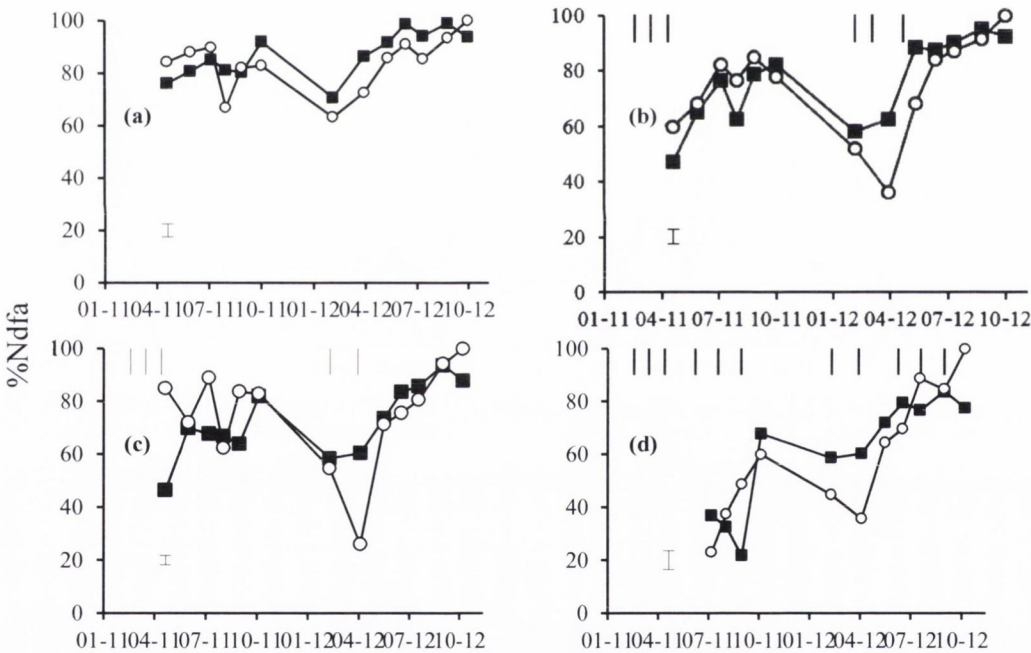


Figure 2.8. Proportion of nitrogen derived from the atmosphere in white clover estimated by ¹⁵N isotope dilution (white circle) and ¹⁵N natural abundance (black box) on each harvest date (mm-yy) in treatments receiving annual fertiliser N inputs of (a) 0, (b) 86, (c) 140 and (d) 280 kg ha⁻¹. Vertical lines indicate dates of fertiliser N application. Error bars represents the interaction S.E.M of technique × harvest date.

There was no significant effect of ¹⁵N technique on annual estimates of %Ndfa. Annual estimates decreased ($P<0.001$) with increasing fertiliser N input (Table 2.4). Annual mean %Ndfa under 0N, 86N and 140N did not differ between years, but was lower ($P<0.001$) in 2011 than 2012 under 280N (Table 2.4).

2.4.7. Biological N₂ fixation of white clover

Annual above ground BNF ranged from 14 kg N ha⁻¹ under 280N in 2012 to 128 kg N ha⁻¹ under 0N in 2011. There was no significant effect of technique on the annual estimates of BNF (Table 2.4). Estimates of BNF declined with increasing fertiliser N input. On average, over the two years and with both ¹⁵N techniques, relative to 0N BNF declined by 19, 17 and 41% under 86N, 140N and 280N, respectively. Annual BNF was lower ($P<0.001$) in 2012 than 2011 under all fertiliser N treatments.

Estimates of BNF using the NDiff technique under 0N were (mean $\pm$ s.e) 73 ± 27.7 kg N ha⁻¹ in 2011 and 54 ± 27.3 kg N ha⁻¹ in 2012 and did not differ significantly between years. There was no difference in estimates of BNF between the three techniques under the 0N treatment. There were significant correlations ($P<0.05$) between annual BNF with herbage and soil N variables (Table 2.5). The strongest correlation was between clover yield and annual BNF.

Table 2.4. Annual proportion of nitrogen derived from the atmosphere (%Ndfa) in white clover and biological N₂ fixation on treatments receiving annual fertiliser N inputs of 0 (0N), 86 (86N), 140 (140N) and 280 (280N) kg ha⁻¹ measured by the ¹⁵N natural abundance (NA) and ¹⁵N isotope dilution (ID) techniques in 2011 and 2012.

2011				2012				S.E.M ^a							
0N	86N	140N	280N	0N	86N	140N	280N	Technique	Fertiliser N	Year	Technique × Fertiliser N	Technique × Year	Fertiliser N × Year	Fertiliser N × Year × Technique	
%Ndffa															
NA	82	68	66	39	90	80	75	71	1.4 ^{NS}	2.1***	1.5***	2.7 ^{NS}	1.9***	2.8***	3.9 ^{NS}
ID	82	75	79	42	82	69	67	64							
Biological nitrogen fixation (kg ha ⁻¹)															
NA	111	83	91	69	49	44	34	23	2.8 ^{NS}	5.3*	2.9***	5.5 ^{NS}	3.3 ^{NS}	5.8*	6.5 ^{NS}
ID	111	90	105	75	47	39	34	21							

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ^{NS} $P > 0.05$

^a standard error of the mean: Differences between means in the table can be deciphered using the presented S.E.M's.

Table 2.5. Pearson correlation coefficients (r) for relationships between annual N₂ fixation with fertiliser N input, herbage yields and soil mineral N.

	Fert N	TotalYld	Grass Yld	Clover Yld	Ndfa NA	Ndfa ID	Soil NH ₄ ⁺ ^a	Soil NO ₃ ^{-a}	BNF NA
BNF NA	-0.38 ^{NS}	0.24*	-0.41*	0.85***	-0.12 ^{NS}	0.26 ^{NS}	0.63**	-0.51*	1
BNF ID	-0.29 ^{NS}	0.31 ^{NS}	-0.35*	0.87***	-0.22 ^{NS}	0.27 ^{NS}	0.59**	-0.53**	0.97***

Analysis carried out on replicate values from each treatment over the two years, 2011 and 2012 (n=24)

BNF NA: annual biological N₂ fixation estimates by the ¹⁵N natural abundance,

BNF ID: annual biological N₂ fixation estimates by the ¹⁵N isotope dilution technique,

Fert N: annual fertiliser N input,

Total Yld: annual total herbage dry matter (DM) yield,

Grass Yld: annual grass DM yield,

Clover Yld: annual clover DM yield,

Ndfa NA: annual mean percentage of N derived from the atmosphere in clover estimates by the ¹⁵N natural abundance technique,

Ndfa ID: annual mean percentage of N derived from the atmosphere in clover estimates by the ¹⁵N isotope dilution technique,

Soil NH₄⁺: annual mean soil ammonium content, Soil NO₃⁻: annual mean soil nitrate content.

^aLog transformed

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ^{NS} $P > 0.05$

2.4.8. B_s -values and influence on %Ndfa in white clover

Greenhouse B_s -values ranged from -1.63‰ to -1.09‰ $\delta^{15}\text{N}$ between clover varieties (Table 2.6). Crusader had a significantly higher B_s -value than cvs Barblanca and Chieftain, which were not different from each other.

Table 2.6. B_s -value estimates of three white clover varieties grown in pots (n=9) in a controlled greenhouse experiment.

	Variety			S.E.M. ^a		
	Barblanca	Chieftain	Crusader	Variety	Harvest	Variety × Harvest
	B_s -value (‰)					
Harvest 1	-1.47	-1.63	-1.24	0.05***	0.04***	0.07 ^{NS}
Harvest 2	-1.30	-1.26	-1.09			

^a standard error of the mean: Differences between means in the table can be deciphered using the presented S.E.M's.

*** $P < 0.001$; ^{NS} $P > 0.05$

The impact of different B_s -values on the estimates of %Ndfa using the NA technique across the range of fertiliser N inputs in the study is presented in Table 2.7. The estimates of %Ndfa generated between the three varieties were not significantly different from each other. Estimates of %Ndfa where no B_s -value was used were always significantly higher than those where a B_s -value was used. The lowest field $\delta^{15}\text{N}$ values generated significantly lower estimates of %Ndfa than the Crusader B_s -value where no fertiliser N was applied.

Table 2.7. Estimates of the proportion of nitrogen derived from the atmosphere (%Ndfa) in white clover plants, according to the ^{15}N natural abundance technique, using B_s -values generated from different white clover varieties and 'other B_s -values'. All estimates were calculated across the range of annual fertiliser N inputs used in the present study.

	Variety			Other B_s -values		S.E.M. ^c		
	Barblanca	Chieftain	Crusader	Lowest field $\delta^{15}\text{N}^b$	No B_s -value	Fertiliser N	B_s -value	Fertiliser N × B_s -value
Fertiliser N input ^a	%Ndfa							
0	85.6ab	84.9ab	88.6a	80.6b	109.3c			
86	73.9a	73.2a	76.4a	69.5a	94.1b	1.29***	1.47***	2.88 ^{NS}
140	70.3a	69.7a	73.1a	65.7a	93.1b			
286	58.3a	57.7a	60.8a	54.2a	80.2b			

*** $P < 0.001$; ^{NS} $P > 0.05$

^a kg N ha⁻¹ year⁻¹

^b lowest clover $\delta^{15}\text{N}$ found in the field in the present study

^c standard error of the mean

B_s -values used in calculations were -1.39, -1.44, -1.17, -1.79 and 0 $\delta^{15}\text{N}$ for Barblanca, Chieftain, Crusader, Lowest field $\delta^{15}\text{N}$ and No B_s -value, respectively.

Values with different letters within rows indicate statistically significant differences

2.5. Discussion

2.5.1. Comparison of the techniques

In general, the two ^{15}N techniques resulted in similar annual estimates of %Ndfa and BNF, which is in agreement with previous studies (Ledgard *et al.*, 1985; Carranca *et al.*, 1999; Huss-Danell and Chaia, 2005). On the other hand, estimates produced using the non ^{15}N -based NDiff technique were very variable, with coefficient of variations of 70% in 2011 and 90% in 2012. Variation in N yields on 0N_G plots was the main cause of the variation in these BNF estimates. The differences in soil N uptake between grass monoculture, clover monocultures and grass/clover stands are known to create errors when using the NDiff technique (Carlsson and Huss-Danell, 2003). While the NDiff technique is cheaper and easier to implement (relative to ^{15}N based techniques) its use in isolation is not recommended and should only be used in conjunction with one of the ^{15}N based techniques (Unkovich *et al.*, 2008).

There was good conformance in %Ndfa between the two ^{15}N techniques throughout the study, particularly under the lower fertiliser N input treatments (0N and 86N). We hypothesised that addition of fertiliser N input, particularly high (280 kg N ha⁻¹ yr⁻¹) fertiliser N regimes, create discrepancies between the ^{15}N isotope dilution and ^{15}N natural abundance techniques. While there was no significant effect of technique on annual estimates (Table 2.4), however, discrepancies occurred following some of the fertiliser N applications under 86N, 140N and 280N. Fertiliser N generally has a slightly negative $\delta^{15}\text{N}$ (Choi *et al.*, 2002; Oberson *et al.*, 2013) and its addition dilutes added ^{15}N fertiliser under the ID technique. Fertiliser N application and subsequent elevation in soil mineral N (often observed at these harvests) also stimulates soil nitrification and denitrification, which are strong N isotope-fractionating processes (fractionation factor: +15 to +35‰) (Boddey *et al.*, 2000; Robinson, 2001; Mathieu *et al.*, 2007). All the above may lead to temporal and spatial variation in soil mineral ^{15}N following fertiliser N addition which disrupts the principal assumption of the ^{15}N techniques i.e. that the isotopic composition of the N derived from soil by the legume and reference plants is the same. Both low levels of N₂ fixation following fertiliser N application (Ndfa <60%) and temporal and spatial variation of soil mineral ^{15}N are known to increase the sensitivity of both ^{15}N techniques to error (Doughton *et al.*, 1995; Chalk and Ladha, 1999; Unkovich *et al.*, 2008), and are the most likely causes for the discrepancies observed following fertiliser N application in this study.

Individual ^{15}N analysis of reference plants of different types, such as buttercups, daisies, docks and ryegrass (relative to using a combination of ryegrass and dicot weed species) would have given a better indication of the isotopic uniformity of the soil mineral N assimilated by clover in this study, and thus allow for improved assessment of the error associated with the principal assumption of the ^{15}N techniques outlined above. However, the lack of difference in ^{15}N between reference plant material (ryegrass and dicot weeds) analysed at the June 2012 harvest (Table 2.2) suggests that the isotopic composition of soil mineral N was relatively uniform, and that the ^{15}N abundance of soil N assimilated by clover would be similar to that of the reference plants used in this study. Nonetheless, it is still recommended to sample multiple reference plants at each individual harvest in future studies, particularly under high fertiliser N regimes.

2.5.2. Potential problems encountered with the ^{15}N techniques and the role of B_s -values

The ID technique produced %Ndfa estimates in excess of 100% on individual replicates in each fertiliser N treatment at the final harvest in October 2012. A supplementary harvest in March 2013 gave similar results (data not shown). Observations of plant available soil and clover ^{15}N enrichment at these harvests (Figure 2.6) suggested that plant ^{15}N enrichments were approaching or at natural abundance. This may have resulted in errors in estimates of %Ndfa due to ^{15}N and ^{14}N fractionation that occurs at natural abundance during the BNF process (Unkovich *et al.*, 2008; Unkovich, 2013).

These small plant ^{15}N enrichments in the ID plots relative to other studies was most likely due to dilution effects associated with N input from fertiliser N and the mineralisation of N in soil organic matter, which was stimulated by the natural drying of these predominantly saturated soils under below average rainfall over the summer of 2011 and by atypically cold temperatures that occurred during the winter of 2010/2011 (Burchill *et al.*, 2014b).

The NA technique also gave estimates of %Ndfa that were greater than 100% on five harvests in one individual replicate of the 0N treatment. The $\delta^{15}\text{N}$ of clover fell below (minimum of $-1.79 \delta^{15}\text{N}$) our estimated mean B_s -value of $-1.33 \delta^{15}\text{N}$ but was within the range of B_s -values (-0.76 to $-1.87 \delta^{15}\text{N}$) encountered in our greenhouse experiment. It was assumed that BNF was the sole source of N for the clover plant in these cases and %Ndfa was set to 100% as recommend by Unkovich *et al.* (2008). Thereafter, NA produced realistic estimates of %Ndfa.

The B_s -values were similar to those presented by Unkovich *et al.* (2008) for white clover. The differences in B_s -values between clover varieties had no significant effect on the NA estimates of %Ndfa and were relatively small compared to other sources of variation encountered when using the NA technique (Holdensen *et al.*, 2007; Carlsson and Huss-Danell, 2014). The results of this study suggest that the lowest derived clover $\delta^{15}\text{N}$ may lead to underestimations of %Ndfa in studies where %Ndfa is high (>80%) which is in agreement with Unkovich *et al.* (2008). Not accounting for fractionation (i.e. using a B_s -value = 0) resulted in large overestimates of %Ndfa under all fertiliser N inputs, including the generation of values of over 100% and so is not recommended.

2.5.3. Annual variation in BNF

Inter-annual variation in BNF was substantial with 2- to 3-fold differences between 2011 and 2012. Differences in clover DM yield, as opposed to %Ndfa, were the largest contributor to annual variation in BNF (Table 2.5). Similar observations have been summarised by Carlsson and Huss-Danell (2003). Variation in clover DM yield may in part be due to differences in weather conditions between years (Ledgard *et al.*, 2001).

Perennial ryegrass (5-6°C) has a lower soil temperature growth threshold than clover (9-10°C) (Davies, 2001). Below average air temperatures in the winter of 2010/2011 and early spring of 2011 depressed the growth of both clover and grass. The sudden and large increase in temperatures in April 2011 created a situation where the growth of both plants was not limited by temperature. In contrast, air temperatures ranged between 6.2 and 8.4°C between December 2011 and April 2012, which allowed for more grass growth relative to clover. This resulted in increased competition from grass over this period with subsequent reductions in clover content and DM yield primarily due to competition for light. Competition from grass over the winter period have been found to have significant effects on both clover content and DM yield and these effects can carry over to the following growing season (Phelan *et al.*, 2014).

The lower clover contents and DM yields in 2012 may also be linked to the high rainfall and saturated soil conditions that prevailed during the growing season of 2012. Observations of reduced clover DM yield under flooded conditions have been previously reported (Huber *et al.*, 2009).

2.5.4. Impact of fertiliser N on BNF and sustainable herbage production

Biological N₂ fixation declined with increasing fertiliser N input in both years of this study (Figure 2.9a) and was primarily due to a reduction in %Ndfa rather than clover DM yield, mostly due to the substitution of biologically fixed N by soil mineral N (Hogh-Jensen and Schjoerring, 1994; Ledgard *et al.*, 2001; Rasmussen *et al.*, 2012).

While fertiliser N reduced BNF it increased herbage production (Figure 2.9b). The cost of producing this additional herbage above 0N was calculated using the equations developed between herbage yield and fertiliser N input (Figure 2.9a) and standard costs for N (€120 t⁻¹), P (€92 t⁻¹), potassium (€288 t⁻¹) and a standard liming and reseeding cost (CSO 2014). The economics of applying fertiliser N to clover pastures was largely dependent on the yield response to fertiliser N, which differed between years due to weather conditions. For example, assuming alternative livestock feeds cost in the region of €200 t⁻¹ DM (€120 to €450 t⁻¹: (O'Donovan *et al.*, 2011; CSO, 2014)), there was little economic benefit of applying fertiliser N to clover pastures above 170 kg N ha⁻¹ under the yield responses achieved in 2012 under poor growing conditions. However, fertiliser N inputs of up to 280 kg N ha⁻¹ were economical under the yield responses achieved in 2011 under relatively good growing conditions (Figure 2.9c).

The proportion of N recovered in herbage diminished with increasing total soil N supply (Figure 2.9d). This was particularly apparent under 280N (82% in 2011 and 76% in 2012). Likely reasons for lower recovery are that the unrecovered N was emitted from the soil as ammonia or nitrous oxide or leached to groundwater, all of which have environmental consequences (Draaijers *et al.*, 1989; Ravishankara *et al.*, 2009; Bouman *et al.*, 2010).

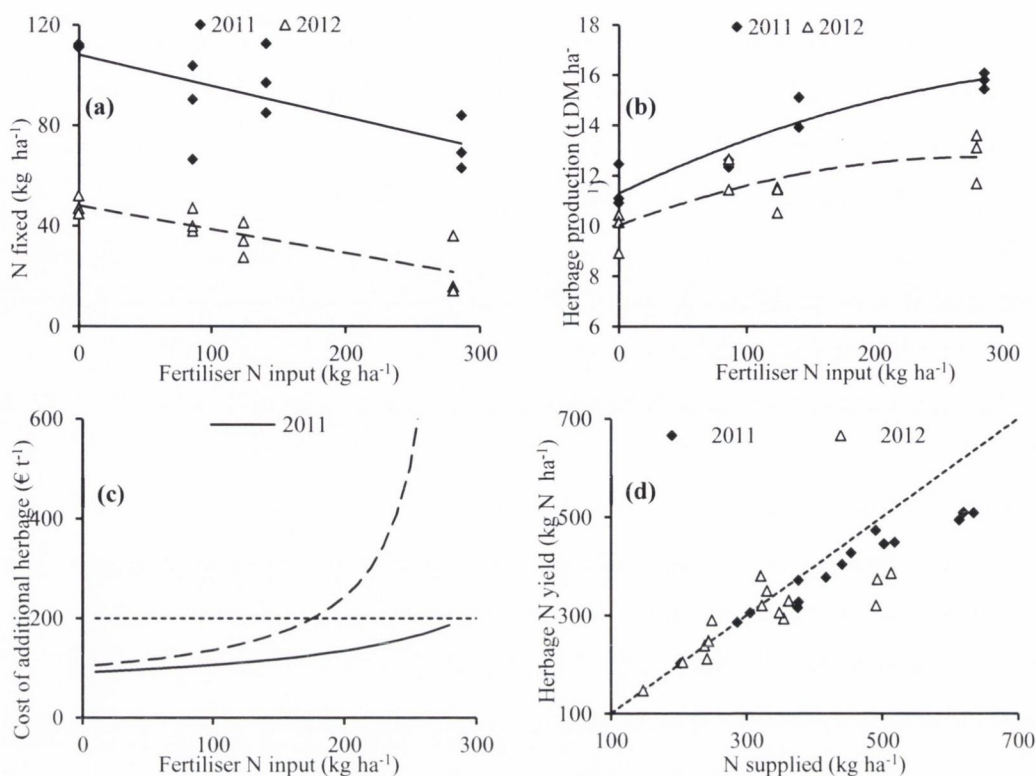


Figure 2.9. Impact of fertiliser N input on biological N₂ fixation (aboveground biomass) associated with white clover (2011: $108.0 - 0.124x$, $R^2 = 0.52$, $P < 0.01$. 2012: $47.9 - 0.094x$, $R^2 = 0.69$, $P < 0.001$) (a), herbage dry matter yield response to fertiliser N input (2011: $11.3 + 0.024x - 0.00003x^2$, $R^2 = 0.86$, $P < 0.001$. 2012: $10.02 + 0.019x - 0.00003x^2$, $R^2 = 0.58$, $P < 0.05$) (b), predicted cost of producing additional herbage above zero fertiliser N input on perennial ryegrass/white clover grassland in 2011 and 2012 (c) and nitrogen uptake in herbage in response to N supplied [Fertiliser N + biological N fixation (aboveground biomass) + background soil N supply (uptake of N in herbage on perennial ryegrass grassland receiving no external N input: $0N_G$)] (d) on perennial ryegrass and perennial ryegrass/white clover grassland in 2011 and 2012. Dotted line in panel (c) indicates a nominal cut off point for fertiliser N input where alternative livestock feeds cost €200 t⁻¹ dry matter.

These results can be viewed from different perspectives. Fertiliser N inputs of 280 kg N ha⁻¹ achieved the highest herbage yield, but they compromised BNF and had the highest potential for N loss. The application of these relatively high fertiliser N inputs on grass/clover pastures may be uneconomical in years with poor growing conditions. On the other hand, grass/clover pastures receiving no fertiliser N input maximised BNF and limited potential N loss, but had relatively low herbage yields. Intermediate fertiliser N inputs would appear to achieve a balance between minimising impact on BNF and potential N loss while producing more herbage DM yield than grass/clover pastures receiving no fertiliser N.

2.6. Conclusions

Annual above ground BNF ranged between 14 to 128 kg N ha⁻¹ in perennial ryegrass/white clover pastures. The two ¹⁵N techniques provided similar annual estimates of BNF across a range of fertiliser N inputs on non-grazed perennial ryegrass/white clover pastures in this study. However, the NDiff technique produced different and variable results. Therefore the hypothesis that techniques used to quantify BNF (¹⁵N isotope dilution, ¹⁵N natural abundance and nitrogen difference) differ in their estimates of BNF is accepted. This study hypothesised that addition of fertiliser N, particularly high (280 kg N ha⁻¹ yr⁻¹) fertiliser N regimes, create discrepancies between the ¹⁵N isotope dilution and ¹⁵N natural abundance techniques used to estimate BNF. Annual estimates of BNF using these ¹⁵N techniques under 280N were very similar. There were, however, some discrepancies between the NA and ID techniques following fertiliser N applications. Care should be taken to negate the effects of ¹⁴N and ¹⁵N fractionation under both techniques i.e. to use appropriate *B_s*-values for the NA technique and to monitor plant ¹⁵N enrichment under the ID technique to ensure that sufficient enrichment is maintained (and that it is uniform). Based on the evidence of this study, the hypothesis that increasing fertiliser N input on grass/clover pastures reduces BNF and increases potential of N losses on such pastures, is accepted. Intermediate fertiliser N inputs in the present study achieved a balance between minimising detrimental impact on BNF and potential N loss while producing more herbage DM yield than grass/clover pastures receiving no fertiliser N.

CHAPTER 3. INTER-ANNUAL VARIATION IN NITROUS OXIDE EMISSIONS FROM PERENNIAL RYEGRASS/WHITE CLOVER GRASSLAND USED FOR DAIRY PRODUCTION

Based on: Burchill, W., Li, D., Lanigan, G., Williams, M., and Humphreys, J. 2014. Interannual variation in nitrous oxide emissions from perennial ryegrass/white clover grassland used for dairy production. *Global Change Biology*. 10, 3137-3146, DOI: 3110.1111/gcb.12595.

3.1. Abstract

Nitrous oxide (N₂O) emissions are subject to intra- and inter-annual variation due to changes in weather and management. This creates significant uncertainties when quantifying estimates of annual N₂O emissions from grazed grasslands. Despite these uncertainties, the majority of studies are short-term in nature (<1 year) and as a consequence, there is a lack of data on inter-annual variation in N₂O emissions. The objectives of this study were to (i) quantify annual N₂O emissions and (ii) to assess the causes of inter-annual variation in emissions from grazed perennial ryegrass/white clover grassland. Nitrous oxide emissions were measured from fertilised and grazed perennial ryegrass/white clover grassland (WC) and from perennial ryegrass plots that were not grazed and did not receive N input (GB), over four years from 2008 to 2012 in Ireland (52° 51' N, 08° 21' W). The annual N₂O-N emissions (kg ha⁻¹; mean ± s.e.) ranged from 4.4 ± 0.2 to 34.4 ± 5.5 from WC and from 1.7 ± 0.8 to 6.3 ± 1.2 from GB. Inter-annual variation in N₂O emissions was attributed to differences in annual rainfall, monthly (December) soil temperatures and variation in N input. Such substantial inter-annual variation in N₂O emissions highlights the need for long term studies of emissions from managed pastoral systems.

3.2. Introduction

Nitrous oxide (N₂O) is a potent GHG and agriculture has been identified as a major source of N₂O accounting for 58% of global emissions, with direct emissions of N₂O from grazed grassland systems comprising between 16 and 33% of this total (de Klein *et al.*, 2008).

Nitrous oxide is primarily produced in soil by microbial processes such as nitrification and denitrification. Other processes such as nitrifier denitrification, co-denitrification and DNRA are also known to produce N₂O (Stevens and Laughlin, 1998). Both nitrification and denitrification are regulated by soil NO₃⁻, NH₄⁺, organic C, pH, temperature, moisture content and oxygen supply (Firestone and Davidson, 1989; Liu *et al.*, 2007; Cuhel *et al.*, 2010). Climatic conditions can directly influence these factors and, hence, N₂O emissions (Cantarel *et al.*, 2012). For example rainfall has a direct impact on soil moisture and oxygen status and can consequently affect N₂O production (Luo *et al.*, 1999). Changes in these controlling factors over time create considerable temporal variation in emissions of N₂O on a seasonal and annual basis, making it difficult to acquire representative estimates of annual N₂O emissions.

Considerable inter-annual variation in N₂O emissions have been observed in previous studies of temperate grassland (two years) (Hyde *et al.*, 2006; Flechard *et al.*, 2007; Rafique *et al.*, 2011), native semi-arid grasslands (Du *et al.*, 2006), arable rotations (Cai *et al.*, 2013) and differing soil types (Webster and Dowdell, 1982). This variation can be driven by differences in (i) management such as stocking densities, N fertilisation rates and drainage and (ii) climatic factors such as total precipitation and temperature (Du *et al.*, 2006; Flechard *et al.*, 2007; Cai *et al.*, 2013; Rees *et al.*, 2013). Despite this, the majority of N₂O studies are focused over relatively short periods (<1 year) and are insufficient to provide robust estimates of annual N₂O emissions or assess the variation that can occur over longer time periods.

The present study measured N₂O emissions from fertilised and grazed perennial ryegrass/white clover grassland and non-grazed perennial ryegrass grassland receiving no N input over four years. The objectives were to (i) quantify annual N₂O emissions and (ii) to access the causes of inter-annual variation in emissions from grazed perennial ryegrass/white clover grassland. This study hypothesises that nitrous oxide emissions from a N fertilised and grazed grass/clover pasture vary inter-annually due to changes in weather conditions over time.

3.3. Materials and Methods

3.3.1. Site description

This study was carried out at the Teagasc Solohead Research Farm (52°51'N, 08°21'W; 95m above sea level). The predominant soils are poorly drained Gleys (90%) and Grey Brown Podzolics (10%) with a clay loam texture (29% clay, 36% silt and 34% sand) and low permeability. The soils are seasonally wet, waterlogged or flooded due to impeded drainage and a shallow water table depth (0 to 2.2m below ground level) at the site. Mean soil organic C, total N content, bulk density and pH at 0 to 10 cm depth are 5.12%, 0.54%, 0.87 g cm⁻³ and 6.2, respectively. The permanent grassland at the site is used predominately for grazing and occasionally harvested for silage. The pastures range from 20 to 30 years in age. This region has a temperate moist climate allowing for pasture based dairy production with a grazing season extending to 9 or 10 months each year. Mean annual rainfall at the site (1997-2008) was 1031 mm. Mean minimum monthly soil temperature at 10cm depth ranged from 4.3 to 8.5°C during winter (November-January) and maximum monthly temperatures ranged from 13.9 to 18.2°C in summer (May-July).

3.3.2. Experiment design and treatments

The experiment was a randomised block design with two treatments and three replicates. The treatments were (i) grazed perennial ryegrass/white clover based pastures (WC) and (ii) background perennial ryegrass plots (GB). The WC treatment formed part of a perennial ryegrass/white clover based system of dairy production at the Solohead Research Farm which consisted of six paddocks, with an annual stocking density of 2.35 cows ha⁻¹. Three of these paddocks were devoted to the WC treatment. The WC paddocks were rotationally grazed by spring calving Holstein-Friesian cows during the main grazing season (March to November) with surplus herbage occasionally removed as baled silage. Paddocks ranged in size from 1.6 to 2.1 ha with a total area of 5.4 ha and were dominated by perennial ryegrass with an annual average white clover content of herbage DM of 24% during the experimental period. The grazing rotation length on WC varied from 21 days in late spring/early summer to 42 days in autumn. Post grazing sward height, maintained at 4 cm, was measured using a rising plate meter (Grasstec, Charleville, Ireland) and used to determine when cows were moved to the next paddock.

Fertiliser N and slurry applications to WC are presented in Table 3.1. Fertiliser and slurry N were applied in a number of applications between early spring and early summer. The additional N input to WC in the form of N deposited by grazing cows was calculated based on the stocking density, residence time and N excretion per cow per day for each grazing event (Table 3.1). Annual N excretion per cow was estimated as the difference between the cow's annual intake of N in feeds and the N output in milk and calves or in live-weight change of the cow as described in detail by Humphreys *et al.*, (2008b). Annual input of N for the WC paddocks due to biological N fixation was estimated to be (mean $\pm$ s.e.) 78.6 $\pm$ 13.2 kg N ha⁻¹ in 2008/09, 112.4 $\pm$ 8.8 kg N ha⁻¹ in 2009/10, 102.6 $\pm$ 10.7 kg N ha⁻¹ in 2010/11 and 65.4 $\pm$ 5.6 kg N ha⁻¹ in 2011/12 based on white clover contents and herbage yields using methodology described by Humphreys *et al.*, (2008b).

Table 3.1. Applications of fertiliser N and cattle slurry, and annual N deposited by grazing animals to perennial ryegrass/white clover pastures (WC) in 2008/09, 2009/10, 2010/11 and 2011/12.

Date	Application	N input (kg ha ⁻¹)
2008/09		
9 th February 2009	Cattle slurry	93.4
25 th March 2009	Urea	28.8
8 th May 2009	CAN ^a	34
	Excreta N ^b	129
2009/10		
10 th February 2010	Cattle slurry	76.9
14 th April 2010	Urea	57.5
	Excreta N	136
2010/11		
21 st January 2011	Cattle slurry	102.9
24 th February 2011	Urea	28.8
22 nd March 2011	Urea	28.8
13 th April 2011	Urea	28.8
	Excreta N	203
2011/12		
23 rd January 2012	Cattle slurry	30.9
15 th February 2012	Urea	28.8
13 th March 2012	Urea	28.8
2 nd May 2012	Urea	28.8
	Excreta N	171

^aCalcium ammonium nitrate

^bAnnual N deposited by grazing livestock

The GB treatment was laid down to measure background soil emissions of N₂O in the absence of N input. The area of each GB plot was 3 × 11 m and was dominated by perennial ryegrass and contained no white clover. The GB plots received no fertiliser N or cattle slurry and were not grazed. Herbage on GB was harvested by cutting during the growing season to a height of 4 cm, with clippings removed.

Both of these treatments were imposed from January 2008 and measurements began in October 2008. Therefore each annual measurement spanned two calendar years and ran from 1st October 2008 to 30th September 2009 (2008/09), 1st October 2009 to 30th September 2010 (2009/10), 1st November 2010 to 31st October 2011 (2010/11) and 1st November 2011 to 31st October 2012 (2011/12).

3.3.3. Soil and weather measurements

Daily weather data including rainfall (mm), air temperature (°C) and soil temperature at 10 cm depth (°C) was recorded at the experimental site as described by Fitzgerald &

Fitzgerald (2004). Effective rainfall (rainfall minus evapo-transpiration) was determined using a water-mass balance model described by Schulte *et al.* (2005). This model is based on a modified version of the Penman-Monteith evapo-transpiration model. Input weather data included daily maximum and minimum air temperatures, rainfall, wind speed and solar radiation.

At each gas sampling volumetric soil water content (VWC) was recorded at 0-5 cm depth using a HH2 moisture meter in conjunction with a Theta Probe sensor (Delta-T Devices, Burwell, Cambridge, England). Soil water filled pore space at 0-5 cm depth was calculated according to Ri *et al.*, (2003).

3.3.4. N₂O flux measurements

A closed static chamber technique (Hutchinson and Moision, 1981) was used to measure N₂O fluxes. The chamber consisted of two parts: a permanent collar and a chamber, both of which were constructed from polyvinylchloride (PVC) pipe (Figure 3.1). The headspace height of the chamber was 40 cm with an internal diameter of 29.5 cm, resulting in a cover area of 0.0683 m² and a headspace volume of 0.0273 m³. A 10 cm long plastic tube (internal diameter of 0.5cm) was permanently inserted into the top of each chamber. One port of a three-way valve (Discofix) was fitted to the top of this tube on the outside of the chamber. A second port of the valve was connected to a needle for extraction of gas samples from the chamber. The third port of the valve could be left open when placing the chamber on the collar to prevent the build-up of air pressure within the chamber. Once the chamber was in place this port was closed to create an air tight seal between the chamber and collar. The collars were permanently inserted into the soil to a depth of 12 cm and extended 4 cm above the soil surface following installation. The collars on WC were exposed to grazing livestock throughout the experiment. Five chambers and collars were deployed in each paddock of WC and one in each of the smaller GB plots. The N₂O sampling was conducted (between 9:30am and 1pm) on a weekly basis with increased frequency (three times *per week*) following N applications on WC. Likewise, N₂O sampling was conducted (between 9:30am and 1pm) on a weekly basis on GB with increased frequency (three times *per week*) following N applications on WC in 2008/09 and 2009/10. Sampling frequency on GB was lower in 2010/11 and 2011/12 and plots were sampled on five occasions in 2010/11 and on thirteen occasions in 2011/12.

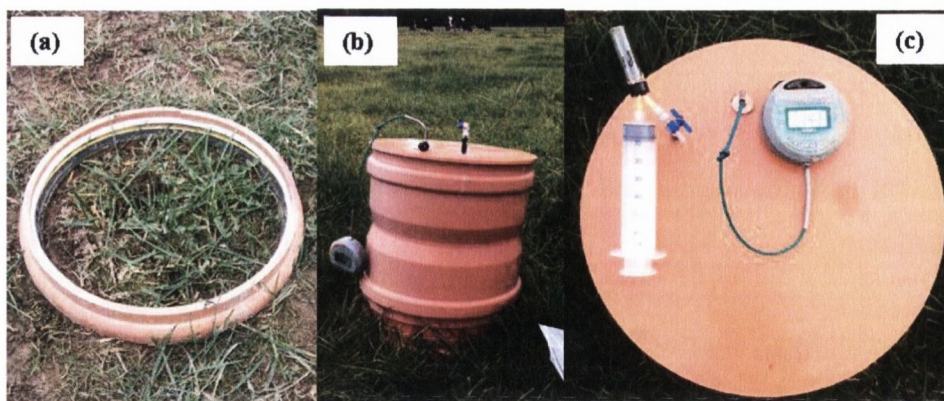


Figure 3.1. Collar (a) and chamber (side view: b and top view: c) used for N_2O measurements in the study.

On each sampling occasion the chamber was placed on the collar. Samples were taken using a gas-tight syringe and the three-way valve fitted to the top of the chamber. Gas samples were taken immediately after chamber closure (T_0) and again after 15 (T_{15}) and 30 (T_{30}) minutes. All gas samples were transferred to 7 ml screw-cap exetainer with Teflon-coated butyl septa (Perbio Science, UK). Air temperature inside the chamber and soil temperature at 5 cm depth beside the chamber was recorded using a thermo-sensor (Sensor-Tech Ltd, Castlebingham, Ireland).

Gas samples were analysed using a gas chromatograph (GC) (Varian GC 450; Netherlands) fitted with an electron capture detector (ECD) and automatic sampler (Combi-PAL auto sampler; CTC Analytics, Zwingen, Switzerland) at Teagasc, Johnstown Castle Environmental Research Centre, Wexford, Ireland. Column and detector temperatures were 60°C and 300°C respectively. The N_2O concentration (parts per million, ppm) of each sample was calculated using calibration curves. These curves were determined using N_2O standards gases with concentrations of 0.2, 0.5, 1, 5, 10, 20, 50 and 100 ppm.

3.3.5. Herbage yield and N uptake in herbage

Herbage yield was measured in each WC paddock before each grazing (or silage harvest) and concurrently on each GB plot. A 10 × 1.2 m strip was harvested to 4cm above ground level using a rotary mower (Etesia UK Ltd., Warick, UK). The fresh herbage was weighed and a sub-sample of 100 g of the fresh herbage was dried for 24 h in an oven with forced-air circulation at 100°C to determine DM content. A second sub-sample was stored at -20°C before being freeze dried and milled through a 0.2 mm sieve prior to N content analysis. The N concentration in the herbage DM was determined using a Leco N analyser

(LECO FP-2000; Leco Coroportiaon, St., Joseph, MI, USA) at the laboratories of the Animal and Grassland Research and Innovation Centre, Teagasc Moorepark, Ireland. Uptake of N in herbage DM (kg N ha^{-1}) was calculated using the N concentration in the herbage DM (g/kg) and the DM herbage yield (kg DM ha^{-1}). Nitrogen uptake in herbage DM for the GB plots was predominantly from the mineralisation of soil organic matter N (MSON) and was therefore used as an indication of the difference in MSON between years but not as a direct measure of MSON. This assumes that there was little difference in the input of N to GB through dry deposition of NH_3 between years.

3.3.6. Calculations

Pre-experimental testing showed a linear increase in N_2O concentration in the chambers over a 60 minute test period. Therefore, nitrous oxide flux calculations ($\text{g N ha}^{-1} \text{ day}^{-1}$) were calculated using the linear change in N_2O concentrations in the chamber between T_0 , T_{15} and T_{30} . Daily N_2O fluxes from each WC paddock were calculated using the arithmetic means of five chambers. Cumulative N_2O emissions were calculated by linear interpolation between sampling occasions. Global N_2O emission factors ($_{\text{G}}\text{EF}$) of all applied N were calculated for WC using:

$$_{\text{G}}\text{EF}(\%) = \frac{(\text{Annual N}_2\text{O-N})_{\text{WC}} \text{ minus } (\text{Annual N}_2\text{O-N})_{\text{GB}}}{\text{Total N applied}}$$

where total N applied is the sum of fertiliser N, N in cattle slurry and N deposited during grazing on WC. Total N applied was adjusted to account for losses of NH_3 and NO_x assumed to be 10% from fertiliser N and 20% from N in cattle slurry and N deposited during grazing (IPCC, 2006). Biologically fixed N (BFN) was not included in the $_{\text{G}}\text{EF}$ calculations in this study as IPCC guidelines do not include BFN as a source of N_2O (IPCC, 2006).

Annual soil N balances (SNB) for WC were calculated to determine available soil N for N_2O emissions in each year using:

$$\text{SNB} = \text{N input}_{\text{WC}} \text{ minus } (\text{N uptake in herbage}_{\text{WC}} \text{ minus N uptake in herbage}_{\text{GB}})$$

where N input is the sum of fertiliser N, slurry N, N deposited during grazing, BFN and rainfall N deposition. Fertiliser N, slurry N and N deposited during grazing were adjusted

for NH_3 volatilisation as above. Rainfall N deposition was calculated by multiplying annual rainfall by rainfall total N concentration as described by Nécipálová *et al.* (2013b). The N outputs from the SNB was the N removed in herbage on WC minus the background N supplied for herbage production i.e. N uptake on GB. The N removed in herbage on WC in the SNB was corrected for N uptake on GB as background supply of N is an important contributor to herbage N uptake at this site (O' Connell, 2005).

3.3.7. Statistical analysis

All data were statically analysed using SAS 9.3 (SAS 2011, Institute Inc., Cary, NC, USA). Data were checked for normality by using residual normality and variance analysis. All N_2O data required a natural log transformation ($y = \log(x+1)$). The value of one was added before log transforming the N_2O data, which was sufficient to prevent the generation of negative transformed values. Substantially larger values of the constant added to the data may affect inference. Annual cumulative N_2O emissions and N uptake in herbage were analysed by ANOVA. The variables included in the model were treatment, year and the treatment by year interaction. Year was included as a repeated measure in the ANOVA. Post hoc Holm-Tukey and Turkey-Kramer multiple comparisons test were carried out to determine differences between treatments within and between years.

Pearson correlation analysis was used to test for relationships between transformed annual N_2O emissions and N input, weather and soil variables using data from each plot in each year ($n = 12$ for each treatment). Single and multiple linear regression (stepwise) analysis were carried out to create simple explanatory models using the same variables to account for variation in annual N_2O emissions. A statistical probability of $P < 0.05$ was considered significant for all statistical tests.

3.4. Results

3.4.1. Soil and weather conditions

Annual rainfall was 1183, 1037, 919 and 1394 mm in 2008/09, 2009/10, 2010/11 and 2011/12, respectively. Monthly rainfall was higher than evapo-transpiration for much of the study particularly in 2008/09 and 2011/12 (Figure 3.2). Soil WFPS ranged between 30 and 100% and remained above 70% for the majority of the study. The only major exception to this was the second half of 2009/10 and the grazing season of 2010/11 (Figure

3.3a). Minimum and maximum mean monthly soil temperatures at 5 cm depth ranged from 3.9 to 18.3°C, 1.9 to 18.4°C, 1.5 to 17.1°C and 5.4 to 17.7°C for each of the four years. There was an exceptional period of cold temperatures in December of 2010/11 (Figure 3.3b). The mean monthly air temperature in December 2010 (0.6°C) was much lower than typical December temperatures at this site (1950 to 2012 mean excluding 2010 $\pm$ s.d.; $6.0 \pm 1.2^\circ\text{C}$). Furthermore, the mean monthly air temperature in December 2010 was the lowest recorded between 1950 and 2012.

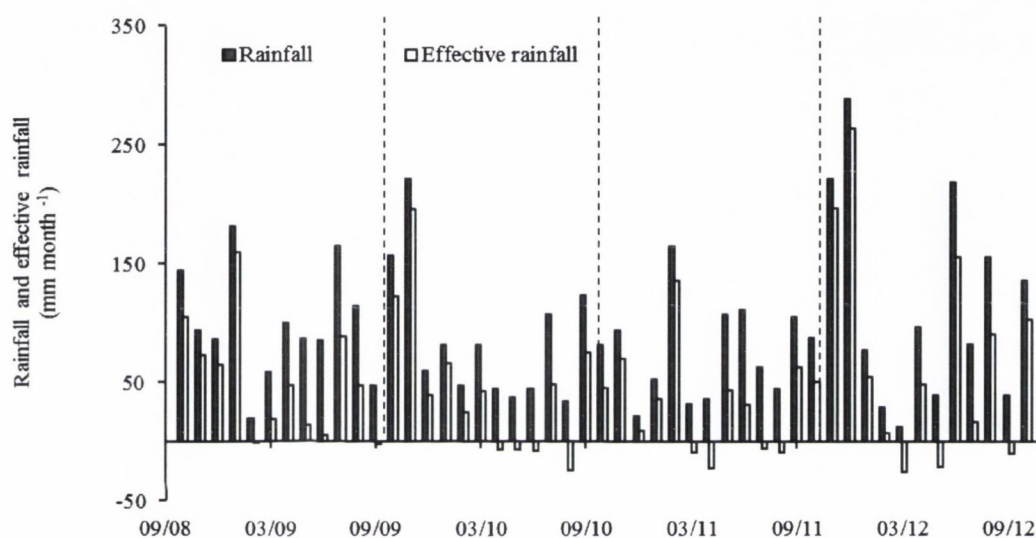


Figure 3.2. Monthly rainfall and effective rainfall (rainfall minus evapo-transpiration) (mm month^{-1}) at Solohead Research Farm during the study period (mm/yy). Dashed vertical lines separate measurement years.

3.4.2. Temporal trends in daily N_2O fluxes

Nitrous oxide fluxes from WC were highly episodic with daily mean values displaying large variation throughout the study and there were clear differences in the rates of daily N_2O fluxes both intra- and inter-annually (Figure 3.3c). Mean daily N_2O -N emissions (g ha^{-1}) from WC ranged from -0.1 to 87.3 in 2008/09, 0.8 to 101.7 in 2009/10, 12.3 to 439.8 in 2010/11 and 1.7 to 44.0 in 2011/12. Highest daily N_2O fluxes from WC were generally found following N applications and during the main grazing season. Fluxes during winter were relatively low except for the winter of 2010/11, where peak fluxes reached $239.3 \text{ g N}_2\text{O-N ha}^{-1} \text{ d}^{-1}$ on the 4 January 2011. Cumulative N_2O -N emissions from WC were 9.2 kg ha^{-1} between 20 November 2010 and 20 January 2011 (period between the end of grazing season 2010 to first application of cattle slurry in 2011: Figure 3.3c) and equated to an 8 to

39 fold increase relative to the cumulative emissions during the same period of each of the other three years of the study.

The range in background emissions of N_2O (from GB plots) was lower than WC, particularly following N applications to WC and during the grazing seasons (Figure 3.3c). Mean daily N_2O -N emissions (g ha^{-1}) from GB ranged from 0.5 to 14.7 in 2008/09, -2.1 to 31.5 in 2009/10, 2.0 to 35.1 in 2010/11 and -0.9 to 12.8 in 2011/12. The daily fluxes on GB were generally higher during the spring to autumn period compared with the winter.

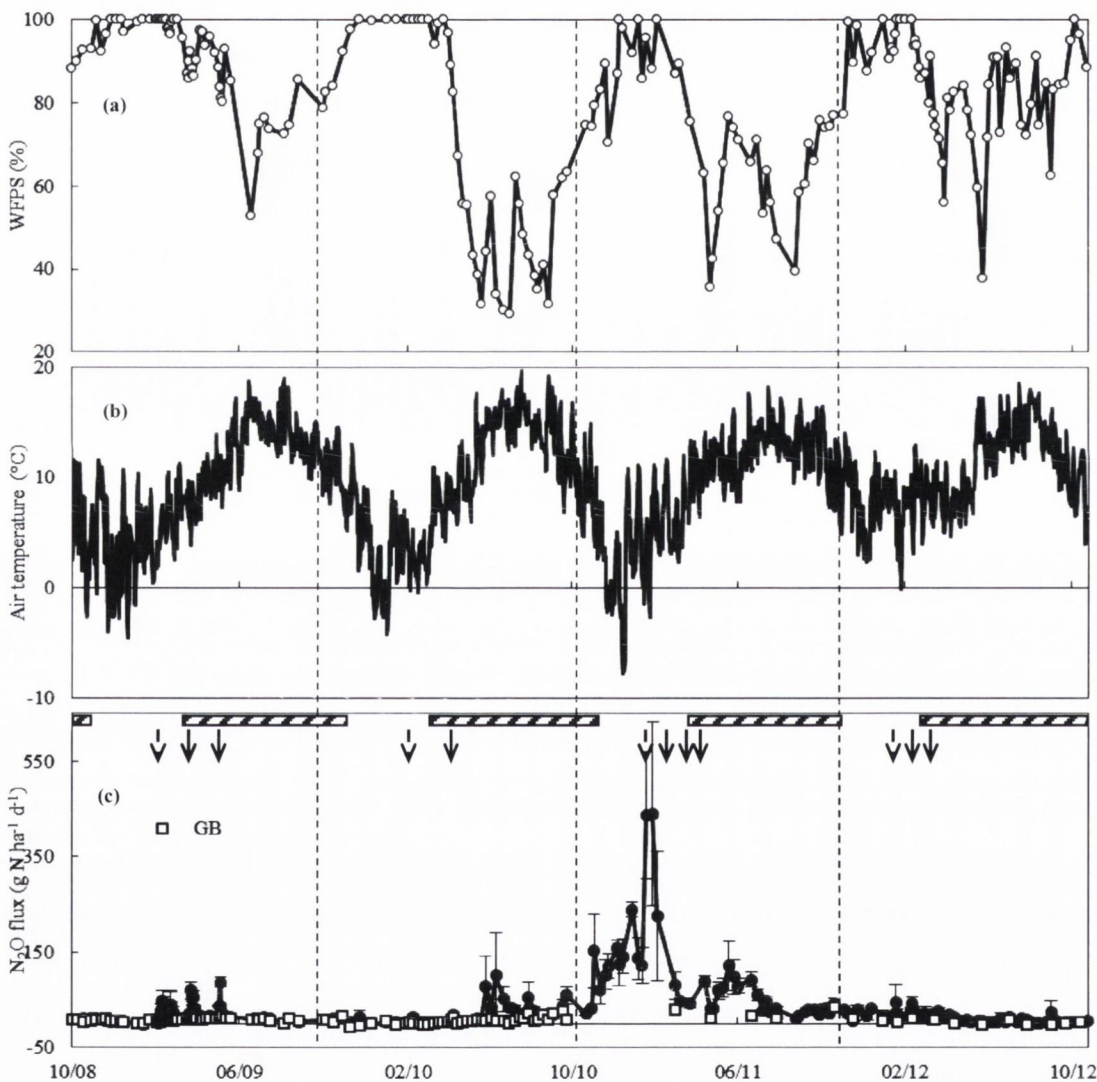


Figure 3.3. Water filled pore space (%) at 0-5cm depth (a), daily air temperature (°C) (b) and daily N_2O fluxes (mean $\pm$ s.e.) from unfertilised perennial ryegrass plots (GB) and grazed and fertilised perennial ryegrass/white clover pastures (WC) (c) during the study (mm/yy). Dashed vertical lines separate measurement years. Dashed arrows indicate dates of cattle slurry application; full arrows indicate dates of fertiliser N application. The vertical edges of the shaded boxes represent the beginning and end of each grazing season.

3.4.3. Annual N₂O emissions

The annual N₂O emissions (kg N₂O-N ha⁻¹; mean ± s.e.) from WC ranged from 4.4 ± 0.2 in 2008/09 to 34.4 ± 5.5 in 2010/11 and from GB ranged from 1.7 ± 0.8 in 2011/12 to 6.3 ± 1.2 in 2010/11 (Figure 3.4). Emissions of N₂O were affected by treatment ($P < 0.01$), year ($P < 0.001$) and an interaction ($P < 0.05$) between treatment and year. There was no difference in annual N₂O emissions between WC and GB in 2008/09, while N₂O emissions were higher ($P < 0.01$) from WC in each of the other years. The exceptionally high annual N₂O emissions from WC in 2010/11 was higher ($P < 0.001$) than emissions from WC in any of the other years, which were not different from each other. On GB, N₂O emissions were higher ($P < 0.05$) in 2010/11 than in 2011/12. Otherwise there were no differences in annual emissions from GB between years. The annual g_{EF} (mean ± s.e.) calculated for WC were 0.5 ± 0.2 in 2008/09, 1.6 ± 0.5 in 2009/10, 7.7 ± 1.6 in 2010/11 and 1.2 ± 0.3 in 2011/12.

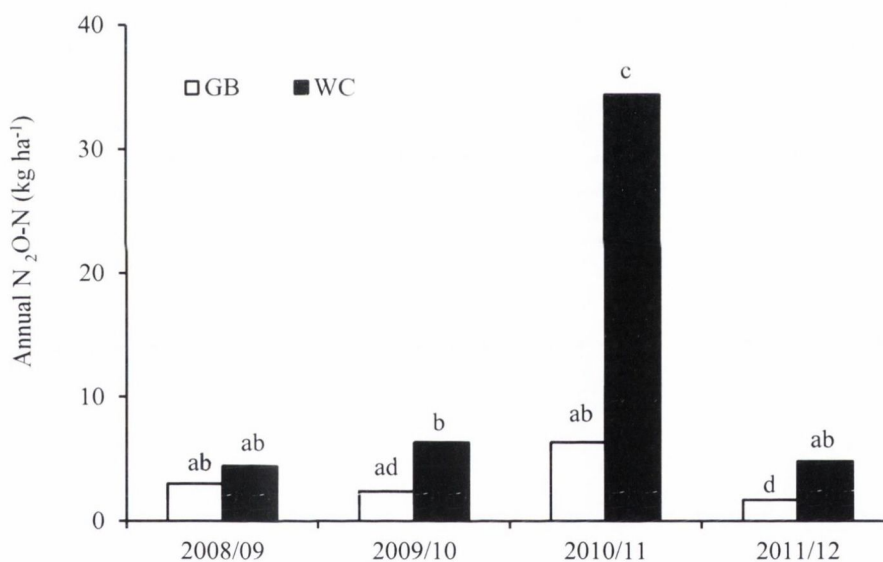


Figure 3.4. Annual N₂O emissions from unfertilised perennial ryegrass plots (GB) and grazed and fertilised perennial ryegrass/white clover pastures (WC) in 2008/09, 2009/10, 2010/11 and 2011/12. Statistically significant differences ($P < 0.05$) between year × treatments are indicated by different letters. Emissions on GB in 2010/11 may be underestimated as no measurements were taken during the winter of 2010, which had elevated emissions on WC.

3.4.4. Uptake of N in herbage

Uptake of N in herbage was affected by treatment ($P < 0.05$), year ($P < 0.001$) and an interaction ($P < 0.001$) between treatment and year (Figure 3.5). Uptake of N was higher ($P < 0.01$) on WC than GB in 2009/10, 2010/11 and 2011/12 but not different in 2008/09.

On GB, uptake of N in 2010/11 was higher ($P<0.05$) than in 2009/10 and 2011/12, while there was no difference in uptake between the other years (Figure 3.5).

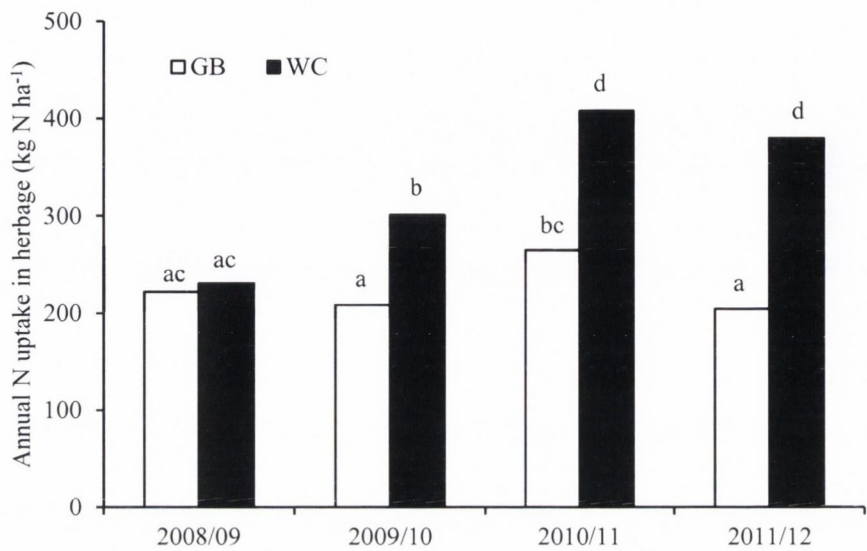


Figure 3.5. Annual N uptake in herbage dry matter on unfertilised perennial ryegrass plots (GB) and grazed and fertilised perennial ryegrass/white clover pastures (WC) in 2008/09, 2009/10, 2010/11 and 2011/12. Statistically significant differences ($P < 0.05$) between year $\times$ treatments are indicated by different letters.

3.4.5. Factors affecting annual N₂O emissions

There were significant correlations ($P<0.05$) between weather, soil variables and N input with annual N₂O emissions (Table 3.2). There was no significant correlation between annual soil N balances and N₂O emissions from WC.

The best regression model for annual N₂O emissions from WC included mean soil temperature in December and annual rainfall (Table 3.3). The inclusion of annual soil temperature instead of annual rainfall also produced a robust model. Mean soil temperature in December accounted for the largest proportion of variation (highest semi-partial R^2) in both of these models and hence variation in annual N₂O emissions from WC.

Single regression models including either effective rainfall or soil temperature in December were the best model to account for variation in annual N₂O emissions from GB (Table 3.3).

Table 3.2. Pearson correlation coefficients (r) between annual N₂O emissions (n=12) on unfertilised perennial ryegrass plots (GB) and grazed and fertilised perennial ryegrass/white clover pastures (WC) with annual and monthly explanatory variables.

	N input ^b	Soil N balance ^c	N uptake WC	N uptake GB	Rainfall	Effective rainfall	Mean WFPS ^d	Temp December ^e	Annual Temp ^f	N ₂ O WC
N ₂ O WC ^a	0.91***	0.29 ^{NS}	0.65*	0.52 [†]	-0.74**	-0.75**	-0.71**	-0.91***	-0.65*	1
N ₂ O GB ^a	-	-	-	0.51 [†]	-0.69*	-0.74**	-0.46 ^{NS}	-0.74**	-0.71**	0.68*

^a N₂O data was transformed using the natural log transformation: ln (N₂O+1)

^b N input = Fertiliser N + slurry N + excreta N + biologically fixed N + rainfall N deposition

^c Soil N balance = N input minus (N uptake WC minus N uptake GB)

^d WFPS = Mean annual soil water filled pore space (0-5cm)

^e Temp December = Mean monthly soil temperature in December (0-5cm)

^f Annual temp = Mean annual soil temperature (0-5cm)

[†] $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ^{NS} not significant

Table 3.3. Multiple and single linear regressions models accounting for variation in annual N₂O emissions from unfertilised perennial ryegrass plots (GB) and grazed and fertilised perennial ryegrass/white clover pastures (WC) using annual and monthly explanatory variables.

Treatment ^a	Variable ^b	Estimate	S.E.	Semi-partial R^2	P value	Model R^2
WC	Temp Dec	-1.011	0.115	0.83	<0.001	0.95
	Rainfall	0.005	0.001	0.12	<0.001	
WC	Temp Dec	-0.831	0.099	0.83	<0.001	0.94
	Annual temp	1.875	0.487	0.11	<0.01	
GB	E. rain	-0.002	0.001	-	<0.01	0.55
GB	Temp Dec	-0.255	0.073	-	<0.01	0.55

^a N₂O data was transformed using the natural log transformation: ln (N₂O+1)

^b Temp Dec = Mean monthly soil temperature in December (0-10cm), Rainfall = annual rainfall, Annual temp = Mean annual soil temperature (0-5cm), N Input = Fertiliser N + slurry N + excreta N + biologically fixed N + rainfall N deposition, E. rain= annual effective rainfall

3.5. Discussion

3.5.1. Annual N₂O emissions

The mean annual N₂O-N emission of 12.5 kg ha⁻¹ recorded between 2008 and 2012 for the WC pastures was very high compared to the annual average of 1.77 kg ha⁻¹ from multiple managed grassland sites across Europe (Flechard *et al.*, 2007). The high level of N₂O emissions in this study were however, similar to other Irish studies on fertilised grazed perennial ryegrass pastures receiving annual N input of between 53 and 390 kg ha⁻¹ (Hyde *et al.*, 2006; Rafique *et al.*, 2011). The emissions in this study were also within the range of emissions (0.85 to 51.3 kg ha⁻¹) found in fertilised grassland in the UK (Cardenas *et al.*, 2010; Rees *et al.*, 2013).

The annual background soil N₂O-N emissions from GB were also high compared with the range of -0.5 to 1.2 kg ha⁻¹ at multiple grassland sites across Europe (Flechard *et al.*, 2007) and with emissions (circa. 1 kg ha⁻¹) from sandy loam-based pastures in Ireland (Abdalla *et al.*, 2009a). However, emissions within the range of those reported in this study (with the exception of the high rates recorded for 2010/11) have been previously reported for gleysol-based grasslands in Ireland (-1.6 to 4.66 kg ha⁻¹) (Hyde *et al.*, 2006; Rafique *et al.*, 2011).

3.5.2. Global N₂O emission factors

The _GEF for all applied N in this study are somewhat different to IPCC EF which calculate EF individually for fertiliser N, manure N and excreta N. Similar calculations have been carried out on other N₂O studies on grasslands, with _GEF ranging from 0.7 to 5.1% (Hyde *et al.*, 2006; Rafique *et al.*, 2011). The reliability of the _GEF estimates in 2010/11 and 2011/12 is questionable due to the lack of N₂O sampling on GB, particularly over the winter of 2010/11. More intensive gas sampling on GB would have improved the accuracy of these annual _GEF estimates and emphasizes the need for regular measurement of background emissions for accurate EF calculation. Nevertheless, the robust _GEF estimates produced in 2008/09 and 2009/10 (0.5% to 1.6%) were relatively low compared with the other studies outlined above.

3.5.3. Inter-annual variation in N₂O emissions

Few published studies have reported inter-annual variation of N₂O emissions from managed temperate grasslands. There was up to 7.8 fold difference in annual N₂O emissions over the four years in this study. This is significantly higher than other studies on managed grassland sites in Ireland, Wales and the Netherlands where 1.1 to 3 fold differences have been reported over two years (Velthof *et al.*, 1996; Hyde *et al.*, 2006; Cardenas *et al.*, 2010; Rafique *et al.*, 2011). Furthermore, background emissions from the GB plots in this study also displayed up to 3.7 fold differences between years whilst previous studies reported 1.1 to 1.9 fold differences between years (Hyde *et al.*, 2006; Rafique *et al.*, 2011).

Highest emissions from both the WC and GB treatments were measured in 2010/11, which contributed to the large variation between years in the present study. During 2010/11 there were exceptionally low temperatures in winter, lower annual rainfall and the highest N inputs.

3.5.4. Influence of N input on N₂O emissions

Input of N had a large impact on annual N₂O emissions in the present study, with significantly higher emissions of N₂O from WC compared to GB in three years of the study (Figure 3.4). Input of N also explains some of the increase in N₂O emissions in 2010/11 relative to the other years. However, the higher N input to WC in 2010/11 (29 and 39% higher relative to other years) was relatively small compared with the large increase in annual N₂O-N emissions (444 to 682% relative to other years; Figure 3.6a).

Input of N, background supply of N from MSON and the rate of N removal in herbage are all crucial factors driving the availability of soil N for N₂O emissions. Annual soil N balance for WC in 2010/11 was similar to 2008/09 and 2009/10 and indicates that despite the higher N additions in this year, the increase in N uptake in herbage on WC removed much of this additional N from the soil (Figure 3.6b).

All the points above indicate that alternations in N management had a significant but small contribution to the higher N₂O emissions during 2010/11 relative to the effects of weather and soil conditions in the present study.

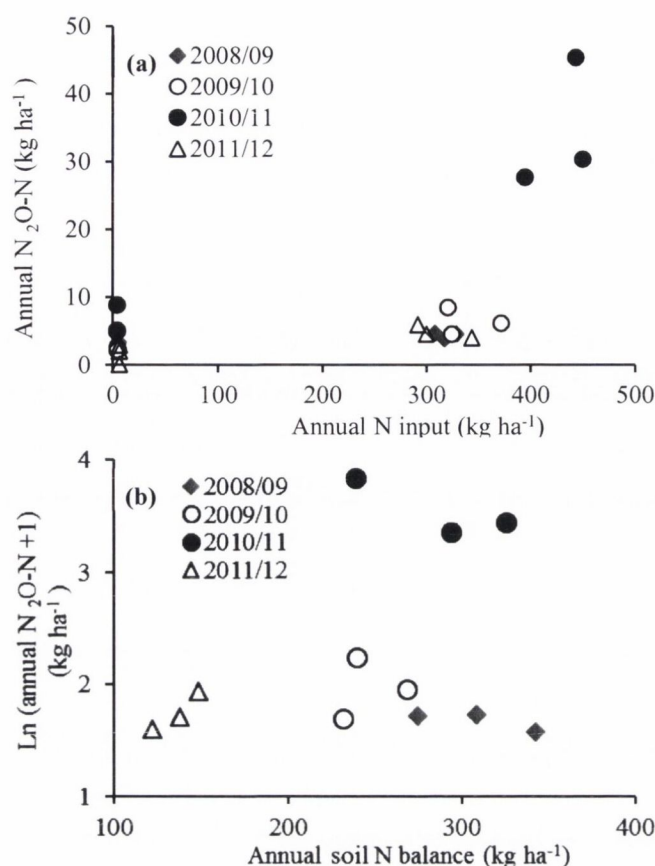


Figure 3.6. Relationship between annual N₂O emissions and N input (a) and lack of relationship between natural log transformed annual N₂O emissions and soil N balances (Pearson correlation $r = 0.29$, $P > 0.05$) (b) using data from unfertilised perennial ryegrass plots (GB) and grazed and fertilised perennial ryegrass/white clover pastures (WC). Annual N input is the sum of fertiliser N, slurry N, N deposited during grazing, biological N fixation and rainfall N deposition. Annual N input was adjusted to account for losses of NH₃ and NO_x assumed to be 10% from fertiliser N and 20% from N in cattle slurry and N deposited during grazing (IPCC, 2006)

3.5.5. Influence of low soil temperatures in December

The low temperatures recorded in December 2010 were atypical of the generally mild winter conditions recorded at this site. Daily mean air temperatures were also low during periods of the winter of 2008/09 and 2009/10 (Figure 3.3b) but maximum daily air temperatures generally remained above zero. In contrast, maximum daily air temperature dropped below zero (minimum of -4.7 °C) for an extended period of six days between 20 and 25 December 2010.

Emissions of N₂O-N during winter 2010 were up to 39 times higher than in other years of this study. The relative cold temperatures in December 2010 are likely to have led to soil freezing and thawing, which have previously been found to stimulate large N₂O emissions

from both grassland and arable soils (Kaiser *et al.*, 1998; Kammann *et al.*, 1998; Lampe *et al.*, 2006). Increased N₂O emissions can be attributed to the release of easily degradable N and C through the lysis of micro-organisms and disrupted soil aggregates and the reduction in N₂O reductase activity (Christensen and Tiedje, 1990; Christensen and Christensen, 1991; van Bochove *et al.*, 2000; Holtan-Hartwig *et al.*, 2002; Müller *et al.*, 2002; Mørkved *et al.*, 2006). Increased emissions during thawing can be explained by the physical release of accumulated N₂O which was produced by active micro-organisms in unfrozen soil water (Teepe *et al.*, 2001) or by a reduction in the diffusion resistance to gases that occurs during thawing (de Bruijn *et al.*, 2009).

3.5.6. Influence of rainfall on N₂O emissions

In the current study N₂O emissions were lower under high rainfall and high WFPS (Table 3.2). These relationships are quite different to most other studies which generally find N₂O emissions to increase following rainfall events and with increasing WFPS (Dobbie and Smith, 2003b; Liu *et al.*, 2007). Taking into account the heavy textured soils and impeded drainage at this site, periods of high rainfall led to a shift from wet to completely saturated soil conditions in the present study, whereas in many other studies it is likely that periods of high rainfall leads to a shift from dry to wet soil conditions. Saturated soil conditions (anaerobic) create more favourable conditions for complete denitrification of NO₃⁻ to dinitrogen gas, rather than nitrification and partial denitrification which would be favoured under lower WFPS (i.e. anoxic soil conditions) which were experienced in 2010/11 in this study (Monaghan and Barraclough, 1993; Rudaz *et al.*, 1999). The relatively low rainfall in 2010/11 allowed the soils at this site to dry out more than normal, which stimulated a transition from completely saturated anaerobic to wet partially aerobic (anoxic) soil conditions (Figure 3.3a). Drying soil conditions is likely to have favoured net mineralisation of soil organic N (Danevčič *et al.*, 2010; Goldberg *et al.*, 2010), which is reflected in the increase in N uptake in herbage DM on GB in 2010/11, and which provided additional substrate for nitrifying and denitrifying bacteria.

Nitrification has been found to be a major contributor to N₂O under WFPS ranging from 40 to 60% (Stevens *et al.*, 1997; Bateman and Baggs, 2005). The reduction of WFPS to between 30-80% during these periods in the present study is likely to have allowed both nitrification and partial denitrification to contribute to N₂O production simultaneously or lead to coupled nitrification-denitrification (Wrage *et al.*, 2001). This may explain some of

the increase in N₂O emissions in 2010/11 relative to other years. The results confirm that N₂O emissions are very much increased under these anoxic soil conditions. Management decisions which support, or lead to, such soil conditions should be avoided if N₂O emissions from grasslands, such as those in the present study, are to be mitigated.

3.5.7. Implications of the study

The results indicate that annual variation in N₂O emissions from WC were regulated by weather and soil variables and N input. The dominance of weather driven variables in regulating inter-annual variation has implications in the context of future climate scenarios where increased weather volatility, including temperature extremes and patterns of rainfall are predicted (Solomon *et al.*, 2007; Miraglia *et al.*, 2009). Based on the evidence gathered, the hypothesis of this study, that nitrous oxide emissions from a N fertilised and grazed grass/clover pasture vary inter-annual due to changes in weather conditions over time, is accepted.

Many studies have stressed the errors and uncertainty associated with N₂O chamber measurements (Smith and Dobbie, 2001; Rochette and Eriksen-Hamel, 2007; Kroon *et al.*, 2008). The results of this study suggest that short-term studies may lead to bias and the underestimation or likewise the overestimation of annual N₂O emissions. At least two to three years of measurement may be required to gain a representative estimate of annual N₂O emissions from an individual site or production system which contrasts with the recommendations of Velthof *et al.* (1996). The significant contribution of the winter of 2010/11 to annual N₂O emissions also highlights the need for frequent N₂O measurements over winter periods even in temperate grazed grasslands as disregarding this period could lead to underestimations of annual N₂O emission estimates. The large variation in emissions that occurred in this study also highlights the need for regular measurement of background emissions to provide robust data for EF calculation.

CHAPTER 4. A COMPLETE FARM N BALANCE FOR A PASTURE-BASED SYSTEM OF DAIRY PRODUCTION UNDER MOIST MARITIME CLIMATIC CONDITIONS

Based on: Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. A complete farm N balance for a pasture-based system of dairy production on a heavy textured soil and under moist maritime climatic conditions. Agriculture, Ecosystems and Environment (under review)

4.1. Abstract

A small proportion (20-40%) of nitrogen (N) input to pasture-based livestock production systems is converted to saleable agricultural products. The remaining N (surplus N) is largely unaccounted for and can be lost from the system through a range of pathways with potential environmental consequences. The objectives of this study were to (i) account for all N inputs and outputs in a pasture-based dairy system and (ii) determine the relative importance of the components of this balance. Detailed N flow measurements were carried out on a pasture-based dairy production system in Ireland (52°51'N, 08°21'W) in 2011 and 2012. The pasture-based dairy production was on a heavy textured soil and under moist maritime climatic conditions. Total N inputs and outputs (N exports: 80 kg N ha⁻¹ and losses: 180 kg N ha⁻¹) were 245 and 260 kg N ha⁻¹, respectively, averaged over both years. Of N lost by various pathways, 6.5% was lost to groundwater, 38.5% as ammonia, 8.5% as nitrous oxide and 46% as dinitrogen gas. Although N surpluses on pasture-based livestock production systems can be substantial, the results of this study suggest that a high proportion of surplus N may be lost as environmentally benign dinitrogen gas. This study also highlights where the emphasis should be (ammonia and nitrous oxide) in terms of minimising environmentally damaging N losses and thereby improving the N-efficiency of such systems of dairy production.

4.2. Introduction

Intensive pasture-based livestock production systems are reliant on imports of nitrogen (N) in fertiliser and purchased feeds to sustain high production. A relatively low proportion (20-40%) of this N is converted to saleable agricultural products (milk and meat) leaving large surpluses of N that are largely unaccounted for (Treacy *et al.*, 2008; Mihailescu *et al.*, 2013). This has raised concerns over environmental impact with many studies highlighting the extent of nitrate losses to water and emissions of nitrous oxide (N₂O) and ammonia (NH₃) from such systems (Jarvis and Ledgard, 2002; Bouman *et al.*, 2010; Li *et al.*, 2011).

Input and output N balance sheets can be used to determine the amount of surplus N generated by a production system. The most popular form is a farm-gate N balance, due to its relative ease of completion, where the N entering (fertilisers, feeds and manures) and leaving (N in agricultural products, such as milk and meat) through the farm-gate is taken into account (Oenema *et al.*, 2003; Schröder *et al.*, 2003). They may also include N inputs such as deposition in rainfall and biological N fixation associated with legumes (Ledgard

et al., 1999; Schröder *et al.*, 2003). Numerous farm-gate N balances have been completed internationally (Neuens *et al.*, 2006; Bassanino *et al.*, 2007; Treacy *et al.*, 2008; Buckley *et al.*, 2013). These balances are useful for estimating potential for losses and for comparing the potential environmental impact of production systems (Oenema *et al.*, 2003). However, they do not differentiate between various components such as N loss to groundwater, N₂O, NH₃, dinitrogen (N₂) gas or accumulation of N in soil organic matter with differing environmental impacts. Many other studies have investigated losses of individual components from pastures and farm building (Misselbrook *et al.*, 1998; Sommer and Hutchings, 2001; Bouman *et al.*, 2010; Necpálová *et al.*, 2012; Burchill *et al.*, 2014b) but despite much research, there is a lack of holistic studies encompassing and accounting for all inputs, exports and losses from the production system.

The objectives of this study, therefore, were to (i) account for all N inputs into and outputs from a pasture-based dairy system on a heavy textured soil and under moist maritime climatic conditions and (ii) determine the relative importance of the components of this balance sheet. This study hypothesises that environmentally benign N₂ gas accounts for between 40 to 65% of the previously unaccounted for farm surplus N on a dairy production system in Ireland under moist maritime climatic conditions on a clay loam soil with impeded drainage.

4.3. Materials and Methods

4.3.1. Site description

This study was carried out at the Teagasc Solohead Research Farm (52°51'N, 08°21'W; 95 m asl). The predominant soils are poorly drained Gleys (90%) and Grey Brown Podzolics (10%) with a clay loam texture (29% clay, 36% silt and 34% sand) and low permeability. The soils are seasonally wet, waterlogged or flooded due to impeded drainage and a shallow water table depth (0 to 2.2m below ground level) at the site. Mean soil organic carbon, total N content, bulk density and pH at 0 to 10 cm depth is 5.12%, 0.54%, 0.87 g cm⁻³ and 6.2, respectively. The permanent grassland at the site is used predominately for grazing and occasionally harvested for silage. The pastures range from 20 to 30 years in age. Mean annual rainfall at the site (1997-2010) was 1036 mm. Mean minimum monthly soil temperature (1998-2010) at 0-10cm depth ranges from 1.7 to 8.5°C during winter (November-January) and maximum monthly temperatures range from 10.2 to 18.2°C in summer (May-July).

4.3.2. Experimental layout and design

All experimental measurements were carried out on a perennial ryegrass (*Lolium perenne* L.)/white clover (*Trifolium repens* L.) based system of dairy production. The system consisted of six paddocks ranging in size from 1.48 to 2.07 ha with a total area of 10.67 ha and an annual stocking density (spring calving Holstein-Friesian cows) of 2.25 cows ha⁻¹ in 2011 and 2.35 cows ha⁻¹ in 2012. Cows were spring-calving Holstein-Friesian; the entire herd was calved in a three-month period in spring (mid-January to mid-April). Cows were turned out to pasture after calving. Three of the paddocks were rotationally grazed during the main grazing season (March to November) with surplus herbage occasionally removed as baled silage. The other three paddocks were rotationally grazed in spring, closed in early April and harvested for silage in late May or early June. Thereafter these paddocks were either closed for a second cut of silage (harvested in late July) or rotationally grazed thereafter until the end of the grazing season in November or December. The grazing rotation length varied from 21 days in spring/summer to 42 days in autumn. Post grazing sward height, maintained at 4 cm, was measured using a rising plate meter (Grasstec, Charleville, Ireland) and used to determine when cows were moved to the next paddock.

Cows were housed during the winter (November to February) in one group containing cows from other experimental systems on the farm. All slurry produced was contained in one tank. To ensure that the appropriate volume of slurry was applied back to the system, standard values for slurry production per cow were used (SI, 610, 2010). The calculated quantity of slurry was applied back to the system in early spring and prior to closing for silage. Fertiliser N was applied in a number of applications between early spring and early summer (Table 4.1).

Table 4.1. Applications of fertiliser N on paddock managed for grazing and first cut silage on the perennial ryegrass/white clover based system of dairy production at Solohead Research Farm in 2011 and 2012.

Date	Fertiliser N (kg ha ⁻¹) ^a	
	Grazing	Silage
2011		
24 th February	28.8	28.8
22 nd March	28.8	28.8
20 th April	28.8	84
Annual	86	141
2012		
15 th February	28.8	28.8
13 th March	28.8	
3 rd April		95
2 nd May	28.8	
Annual	86	124

^aFertiliser N was applied as Urea (46% N)

The system boundary was the six paddocks within the dairy production system and the herd of dairy cows (24 in 2011 and 25 in 2012) contained within the system. The rearing of replacement heifers was not included. This dairy production system was established on the Solohead Research Farm in 2000 and has been under consistent management since its establishment. Measurements in this study commenced in October 2010 and continued for two years of production: 2011 and 2012.

4.3.3. Soil and weather measurements

Daily weather data including rainfall (mm), air temperature (°C) and soil temperature at 10 cm depth (°C) were recorded at the experimental site as described by Fitzgerald & Fitzgerald (2004).

4.3.4. N inputs

Annual rainfall N deposition was calculated by multiplying annual rainfall by its total N concentration, determined bi-weekly, as described by Necpálová *et al.* (2013b). Nitrogen imported in feed was calculated by multiplying the quantity of imported feed, mainly concentrates, by its crude protein content and dividing by 6.25 (McDonald *et al.*, 1995). Above ground biological N₂ fixation was determined in each paddock using the ¹⁵N isotope dilution and the ¹⁵N natural abundance technique and clover yield using

methodology described in full by Burchill *et al.* (2014a) (Chapter 2). A correction factor of 1.28 was used to account for N₂ fixation in belowground clover biomass (Vinther, 2006).

4.3.5. N exports in product

Output of N in milk was calculated by multiplying individual cow milk yield by milk protein content assuming the milk protein contained 0.156 g g⁻¹ N as described by Humphreys *et al.*, (2008b). Nitrogen exported in calves was estimated as the average birth weight of the calves multiplied by 0.0295 kg N kg⁻¹ live-weight. The change of N in cow live-weight was calculated as described by Humphreys *et al.*, (2008b).

4.3.6. Nitrogen losses

Nitrogen losses to groundwater were measured using 20 screened wells (1 to 2 m depth) per paddock using methodology described by Necpálová *et al.*, (2012). Wells were sampled on a bi-weekly basis during the winter drainage period (October to March) and, during and following periods of heavy rainfall during the remainder of the year.

A closed static chamber technique was deployed to measure N₂O emissions from each paddock using methodology described in full by Burchill *et al.* (2014b) (Chapter 3): There were five static chambers per paddock. The N₂O sampling was conducted (between 9:30 am and 1 pm) on a weekly basis with increased frequency (three times *per week*) following N applications on each paddock during both years.

Dinitrogen emissions were determined using a combination of N₂O emissions (see above) and measured bi-monthly N₂:N₂O ratios. The N₂:N₂O ratio was measured using the ¹⁵N gas flux method (Stevens and Laughlin, 1998) on twelve bi-monthly occasions between February 2011 and December 2012. Four static chambers were deployed in each paddock. The chambers consisted of two parts: a permanent collar and a chamber, both of which were constructed from PVC pipe. The headspace height of the chamber was 9 cm with an internal diameter of 29.5 cm, resulting in a cover area of 0.0683 m² and a headspace volume of 0.0065 m³. The collars were inserted into the soil to a depth of 12 cm and extended 4 cm above the soil surface following installation. The collars were exposed to grazing livestock throughout the experiment. A rubber septum was permanently inserted into the top of each chamber for gas sample extraction. A rubber bung with a 5mm diameter hole was also permanently inserted into the top of each chamber. This hole was

left open when placing the chamber on the collar to prevent the build-up of air pressure inside the chamber. A thermo-sensor (Sensor-Tech Ltd, Castlebellingham, Ireland), was inserted into this hole following chamber closure which created an air tight seal between the chamber and the collar and allowed for air temperature recording within the chamber.

Double labelled $^{15}\text{NH}_4^{+15}\text{NO}_3^-$ enriched to 98 atom% ^{15}N was applied at a rate of 0.2 g N per collar (10 kg N ha^{-1}) at the beginning of each measurement period. The ^{15}N labelled fertiliser was applied to the collars in an aqueous solution (100 ml per collar) using an atomiser. The atomiser was subsequently rinsed twice with a total of 200 ml of deionised water to ensure the complete transfer of the ^{15}N labelled fertiliser from the atomiser to the soil. The collars were moved to new random locations in each replicate one week before each ^{15}N application. Grass within the collars was cut to 3 cm above ground level and removed three days before the applications, to improve transfer of the ^{15}N fertiliser to the soil.

Sampling of N_2O and N_2 was conducted every 24 hours (between 9:30 am and 1 pm) for a period of 10 days after each ^{15}N application. At each sampling the chambers were placed on the collars. Gas samples were extracted from the chamber using an air tight syringe by inserting a needle through the rubber septum on the top of each chamber (Figure 4.1). Gas samples (12 ml) were taken immediately after chamber closure (T_0) and again after ten minutes (T_{10}) and two hours (T_{120}). These samples were transferred to 7 ml screw-cap exetainers with Teflon-coated butyl septa (Perbio Science, Cambridge, UK). A second gas sample (15 ml) was taken, using the same procedure, at T_{120} and transferred to a 12 ml evacuated septum capped glass vial with the same cap and septa (Labco Limited, Ceredigion, UK). Atmospheric gas samples were taken at each sampling and transferred to 12 ml evacuated septum capped glass vials.



Figure 4.1. Extraction of gas samples, for N_2O and N_2 concentration and isotopic composition, from the static chambers used in the study.

Pre-experimental testing showed a linear increase in N_2O concentration in the chambers over a 60 minute test period. Therefore, daily N_2O fluxes were calculated using T_0 and T_{10} samples as described by Burchill *et al.* (2014b). The isotopic composition of the N_2 and the concentration and isotopic composition of N_2O , in samples transferred to 12 ml screw-cap exetainers, were determined using a ThermoFinnigan GasBench + PreCon trace gas concentration system interfaced to a ThermoScientific Delta V Plus isotope-ratio mass spectrometer (Bremen, Germany) at the Stable Isotope Facility of the University of California, Davis, USA. The daily N_2 flux (kg N ha^{-1}) was calculated according to the methodology and equations of (Mulvaney, 1984; Mulvaney and Boast, 1986; Mosier and Schimel, 1993; Stevens and Laughlin, 2001b).

The mean $\text{N}_2:\text{N}_2\text{O}$ ($\text{N}_2 \div \text{N}_2\text{O}$) ratio of each measurement period was calculated as the mean of daily ratios recorded in each measurement period. A daily N_2 flux from each paddock was calculated by multiplying daily N_2O fluxes, determined above, by a daily $\text{N}_2:\text{N}_2\text{O}$ ratio. Daily $\text{N}_2:\text{N}_2\text{O}$ ratios were calculated by linear interpolation between mean $\text{N}_2:\text{N}_2\text{O}$ ratios of each measurement period. Annual N_2 emissions were calculated as the sum of calculated daily N_2 fluxes.

Losses of ammonia gas during winter housing, grazing, milking periods, land application of slurry and slurry storage from the system were estimated using a mass flow approach based on the flow of total ammoniacal N (TAN) through the farm system (EMEP/EEA, 2013a; EPA, 2014). Ammonia emissions were calculated using the following NH_3

emission factors: 6% of TAN deposited on grazed pastures, 32% of TAN deposited indoor during winter, 75% of TAN deposited on concrete yards during milking periods and 5% of TAN stored as slurry that formed a crust during the winter. Losses during land application of slurry were calculated assuming that 48% of TAN in summer applied slurry is volatilised and 26% of TAN in winter, spring and autumn applied slurry (EPA, 2014). Losses of N_2O , NO and N_2 from slurry storage were estimated using emission factors as described by EMEP/EEA (2013a). Emissions were based on annual N excreted by the dairy cow which was calculated considering the annual intake of N in feed and exported in product from the cow as described by Humphreys *et al.* (2008b). A TAN content of 52% of total N excreted was used based on the slurry generated on the farm. Losses of NH_3 from fertiliser N application were estimated using fertiliser specific emission factors (EMEP/EEA, 2013b).

4.3.7. Farm N balances and calculations

The farm N balance for the dairy production system was calculated, to determine the farm N surplus, considering the difference between N inputs (rainfall N deposition, fertiliser N, N in imported feeds and biological N fixation) and the N exported in agricultural products (milk, sold calves and the change in live-weight of dairy cows between entering and leaving the herd). The efficiency of N use was calculated as the N exports divided by the N inputs. A complete farm N balance was calculated for the dairy production system as the difference between N inputs and total N outputs where total N outputs is the sum of N exports and measured N losses (sum of N_2O , N_2 , NH_3 , N losses to groundwater and gaseous N emissions from slurry storage).

4.3.8. Statistical analysis

Data were statistically analysed using SAS 9.3 (SAS 2011, Institute Inc., Cary, NC, USA). Data were checked for normality by using residual normality and variance analysis. The dairy production system was not replicated, therefore, differences between year were not determined for variables with single values such as NH_3 volatilisation, total N input, total N exports, farm N surplus and complete farm N balance. The variables BNF, N losses to groundwater, N_2O emissions and N_2 emissions were analysed using ANOVA to determine the effect of year using variance between replicate paddocks ($n=6$). Likewise, the difference between years in N input in concentrates fed and output in milk was analysed using the variance between cows ($n=24$ in 2011 and $n=25$ in 2012).

Single and multiple linear regression (stepwise) analysis were carried out to create simple prediction equations for annual N₂O and N₂ emissions using N input, weather and soil variables. Simple statistics (mean, standard deviation, maximum and minimum) were performed on a collection of measured and predicted data to determine the effect of including denitrification N losses on complete farm in balances over an eleven year period (2001 to 2012). Annual N losses to groundwater, N₂O emissions and N₂ emissions required a natural log transformation. A statistical probability of $P<0.05$ was considered significant for all statistical tests.

4.4. Results

4.4.1. Soil and weather conditions

Annual total rainfall was 1315mm in 2011 and 1147mm in 2012. The majority of annual rainfall occurred over three months (November, December and February) in 2011, with relatively low rainfall for the remainder of the year (Figure 4.2a).

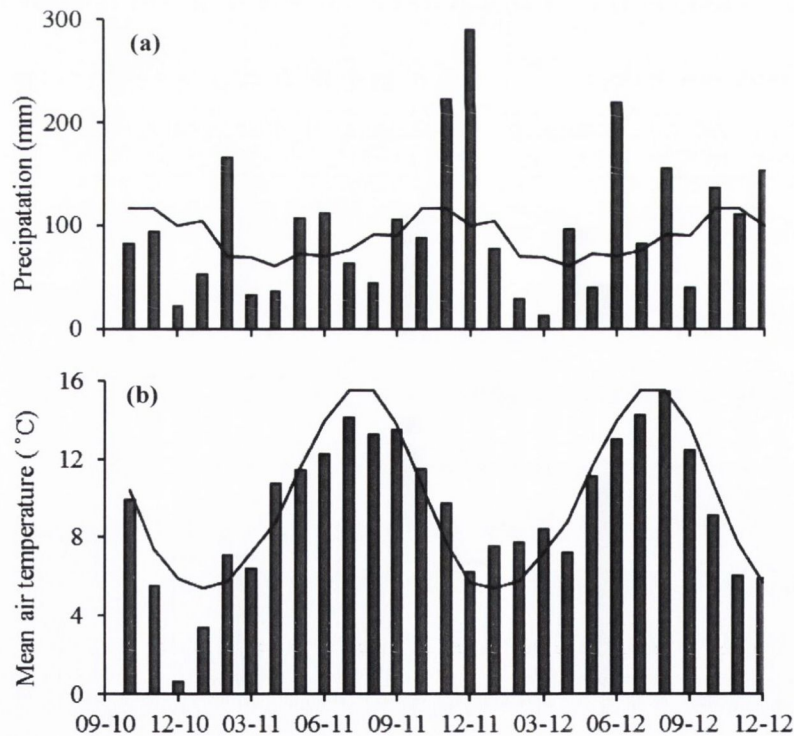


Figure 4.2. Monthly cumulative precipitation (a) and mean monthly air temperature (b) at Solohead Research Farm during the experimental period (mm-yy). Solid lines indicate long-term mean monthly rainfall and air temperature.

In 2012, the majority of the rainfall occurred from June onwards. Monthly mean air temperatures were generally similar to the long-term average with the exception of the

winter of 2010/2011 (below average) and winter/spring of 2011/2012 (above average) (Figure 4.2b).

4.4.2. N inputs and exports in products

Fertiliser N and biological N fixation were the largest contributors to N imports (Table 4.2). Biological N₂ fixation was higher in 2011 than 2012, while N imported in concentrates was higher in 2012 than 2011. Removal of N in milk was the dominant export of N from the system. Total N exported in products accounted for 29% and 37% of total N inputs in 2011 and 2012, respectively. The annual farm N surplus (kg ha⁻¹) was 193 in 2011 and 138 in 2012.

4.4.3. Nitrogen losses

Losses of N to groundwater, N₂O and N₂ emissions from the system were higher ($P<0.001$) in 2011 than 2012 (Table 4.2).

Table 4.2. Farm N balance for the perennial ryegrass/white clover based system of dairy production at Solohead Research Farm in 2011 and 2012.

	2011	2012		2011	2012
	kg N ha ⁻¹			%	
N inputs					
Rainfall N deposition	6	5		2	2
Fertiliser N	114	105		42	48
Biological N ₂ fixation	118 (21) ^a	49 (9)	***	43	22
Purchased concentrates	34 (5)	59 (7)	***	13	27
Total N inputs	272	218			
N exports in products					
Milk sales	74 (13)	75 (10)	N.S.	94	94
Livestock sales + live-weight change	5	5		6	6
Total N exports	79	80			
Farm N surplus	193	138			
Nitrogen losses					
N lost to groundwater	21 (8)	5 (2)	***	9	4
NH ₃ volatilisation	63	64		27	50
Nitrous oxide	25 (10)	8 (6)	***	11	6
Dinitrogen	123 (97)	50 (19)	***	53	39
NO, N ₂ O and N ₂ from slurry storage	0.8	0.9		<1	<1
Total N outputs	312	208			
Farm N balance	-40	10			

^a values in parenthesis represent the standard deviation of either six replicate paddocks or 24/25 replicate cows on the production system.

*** and N.S. indicate differences in values between 2011 and 2012 at $P<0.001$ and $P>0.05$, respectively.

Ammonia volatilisation was the largest environmentally damaging N loss from the system in both years. Losses from denitrification ($\text{N}_2 + \text{N}_2\text{O}$) accounted for 64% and 45% of total N losses in 2011 and 2012, respectively. Dinitrogen emissions were much higher than N_2O emissions in both years and accounted for 46% of total N losses on average, over the two years. Estimated gaseous N losses from slurry storage was negligible at $<1 \text{ kg N ha}^{-1} \text{ yr}^{-1}$. Total N outputs exceeded total N inputs by 40 kg N ha^{-1} in 2011 while 10 kg N ha^{-1} was unaccounted for in 2012.

4.5. Discussion

This study measured N imports, exports in products and losses to determine a complete farm N balance over two years under contrasting weather conditions. The accuracy of N balances will depend on the methods used to quantify N flows in the balance (Ledgard *et al.*, 1999; Watson and Atkinson, 1999). The N flows associated with fertiliser N, concentrates fed to livestock, N exported in milk and live-weight change were relatively easy to determine accurately. Biological N_2 fixation, N losses to groundwater, N_2O emissions and N_2 emissions were measured on each of six replicate paddocks using the most up-to-date and appropriate techniques for field measurements of these N flows (Hutchinson and Moir, 1981; Stevens and Laughlin, 1998; Unkovich *et al.*, 2008; Necpálová *et al.*, 2012). Ammonia volatilisation from the system and gaseous N losses from slurry storage were estimated in this study and are likely to be the least accurately determined N flow. However, the estimated annual $\text{NH}_3\text{-N}$ losses were within the range (25 to 78 kg ha^{-1}) of emissions measured from other pasture-based livestock production systems (Ledgard *et al.*, 1999; Eckard *et al.*, 2007) and within those reported for intensive dairy production systems in the northwest of Europe (36 to 105 kg ha^{-1}) (Bussink and Oenema, 1998; Jarvis and Ledgard, 2002).

4.5.1. N inputs

Fertiliser N was the largest contributor (45%) to N inputs when averaged across the two years, however, this proportion was relatively low compared to the average values of between 80% and 86% previously reported for pasture based dairy production systems in Ireland (Treacy *et al.*, 2008; Buckley *et al.*, 2013; Mihailescu *et al.*, 2013). This system was designed to exploit biological N_2 fixation, therefore, fertiliser N input was restricted as BNF has been found to decline with increasing fertiliser N input (Hogh-Jensen and Schjoerring, 1994; Ledgard *et al.*, 2001; Burchill *et al.*, 2014a). Biologically fixed N was

the second largest contributor to N inputs but varied between years. Differences in weather conditions between years led to a decline in clover yield and hence BNF from 2011 to 2012 (Burchill *et al.*, 2014a). The input of N from BNF of free-living non-symbiotic bacteria was not measured in this study and is usually relatively small ($<5 \text{ kg N ha}^{-1}$) in agricultural ecosystems (Unkovich *et al.*, 2008). Lower pasture production and utilisation in 2012, as a result of high rainfall and saturated soil conditions during the main growing season, led to higher N input in the form of purchased concentrates in 2012 compared to 2011. Atmospheric N deposition was a minor contributor to N input in both years.

4.5.2. N removed in products

The N removed in milk accounted of 96% of N output in products in both years and was relatively similar to previous studies with similar system boundaries (87.9 to 94%) in Ireland (Humphreys *et al.*, 2008b; Ryan *et al.*, 2012) and other European countries (Raison *et al.*, 2006). This percentage was, however, somewhat higher compared to the mean figures of 78% (Treacy *et al.*, 2008), 76% (Buckley *et al.*, 2013) and 76% (Mihailescu *et al.*, 2013) previously reported for commercial pasture based dairy production systems in Ireland. The system in the current study did not include the rearing of replacement heifers, which is common practice on commercial farms and explains this discrepancy. Furthermore, the N exported in livestock sales and live-weight change was relatively small from this system. Firstly, the main livestock sales were those of young calves at an average live-weight of 42 kg and much of the N removed in the sale of cull cow was offset by the replacement of these cows in the system with two year old pregnant heifers.

4.5.3. N losses

Losses of N to groundwater were relatively small in this study and have been consistently low ($<35 \text{ kg N ha}^{-1}$) at this site for up to ten years (Humphreys *et al.*, 2007; Humphreys *et al.*, 2008a; Necpálová *et al.*, 2012). Nitrate-N concentrations (mg l^{-1}) (0.8 to 5.5: mean 2.7) in groundwater in 2011 and 2012 were well below the maximum allowable concentration of $11.3 \text{ mg NO}_3\text{-N l}^{-1}$ under the European ND (EC, 1991).

The impermeable nature of the soil and predominantly saturated soil conditions were more conducive to soil processes such as denitrification, which is indicated by the substantial emissions of N_2 and N_2O from the system. Denitrification losses were within the range found in other clay soils in the Netherlands (van der Salm *et al.*, 2007) but higher than

those on free draining silt loams in New Zealand (Ledgard *et al.*, 1999). Estimated N losses from NH₃ volatilisation were the highest environmentally damaging N loss from the system. Land spreading of cattle slurry (32%), N deposited by cows while indoors (26%) and fertiliser N application (24%) were the major sources of NH₃-N losses.

There was substantial variation in measured N losses between years with between 2 and 4 fold higher N₂O, N₂ and N leaching losses in 2011 than 2012 in this study. This may be due to the higher total N inputs and farm N surplus in 2011 as many studies have found these N losses to increase as N input increases (Ruz-Jerez *et al.*, 1994; Bouman *et al.*, 2010; Cardenas *et al.*, 2010). Temporal variation in weather conditions also contributed to differences in N losses between years (Burchill *et al.*, 2014b). The natural drying of these predominantly saturated soils under below average rainfall over the summer of 2011, and atypically low temperatures that occurred during the winter of 2010/2011 is likely to have stimulated the release of soil organic matter N through mineralisation providing additional N for these losses (Burchill *et al.*, 2014b).

4.5.4. Complete farm N balance

Within the two year duration of the experiment the N inputs more-or-less equalled N outputs within experimental error. To our knowledge, only one other N balance study has carried out detailed N flow measurements which accounted for all N inputs (Ledgard *et al.*, 1999). Other N balances have been largely based on estimates of N flows or have been unable to account for all N inputs (Watson and Atkinson, 1999; Eckard *et al.*, 2007; Humphreys *et al.*, 2008a).

For example, Humphreys *et al.* (2008a) was unable to account for between 45 to 60% of the N balance at this site where measurements of N leaching and estimates of ammonia volatilisation were made and suggested that denitrification was the most likely N loss processes for this unaccounted for N, given the heavy textured soils with high soil organic C and moist maritime climate at this site.

As pointed out above the system of dairy production described in this study was established on Solohead Research Farm in 2000 and has since been under consistent management. Data from this system between 2001 and 2010 (excluding 2007 due to lack of data) were collated and compared with the results of the current study (Humphreys *et al.*, 2007; Humphreys *et al.*, 2008a; Humphreys *et al.*, 2008b; Necpálová, 2012; Burchill *et*

al., 2014b). The existing data included measurements of N inputs, N surpluses, N losses to groundwater and N₂O emissions. Ammonia volatilisation was recalculated using the up-to-date methodology described above. Emissions of N₂O were measured for two of these years (Burchill *et al.*, 2014b) and emissions for the other years were estimated using a multiple regression equation (Table 4.3) developed from four years of data. Emissions of N₂ were estimated using an exponential equation developed with N input from data from individual paddocks in the present study and non-grazed perennial ryegrass/white clover plots receiving no N input (unpublished data) (Table 4.3).

Table 4.3. Single and multiple regression models used to predict annual N₂O and N₂ emissions on the perennial ryegrass/white clover based system of dairy production at Solohead Research Farm.

Dependant variable	Independent variable ^a	units	n	Relationship	P value	Model R ²
N ₂ O	N input (x)	kg N ha ⁻¹ yr ⁻¹	23	Y= -0.162 + 0.004x + 0.004w -0.005z	<0.001	0.74
	Rainfall (w)	mm				
	GS rainfall (z)	mm				
N ₂	N input (x)	kg N ha ⁻¹ yr ⁻¹	15	Y= 3.6606e ^{0.0069x}	<0.001	0.90

^aN input = fertiliser N + biologically fixed N + N deposited by grazing livestock + slurry N + rainfall N deposition; Rainfall = total annual precipitation; GS rainfall = precipitation during the main growing season (March to October)

Inclusion of estimated N₂O and N₂ emissions virtually closed the farm N balance and accounted for 40% of the farm N surplus on average over the eleven years (Table 4.4). Both positive and negative N balances were calculated in individual years but averaged 10 kg N ha⁻¹ yr⁻¹ over 11 years. The management of the dairy system in the present study was relatively consistent over a number of years. In such a situation it is expected that the soil organic N pool will be relative stable. However, net soil organic N mineralisation or likewise immobilisation can take place in individual years due to temporal variations in these processes. These flows of N can act as an additional N source or sink in individual years and may account for the fluctuations between negative and positive farm N balances between years. For this reason, it is important that farm N balances should be studied over a number of years.

Table 4.4. Effect of including a combination of measured and predicted annual N₂O and N₂ emissions on farm N balances over 11 years on a perennial ryegrass/white clover based system of dairy production at Solohead Research Farm between 2001 and 2012.

	Period of time	Mean	Stdev ^a	Min ^b	Max ^c	Mean
	Years	kg N ha ⁻¹ yr ⁻¹				% of farm N surplus
Farm N surplus	11	157	18	136	193	100
N losses to groundwater	11	22	7	5	32	14
NH ₃ volatilisation	11	61	8	48	73	39
NO, N ₂ O and N ₂ from slurry storage	11	0.8	0.1	0.6	1.1	<1
Farm N balance excluding N₂O+N₂	11	74	19	48	108	54
N ₂ O	11	8	6	3	25	5
N ₂	11	56	27	32	123	35
Farm N balance including N₂O+N₂	11	10	31	-40	55	93

^a standard deviation

^b Minimum

^c Maximum

4.5.5. Implications

Globally, permanent pastures account for up to 68% of all agricultural land. Agricultural land in countries/regions such as Ireland (90%), northwest Europe (50%), New Zealand (95%), Australia (88%) and South America (76%) are dominated by permanent grassland (FAO, 2014). Areas with high precipitation and heavy soils tend to have pasture-based livestock production systems due, in part, to the inappropriateness of the soil and climate conditions for alternative land uses (Humphreys *et al.*, 2009a). Pasture-based systems require large inputs of N to sustain high levels of herbage production. The N content of such herbage often exceeds the dietary N requirement of ruminants resulting in inefficient use of N by the grazing animal (75 to 95% of dietary N intake is excreted) (Lantinga *et al.*, 1987; Humphreys *et al.*, 2003), as a consequence pasture-based systems tend to have high N surpluses. However, as this study illustrates, once the system is based on heavy soil in high rainfall areas, damaging losses to water can be minimal and a large proportion of surplus N can be lost by denitrification (attenuation of nitrate in the soil before it is leached), most of which is environmentally benign N₂ gas.

The results of this study identified losses of NH₃ and N₂O to be the most prevalent environmentally damaging N losses. Mitigation of these N losses should be investigated and prioritised to improve the sustainability of such systems. Mitigation strategies should focus on the major sources of such N losses, for example, land-spreading of manure and housing emissions for NH₃ and emissions associated with N management and grazing for N₂O in this study. The effectiveness of numerous mitigation strategies for both N losses

have been reviewed on many occasions (Saggar *et al.*, 2004; Ndegwa *et al.*, 2008; Webb *et al.*, 2010; Li *et al.*, 2013; Saggar *et al.*, 2013; Schils *et al.*, 2013). The aim of any N₂O or NH₃ mitigation option should be to reduce losses in a sustainable and cost effective manner while not increasing other N losses or greenhouse gasses such as methane or CO₂.

Further N balance studies of a similar nature to the present study are required to identify the most important N loss pathways under different soil, climatic and management conditions. This will allow targeted mitigation strategies to reduce the most prevalent N losses which should ultimately lead to improved sustainability of pasture-based livestock production systems.

4.6. Conclusions

Measured N outputs accounted for measured N inputs within experimental error in this study. Fertiliser N and BNF were the largest contributors to N inputs and N removed in milk sales was the largest export. Complete farm N balances ranged from -40 to 10 kg N ha⁻¹ yr⁻¹. Nitrogen losses to water were relatively low and NH₃ volatilisation was the largest environmentally damaging N loss from the system. Denitrification was the largest N loss pathway from this production system under the heavy textured soils and moist maritime climatic conditions at this site. This study hypothesised that environmentally benign N₂ gas would account for between 40 to 65% of the previously unaccounted for farm surplus N on a dairy production system in Ireland based on a clay loam soil under moist maritime climatic conditions. The results suggest, indeed, that a high proportion (46%, on average over two years) of surplus N was removed from this pasture based livestock production systems as environmentally benign N₂ gas. This hypothesis is, therefore, accepted. This study also highlights where the emphasis should be (NH₃ and N₂O) in terms of minimising environmentally damaging N losses on such systems of dairy production.

CHAPTER 5. GENERAL DISCUSSION & CONCLUSIONS

5.1. Findings of the study

5.1.1. Biological N₂ fixation on white clover based pastures in Ireland

Chapter 2 quantified the N input from BFN on cut grass/clover pastures with differing fertiliser N inputs under Irish conditions. Annual above-ground BNF ranged from 14 kg N ha⁻¹ to 128 kg N ha⁻¹ depending on fertiliser N input and year. There was substantial variation in BNF between years. Similar observations have been made elsewhere (Ledgard *et al.*, 2001; Carlsson and Huss-Danell, 2003). This inconsistent and variable supply of N from BNF may be a deterrent to the adoption of grass/clover pastures for farmers. A large proportion of the variation in BNF (y: kg N ha⁻¹) was accounted for using a multiple regression model including both annual clover yield (x: kg DM ha⁻¹) and annual fertiliser N input (z: kg N ha⁻¹) in this study ($y = 24.999 + 0.022(x) - 0.1138(z)$, $R^2=90$, $P<0.001$). Fertiliser N input can be easily controlled and can be applied strategically to grass/clover pastures. Moderate applications (<90 kg N ha⁻¹) in spring have been recommended as, clover is dormant and thus N supply from BNF is low in this period (Humphreys and Lawless, 2008). Intermediate applications (86 to 140 kg N ha⁻¹ yr⁻¹) of fertiliser N in spring increased annual herbage production while having a substantially lower effect on BNF compared to higher fertiliser N inputs (280 kg N ha⁻¹ yr⁻¹) in this study. Clover yield, on the other hand, is much more difficult to control as it can be influenced by factors such as weather and competition from companion grasses (Frame and Newbould, 1986), which was evident in this study. Adjusting defoliation frequency and post-grazing sward height to suit clover growth can be used to reduce competition from companion grasses (Humphreys and Lawless, 2008; Phelan *et al.*, 2013a, 2014) and thus may help to reduce variation in clover yield and thus BNF between years.

A comparison of techniques to measure BNF was also carried out in Chapter 2. While studies have compared these techniques, few have compared them over a long term period of up to two years and across different N inputs as in this study. This study found that the two ¹⁵N techniques, in general, resulted in similar annual estimates of %N_{fda} and BNF, which is in agreement with previous shorter term studies (Ledgard *et al.*, 1985; Carranca *et al.*, 1999; Huss-Danell and Chaia, 2005). However, the ND technique produced variable results mostly due to variation in N uptake on 0N_G (grass only plots). Such difficulties with this technique have been previously reported (Carlsson and Huss-Danell, 2003) and for this reason its use is only recommended in conjunction with one of the ¹⁵N based techniques

(Unkovich *et al.*, 2008). However, a number of issues were also encountered with the ^{15}N techniques in this study. The longevity of this study revealed that soil ^{15}N levels, when using the ID technique, can return to natural abundance levels and therefore create problems when ignoring ^{14}N and ^{15}N fractionation when using the ID technique. These techniques require expertise, are expensive and time consuming. In many situations, for example grassland/milk production studies where the focus is not on BNF, a rough estimate of BNF can be useful. In such a situation the use of a simple model to predict BNF, such as the model presented above, could be quite useful.

The dairy production system described in Chapter 4 was designed to exploit BNF and therefore fertiliser N inputs were restricted to between 86 and 140 kg N ha⁻¹. The rationale behind this restriction was that BNF has been found to decline as fertiliser N input increases in grass/clover grasslands as outlined in section 0. Also the substitution of N fertiliser with biologically fixed N is seen as way to mitigate N losses (Li *et al.*, 2011; Li *et al.*, 2013). The finding of Chapter 2 supports this rationale as BNF declined as N fertiliser input increased. These restricted fertiliser N inputs also appeared to achieve a balance between herbage production and BNF, while limiting potential N loss to the environment compared to higher N fertiliser inputs (i.e. 280 kg N ha⁻¹ yr⁻¹) while growing more herbage than grass/clover pastures receiving no N fertiliser. Previous experiments have shown that this white clover based system has similar milk production and net margin *per* hectare compared with grass-only fertiliser N based systems receiving 220 kg N ha⁻¹ while at the same time having lower N₂O emissions and carbon footprint (Humphreys *et al.*, 2009b; Li *et al.*, 2011; Humphreys *et al.*, 2012; Yan *et al.*, 2013). Therefore, the substitution of fertiliser N for biologically fixed N appears to be a sustainable and cost effective option for lowering the environmental impact of pasture-based production systems. The results of this study highlights that volatility in weather conditions (predicated to increase in the future (Solomon *et al.*, 2007; Miraglia *et al.*, 2009)), however, is a major concern for this type of system. Changes in weather conditions between years had major impacts on annual BNF and also on N losses, in particular N₂O emissions in this study.

5.1.2. Nitrogen loss as N₂O from white clover based pastures

Nitrous oxide emissions from grass/clover pastures in this study, with the exception of 2010/11, were relatively low at 4.4 to 6.3 kg N ha⁻¹ yr⁻¹ compared to grass only N fertilised pastures (6.45 to 28.93 kg N₂O-N ha⁻¹ yr⁻¹) receiving between 225 and 390 kg N ha⁻¹ in

Ireland (Hyde *et al.*, 2006; Rafique *et al.*, 2011). In general, pastures based on grass/clover receiving relatively small amounts of N ($<90 \text{ kg N ha}^{-1}$) tend to have lower emissions of N_2O compared to grass only pastures reliant on higher (226 kg N ha^{-1}) N fertiliser inputs (Li *et al.*, 2011). Lower emissions can be explained by the lower requirement for N fertiliser, the fact that the actual biological N fixation process *per se* is only a negligible source of N_2O (Carter and Ambus, 2006; Zhong *et al.*, 2009) and the greater efficiency of grass/clover mixtures to transform N into biomass than monocultures of grass (Nyfeler *et al.*, 2011). Biologically fixed N may, however, increase N_2O emissions in pasture-based systems, via increased excreta N deposition, if it leads to an increase in stocking rates. The breakdown of N-rich legume residues with low C:N ratios can also be an important source of N and C for micro-organisms producing N_2O (Baggs *et al.*, 2000a; Baggs *et al.*, 2000b; Baggs *et al.*, 2003; Pappa *et al.*, 2011). However, this is more prominent for crop legumes rotations, which generally undergo some form of tillage on an annual basis, which contrasts to permanent pastures.

Of concern, in this study, was the atypically high emissions ($34.4 \text{ kg N ha}^{-1}$) recorded in 2010/11. These relatively high emissions, however, appear to be a rare event in the context of the four years of measurements carried out in this study. The extensive dataset gathered in this study allowed for an assessment of the drivers of inter annual variation using annual data. While many studies have found N input to be the major driver of emissions (Rees *et al.*, 2013; Rafique *et al.*, 2011), this study, found annual emissions to be primarily regulated by weather and soil conditions. Another interesting result arising from this study is that the high annual emissions of 2010/11 appear to have been regulated by atypical weather conditions, most notably the record breaking cold temperatures in December and the drier than normal summer (Table 3.3). Extreme weather events are predicted to increase in future climate prediction models including those presented in the most recent IPCC assessment in 2013 (Solomon *et al.*, 2007; Collins *et al.*, 2013). These assessments have stressed that global climate change, which include extreme weather events, will continue in line with the projected upward trends in GHG concentrations in the atmosphere. The results of this study suggest that this may lead to a so called ‘vicious cycle’ between GHG emissions and extreme weather events.

The relatively small increase in N inputs relative to the increase in emissions as well as the results of SNB for the four years of this study indicate that variations in N input (both in terms of quantity and timing of application) was not the major driver of the increase in

N₂O emissions experienced in 2010/11. A more important source of N was that from mineralisation of SOMN stimulated by the cold temperatures (freeze thawing) and natural drying of this soils under relatively low rainfall experienced in 2010/11. The elevation in N uptake in background grass plots (GB) in 2010/11 relative to other years (Chapter 3), rapid depletion of grass and clover atom% ¹⁵N excess on ID plots (Chapter 2) and negative complete farm N balance (Chapter 4) further strengthens the argument that net N mineralisation occurred in 2010/11 and contributed to increased N₂O emissions. A shift from complete to partial denitrification under drying soil conditions was also discussed as a possible cause of increased N₂O emissions in this year (Chapter 3). However, subsequent results from Chapter 4 revealed that N₂ emissions were also highest during this year. This suggests that N₂O and N₂ emissions were higher in this year as opposed to a shift from complete to partial denitrification in this study. This finding warrants further investigation.

5.1.3. Nitrogen loss as N₂ from a pasture-based system of dairy production

The losses of N₂ ranged between 50 to 123 kg N ha⁻¹ in this study using a relatively new approach of combining periodically determined N₂:N₂O ratio using ¹⁵N with frequent static chamber measurements of N₂O. These results provide valuable data as annual estimates of N₂ under field conditions are scarce in the literature primarily due to the difficulties involved in measuring this gas (Groffman *et al.*, 2006). A recent review of denitrification emissions from temperate grassland soils indicated a median daily emission of 17 g N ha⁻¹ (Saggar *et al.*, 2013). Emissions in this study were relatively high compared to this median value and those reported for N fertilised and grazed pastures in New Zealand (11 to 34 kg N ha⁻¹) (Ruz-Jerez *et al.*, 1994; Ledgard *et al.*, 1999) and Australia (13 to 15 kg N ha⁻¹) (Eckard *et al.*, 2003) but similar to those found on clay soils in the Netherlands (van der Salm *et al.*, 2007). The heavy textured soils with high soil organic carbon and moist maritime climate at this site created conditions conducive to denitrification which may explain the relatively high rates of emissions. Furthermore, N losses to water in this study at 5 to 21 kg N ha⁻¹ were at the lower side of reported leaching losses (4 to 204 kg N ha⁻¹ yr⁻¹) for grazed and N fertilised pastures (Ledgard *et al.*, 1999; Di and Cameron, 2002; Eckard *et al.*, 2007; Bouman *et al.*, 2010). Leaching losses at this site have been consistently low over the last ten years. The low N leaching on this fine textured soil was most likely due to the removal of the soil surplus N through denitrification (Di and Cameron, 2002; Kroeze *et al.*, 2002). Indeed an exponential relationship was found between N₂ emissions and N input (Table 4.3). An interesting finding in this study, which has previously been reported at the Solohead Research Farm (Necpálová *et al.*, 2012), was

the relatively high proportion of N leached as NH_4^+ and DON. This finding, along with the relatively high N_2 emissions found at this site requires further investigation.

5.1.4. Farm N balance for a pasture-based system of dairy production

Measured and estimated N inputs accounted for N outputs within experimental error from a dairy production system in this study (Chapter 4). To the authors' knowledge, the majority of previous N input and output balances including losses by leaching, volatilisation and possible N accumulation have never closed the N balance. ^{15}N balance studies of urine patches have also failed to account for all N inputs (Clough *et al.*, 1998b; Wachendorf *et al.*, 2008). Unaccounted for N (25 to 45%) is generally assumed to be lost through denitrification (Kroeze *et al.*, 2002; Humphreys *et al.*, 2008b). This N balance study (for the first time), included measurements of N_2O and N_2 using the static chamber technique and ^{15}N gas-flux method, respectively, in order to include these flows in the N balance. Previous N balance studies have relied heavily on estimates of many N inputs and losses, and have used the C_2H_2 technique to quantify $\text{N}_2+\text{N}_2\text{O}$ losses, which is now known to underestimate these losses and have other major disadvantages (Eckard *et al.* 2007; Groffman *et al.*, 2006; Ledgard *et al.*, 1999; Watson and Atkinson, 1999). For this reason the detailed measurements of N flows, in particular losses of N_2 and N_2O , and relative closure of the N balance for this production system sets this study apart from those previously conducted.

Closing of the farm N balance has been a topic of research at the Solohead Research Farm for over 10 years. Accumulated results suggested that denitrification may account for between 45 and 60% of the N surplus. The collation of results from this study with previous results suggests that 40%, on average, of the N surplus was lost by denitrification products N_2 and N_2O at this site over the last 10 years (Chapter 4). The results of this study and postulations of many other studies indicate that denitrification and in particular N_2 may be the key to explaining unaccounted for N in many unclosed agricultural N balance studies (Clough *et al.*, 1998b; Kroeze *et al.*, 2002; Humphreys *et al.*, 2008b; Wachendorf *et al.*, 2008; Krol, 2013; Necpálová *et al.*, 2013a; Necpálová *et al.*, 2013b). The results of this study, also highlights the need to adopt management practices to reduce losses of N_2O and N_2 . Losses of N_2 are environmentally benign but they still represent a substantial economic loss for farmers. If losses via these pathways are unavoidable, care should be taken to insure that the $\text{N}_2:\text{N}_2\text{O}$ ratio is kept as high as possible. Management practices/rules which

can reduce these losses are described in detail by Saggar *et al.*, (2012) and include (i) using stand-off pads to reduce N deposition at pasture during periods of high soil moisture (late autumn, winter and early Spring), (ii) diet manipulation to lower urine N excretion (i.e. reduce crude protein intake or increase energy intake), (iii) the use of nitrification inhibitors, (iv) avoiding soil compaction, (v) improving soil drainage. These practices may all come at an economic cost for farmers. Improving soil drainage to reduce WFPS may, however, increase N₂O emissions on soil types such as those in the present study and requires further investigation. One management practice which can increase the N₂:N₂O ratio is liming to rise soil pH levels. This facilitates the reduction of N₂O to N₂ as nitrous oxide reducing bacteria are sensitive to low soil pH. Increasing soil C availability, for example, by applying biochar to land, may also increase the N₂:N₂O ratio (Saggar *et al.*, 2012).

5.1.5. Partitioning of N losses on a pasture-based system of dairy production

The relative closure of the N balance in this study allows us to, for the first time, to evaluate how different N loss pathways contributed to surplus N removal from a dairy production system. Total N losses were 233 and 128 kg N ha⁻¹ in 2011 and 2012, respectively. The contribution of N loss pathways, on average over the two year, decreased in the order: N₂ (46%) < NH₃ (38.5%) < N₂O (8.5%) < N lost to groundwater (6.5%) < gaseous N loss from slurry storage (0.5%). The major finding of this study is that almost half of the losses of surplus N from in this system were environmentally benign N₂ gas. This has major implications for pasture-based production systems under similar soil and climatic conditions which are outlined below. This proportion is similar to the modelled results of Velthof *et al.* (2014) where N₂ was estimated to account for 53% of total N losses from agriculture in the EU-27. Losses of N₂O accounted for a much smaller proportion of N losses which indicates that N₂ is the dominant product of denitrification processes under the heavy textured soils with impeded drainage and moist maritime climatic conditions at this site. Losses to water were relatively low, due to the reasons discussed above, and therefore accounted for the lowest proportion of environmentally damaging N losses.

The largest damaging N loss from the system was NH₃ gas (64 kg N ha⁻¹ yr⁻¹) accounting for between 27% and 50% of N losses in 2011 and 2012, respectively. Therefore, mitigation strategies to reduce N losses from this system should play particular attention to NH₃. Emissions from land-spreading slurry, housing and fertiliser N applications were the

largest sources of NH_3 emissions. There are a number of mitigation options available to reduce NH_3 emissions from these sources. Application techniques such as band spreading, trailing shoe and slurry injection have all been found to reduce NH_3 emissions (Huijsmans *et al.*, 2001; Misselbrook *et al.*, 2002; Lanigan, 2009) during land spreading compared to the splash plate technique used in this study and in the vast majority of Irish farms. The timing of slurry spreading in terms of prevailing weather conditions is known to be a more cost effective mitigation option compared to the use of low emission application techniques outlined above. Lanigan (2009) reported NH_3 emissions of 78% of TAN applied on hot dry days in summer while emissions in colder periods such as spring were up to 30% lower. Pressures washing yard surfaces (Misselbrook *et al.*, 2006), segregation of urine and feces, use of ammonium binders, acidify slurry and covering slurry store are all possible mitigation options for winter housing emissions (Ndegwa *et al.*, 2008). Finally, appropriate timing of application (matching plant demand and weather condition) and use of urease inhibitors have been found to be effective mitigation options for emissions associated with N fertiliser application (Singh *et al.*, 2013; Suter *et al.*, 2013).

5.1.6. Environmental Impact of legume-based cropping systems

The environmental impact of legume crops were assessed in the Legume Futures Project by comparing (i) nitrous oxide emissions, (ii) N leaching and (iii) biodiversity between legume and conventional crops across a number of European sites. Nitrous oxide emissions from legume crops were consistently low and often lower than emissions reported from conventional crops such as wheat and barley (Table 5.1). The conventional crops generally received more inorganic N to meet their N demand for growth, which is well documented to stimulate large fluxes of N_2O from arable soils (Kaiser *et al.*, 1998; Pappa *et al.*, 2011). On the other hand, the legume crops supplied their own BFN which is not seen as a major source of N_2O emissions as discussed above. In fact, background N_2O emissions from control plots with no legumes were often higher than those recorded from plots containing legume crops (data not shown). Calculated N_2O emissions factors for BFN across the range of legume crops were minor with a median of 0.03% (mean: 0.14%, max: 2.03%, min - 0.40%). Overall, legume crops seem to have lower N_2O compared to conventional crops during the growing season that they are cultivated in. However, the legacy of N-rich legume residues may stimulate N_2O emissions in subsequent growing seasons and requires further investigation (Pappa *et al.*, 2011).

Nitrous oxide emissions from the legume and conventional crops in the Legume Futures Project were substantially lower than the emissions recorded from grazed and N fertilised grass/clover pastures in this study. While differences in climatic condition between sites is likely to have contributed to the difference in emissions, this is still not a surprising result and agrees with the trend in the literature (Rees *et al.*, 2013). First of all, pastures require higher N inputs than crops (section 1.2.3.3). The presence of grazing cows on pastures also leads to higher N recycling and inefficient use of N (section 1.2.3.4). While pastures may have higher emissions of N₂O than arable soil, they have higher potential (particularly permanent pastures) for carbon sequestration than arable soil which are cultivated annually (Hutchinson *et al.*, 2007). Pasture based systems, in particular those based on white clover, have also been found to have a lower carbon footprint of milk production compared to confinement based milk production systems reliant on the harvesting of crops to supply feed for livestock (O'Brien *et al.*, 2012; Yan *et al.*, 2013). This is important in the context of projected increases in global demand for meat and dairy products (Alexandratos and Bruinsma, 2012).

Nitrogen leaching data from the Legume Futures Project is still in the raw data stage but will become available on the Legume Futures website (www.legumefutures.de) overtime.

Biodiversity was assessed on eleven of the Legume Futures sites and considered primary producers (vegetation communities), decomposers (earthworms), and secondary consumers (predatory ground-dwelling invertebrates). No clear difference in biodiversity was found between legume and conventional crops using these three indicators. Insufficient replication at the site scale was a limitation of the study and made it difficult to infer differences. Legume crops can be somewhat perceived as being beneficial for biodiversity compared to conventional crops, however, the results of the Legume Futures Project found no clear evidence of this, albeit the drawbacks of the poor field site design (Futures, 2014).

Table 5.1 Nitrous oxide emissions from a range of legume and conventional crops across the Legume Futures sites. Source: Legume Futures Project

Location	Plant/crop	Mean N ₂ O fluxes	Cumulative N ₂ O ^c	Inorganic N	BFN ^d	Emission intensity
		g N ₂ O-N ha ⁻¹ d ⁻¹	kg ha ⁻¹ period ⁻¹	kg N ha ⁻¹ yr ⁻¹	kg N ha ⁻¹ period ⁻¹	kg N ₂ O-N tonne grain yield ⁻¹ yr ⁻¹
Greece	¹ Common bean	5.95	0.326	120	25	0.02
	Common bean	4.72	0.228	0	56	0.02
Italy	² Barley	0.67	0.079	36	0	0.06
	³ Durum wheat	7.52	0.988	0	0	0.32
	⁴ Faba bean	0.59	0.128	36	155	0.06
	Faba bean	0.93	0.123	36	161	0.05
	⁵ Pea	0.46	0.073	36	121	0.03
Romania	⁶ Lentil	0.83	0.048	0	N/D	N/D
	Lentil	1.22	0.071	0	N/D	N/D
	⁷ Lupine	6.67	0.387	0	N/D	N/D
	⁸ Mung bean	3.10	0.390	0	N/D	N/D
	⁹ Soybean	13.62	1.226	0	N/D	N/D
	Soybean	10.31	0.928	0	N/D	N/D
Scotland ^a	Common bean	2.25	0.159	0	185	0.04
	² Spring barley	8.33	0.222	111	0	0.12
	¹⁰ Winter wheat	7.50	1.623	180	0	0.11
Scotland ^b	⁵ Combinable Pea	2.21	0.409	0	N/D	N/D
	¹ Spring beans	1.09	0.252	0	N/D	N/D
	⁵ Vining pea	1.22	0.170	0	N/D	N/D
	¹ Winter bean	1.68	0.655	0	N/D	N/D
Spain	Faba bean	20.91	3.939	50	142	N/D
	¹¹ Sunflower	21.82	3.015	50	0	N/D
	¹⁰ Wheat	N/D	0.013	50	0	N/D
UK	Common bean ^e	4.23	0.850	0	N/D	N/D
	Peas ^f	7.49	1.224	0	N/D	N/D
Mean	Legume crops	4.71	0.61	14.63	76.74	0.04
Mean	Conventional crops	6.00	0.58	75.30	0.00	0.15

¹Phaseolus vulgaris; ²Hordeum vulgare; ³Triticum durum; ⁴Vicia faba; ⁵Pisum sativum; ⁶Lens culinaris; ⁷Lupinus; ⁸Vigna radiate; ⁹Glycine max; ¹⁰Triticum; ¹¹Helianthus annuus.
^aBalruddery
^bBush
^cMeasuring periods differ between crops and sites
^dBiologically fixed N
^eMean of five varieties
^fMean of seven varieties
N/D: not determined

5.2. Implications

Nitrogen fertiliser is the dominant N input on Irish pastures-based production systems. Its application to grassland is generally a cost effective way of increasing herbage production. Its cost compared to farm level product prices, however, has increased steadily over the

last two decades and this upward trend is projected to continue in the near future. Current N fertiliser usage stands at, on average, 75 and 148 kg N ha⁻¹ on Irish ‘suckler to beef’ and dairy farms, respectively (Teagasc, 2013a, b). The measured range of BFN input reported in this study can be worth between €17 to €154 ha⁻¹ yr⁻¹ considering current fertiliser N price in Ireland of €120 t⁻¹. This makes it a competitive alternative to mineral N fertiliser and BNF may be worth substantially more if belowground BNF is considered. If only 5% of grasslands in Ireland (excluding rough grazing) were established as white clover based swards, and achieved similar levels of BFN as in this study, it would be worth between €3.2 and €29 million yr⁻¹ in terms of BFN at a national level. At present, pasture-based systems in Ireland are dominated by monocultures of grass with present N fertiliser recommendations based solely for grass monocultures. The results of this study may be useful when drawing up future N fertiliser recommendations for grass/clover pastures, which should account for the N supplied by BNF. There was large variation in BNF between years in this study due to volatility in annual weather conditions. This will have to be considered and planned for, when establishing clover based production systems. The results of this study also highlight the importance of sward clover content and yield to achieve high levels of BNF. Some management practices which can help to maintain sward clover content are discussed in section (5.1.1). The reader is referred to the thesis of Phelan, (2012) and booklet of Humphreys and Lawless, (2008) for further information on these management practices.

A number of methodological concerns and recommendations related to measurements of N₂O and BNF were highlighted in this study. These will serve as an insight for future studies aiming to measure these N flows. Another concern arising from this study is the usefulness of farm-gate N balances to predict the environmental impact of production systems. For example, a production system with a low farm-gate N balance may lose a greater proportion of its N surplus to groundwater or as N₂O and ultimately have a higher environmental impact than a system with a higher surplus that loses a large proportion of its N surplus as N₂. Up to half of N surplus on this production system was lost as environmentally benign N₂.

The results of this study highlight the dominance of weather driven variables in regulating inter-annual variation in N₂O emissions. This highlights the uncertainty associated with generating higher tier N₂O emission factors. The results also indicate that it may be possible to model these inter-annual variations in N₂O emissions using weather data and

therefore to predict emissions based on weather conditions in the future. The results also have major implications in the context of future projected climate change where increased weather volatility, including temperature extremes and patterns of rainfall are predicted (Solomon *et al.*, 2007; Collins *et al.*, 2013). These changes in weather conditions had a direct impact on soil moisture conditions. N₂O emissions were highest under anoxic soil conditions in this study. While the influence of weather on soil moisture conditions cannot be controlled, management decisions which support, or lead to, anoxic soil conditions should be avoided if N₂O emissions from grasslands, such as those in the present study, are to be mitigated.

Globally, permanent pastures account for up to 68% of all agricultural land. Areas with high precipitation and heavy soils tend to have pasture-based livestock production systems due, in part, to the inappropriateness of the soil and climate conditions for alternative land uses (Humphreys *et al.*, 2009). These systems tend to have high N surpluses. However, as this study illustrates, once the system is based on heavy soil in high rainfall areas, damaging losses to water can be minimal and a large proportion of surplus N can be lost as environmentally benign N₂ gas. These findings may have implications in terms of policy. For example, pasture based livestock production systems in Ireland have the option to apply for a 'derogation' to apply up to 250 kg organic N ha⁻¹ yr⁻¹ instead of the standard limit of 170 kg organic N ha⁻¹ yr⁻¹ under the ND. This allows for an increase in stocking rates from 2 to 2.9 livestock units ha⁻¹ (EC, 1991; Humphreys, 2008). Ireland's 'derogation' agreement is subject to review every four years. One of the original arguments for Ireland's 'derogation' agreement was based on the hypothesis that N losses to groundwater would be relatively low due to the high denitrification potential of Irish soils (heavy textured soils with high organic carbon content and moist maritime climate) (Teagasc, 2012, 2013c). The results of this study provides substantial evidence to support this hypothesis and also indicates that benign N₂ as opposed to N₂O is the dominant end product of denitrification under heavy textured soil and wet maritime climatic conditions. The results also identified losses of NH₃ and N₂O to be the most prevalent environmentally damaging N losses from this pasture-based system of dairy production. These should, therefore, be given priority when mitigating N loss. Mitigation strategies should focus on the major sources of such N losses, for example, land-spreading of manure and housing emissions for NH₃ and emissions associated with N management and grazing for N₂O from this system.

The substantial datasets (i.e. four years of N₂O data) of N flows and fluxes gathered during this study are quite rare. While useful, the results are site and condition specific. However, these detailed datasets may be invaluable for future modelling validation purposes. Many biogeochemical computer simulation models are available for N and C dynamics prediction in agro-ecosystems. Models such as DNDC (DeNitrification-DeComposition) and DAYCENT have been commonly used to simulate emissions of N₂O, for example, (Abdalla *et al.*, 2009b; Abdalla *et al.*, 2010b; Li *et al.*, 2011). The validation of such models has a number of benefits. Validated models can be used to upscale emissions estimates from the field and system scale (which was the case in this thesis) to the catchment/regional or even national scale. Validated models will also allow the research community to assess the effect of future predicted climate change and to access mitigation strategies without the need to carry out time consuming field measurements of N₂O emissions. Irelands' agricultural GHG emissions are currently estimated using the IPCC Tier 1 approach for national GHG inventory reporting required under the EC Climate and Energy Package (See section 1.6.2). The Tier 1 approach uses default IPCC EF of N applied in different forms, which have been shown to overestimate emissions (Lesschen *et al.*, 2011). Ireland is currently in the process of generating country specific EF for the Tier 2 approach. Emissions can be estimated using validated models under the Tier 3 approach. This allows for more refined emission estimates and the inclusion of more mitigation strategies. The N₂O flux data from this study is currently being used in a Trinity College Dublin co-ordinated project called 'Scaling soil process modelling to the national level'. The aim of the project is to use three models (DNDC, DayCent, ECOSSE) to predict GHG emissions from agriculture at the Tier 3 level and to ultimately produce a map of the modelled data.

5.3. Conclusions

Comparisons of biological nitrogen fixation in association with white clover (*Trifolium repens* L.) under four fertiliser nitrogen inputs as measured using two ¹⁵N techniques (Chapter 2).

Above-ground biological N fixation was an important N input on grass/clover pastures in this study supplying between 14 to 128 kg N ha⁻¹ yr⁻¹. This BFN can allow for a reduction in N fertiliser input while maintaining a reasonable level of herbage production. Both ¹⁵N based techniques, in general, gave similar reliable estimates of BNF in this study and are recommended for measurement of BNF in N fertilised and cut pastures once appropriate

measures are taken to prevent methodological issues associated with ^{14}N and ^{15}N fractionation. The use of the ND technique, however, is only recommended when used in conjunction with one of the ^{15}N based techniques. Fertiliser N input increased herbage yields but reduced BNF and increased potential N loss to the environment on grass/clover pastures. Intermediate N fertiliser inputs of between 86 and 140 kg N ha⁻¹ in spring appear to achieve a compromise between herbage production and BNF on grass/clover pastures.

Inter-annual variation in nitrous oxide emissions from perennial ryegrass/white clover grassland used for dairy production (Chapter 3).

The long-term dataset recorded in this study allowed for a comprehensive evaluation of inter-annual variation in N₂O emissions from a pasture based system. Nitrous oxide emissions from grass/clover pastures in this study displayed substantial inter-annual variation with emissions ranging from 4.4 to 34.4 kg N ha⁻¹. The high emissions of 2010/11 were the main contributor to inter-annual variation and were uncharacteristic of these grass/clover pastures. The peak in emissions encountered in 2010/11 were more a function of atypical weather induced emissions such as the record breaking cold temperatures in December and drier than normal summer than the actual design and management of the pastures.

A complete farm N balance for a pasture-based system of dairy production under moist maritime climatic conditions (Chapter 4)

Nitrogen loss as N₂ gas at 50 to 123 kg N ha⁻¹ was substantial from the grass/clover pastures in this study. The relatively high emissions, however, are not surprising given (i) the heavy textured soils with high organic carbon at the site, (ii) the moist maritime conditions at the site and (iii) the relatively low levels of N losses to groundwater at the site during the study and in general at this site. The measurement and estimate of multiple N flows in this study allowed for the completion of a detailed N balance where all N inputs more or less accounted for N outputs within experimental error. Collation of the results of this study with previous finding at this site suggest that losses as N₂ was the key to closing the N balance on this site not only over the duration of this study but over the last decade at this site and may well be the missing sink in many unclosed N balances studies carried out on similar soil types and under moist maritime climatic conditions. Dinitrogen gas accounted for the largest proportion of the surplus N (46% on average over two years).

Nitrogen losses to groundwater accounted for a relatively small proportion of the N surplus while NH_3 emissions were the largest environmentally damaging N loss.

Overall conclusions

Biological N_2 fixation was a substantial N input on the white clover based dairy production system in this study and is a sustainable alternative to N fertiliser. The grass/clover pastures in this study also, in general, had relatively low emissions of N_2O . However, volatility in weather conditions, predicted to increase in the future, is a major concern for such systems, which resulted in increased N_2O emissions and a major reduction in BNF in individual years of this study. The thesis hypothesis, that measurement of nitrous oxide, dinitrogen and N losses to groundwater and estimates of ammonia volatilisation will account for farm surplus N, is accepted. Although N surpluses on pasture based livestock production systems can be substantial, the results of this study for the first time provide evidence that a high proportion of surplus N may be lost as environmentally benign dinitrogen gas when the system is on heavy textured soils with impeded drainage and under moist maritime climatic conditions. Such soil types are representative of around 23% of Irish soils. Estimated ammonia emissions were the largest environmentally damaging N losses from this system and will require further research. This study highlights where the emphasis should be (NH_3 and N_2O) in terms of minimising environmentally damaging N losses and improving the N-efficiency of such systems of dairy production.

5.4. Recommendations for future work

- BNF apportioned to above-ground biomass was measured in this study. It would be useful to have an estimate of below-ground BNF and N transferred to companion grasses under Irish conditions to account for the total N supplied by BNF. It would also be interesting to assess the effects of N fertiliser inputs on below-ground BNF and N transferred to companion grasses.
- BNF was assessed under cutting in this study. It would be interesting to investigate BNF and the appropriateness of techniques to measure BNF under grazing conditions.

- White clover yield was the dominant factor driving BNF in this study. It would be useful to investigate management practices which improve clover yield in Irish pastures in order to increase BNF.
- Inter-annual variation was substantial in most of the N flow measured in this study. Future studies should aim to conduct measurements over a number of years in order to gain representative estimate of these N flows from an individual site or production system.
- N₂O emissions during the atypically cold winter of 2010 in this study were substantial (>9 kg ha⁻¹). These emissions can be explained by soil freezing and thawing events. Future work should be carried out to gain a better understanding of the processes producing N₂O under these events and potential mitigation options should be investigated.
- Further N balance studies of a similar nature to the present study are required to identify the most important N loss pathways under different soil, climatic and management conditions. This will allow for targeted mitigation strategies to reduce the most prevalent N losses which should ultimately lead to improved sustainability of pasture-based livestock production systems.
- Ammonia emissions were estimated in this study and were found to be the largest detrimental N loss. There is a lack of studies in Ireland investigating NH₃ emissions from agricultural production systems. There is a need for baseline measurements of NH₃ and also investigation of ammonia mitigations options from various farm sources following the results of this study.
- Dinitrogen was the dominant N loss in this study. There is a need for further field studies investigating the processes producing and management, soil and climatic factors which regulate N₂ emissions and the N₂:N₂O ratio.
- Soil organic N mineralisation/immobilisation was not measured in this study and would have helped to explain the difference in N losses and complete farm N balances between years.

- Measurements of soil inorganic N would have been useful to interpret the temporal variation in N₂O emissions experienced in this study.
- While the dataset presented in this study gives very good detail, it is only specific to one management, climate and soil type. Going forward this dataset should be used, perhaps with other datasets, to valid models in order to predict N fluxes and flows under different management and environmental conditions and therefore increase our understanding of N fluxes and flows at the catchment or regional scale.
- The results of this study highlights the importance of using appropriate B_s -values when using the NA technique and monitoring of plant ¹⁵N enrichment under the ID technique to ensure that sufficient enrichment is maintained (and that it is uniform) in future studies of BNF. The use of more than one reference plant is also recommended.

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APPENDIX

Scientific publications and disseminations

Scientific papers (published and submitted for publication)

- Burchill, W., Li, D., Lanigan, G., Williams, M. and Humphreys, J. (2014) Inter-annual variation in nitrous oxide emissions from perennial ryegrass/white clover based grassland used for dairy production. *Global Change Biology*. 10, 3137-3146, DOI: 3110.1111/gcb.12595.
- Necpálová, M., Li, D., Lanigan, G., Casey, I. A, Fitzgerald, E., Burchill, W., and Humphreys, J. (2013). Changes in soil organic carbon in clay-loam soil following ploughing and reseeded of permanent grassland under moist temperate climatic conditions. *Grass and Forage Science*. 69, 611-624, DOI: 10.1111/gfs.12080
- Burchill, W., James, E., Lanigan, G., Li, D., Williams, M., Iannetta, P. and Humphreys, J. (2014). Comparisons of biological nitrogen fixation in association with white clover (*trifolium repens* L.) under four fertiliser nitrogen inputs as measured using two ¹⁵N techniques. *Plant and Soil*. 385, 287-302, DOI 10.1007/s11104-014-2199
- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. A complete farm N balance for a pasture-based system of dairy production under moist maritime climatic conditions. *Agriculture, Ecosystems and Environment* (under review)

Research article

- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. (2013) Nitrous oxide production from Irish grassland. *TResearch*, Volume 8: number 2. Summer 2013. pp 28-29: Teagasc.

Conferences presentations

- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. (2013). Comparisons of biological N fixation of white clover under four fertiliser nitrogen inputs measured using two ^{15}N techniques. Poster presented at the Sustainable intensification conference: Pathway to low carbon farming. Edinburgh, Scotland.
- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. (2013). Inter-annual variation in nitrous oxide emissions from white clover based grassland used for dairy production. Poster presented at the proceedings of the Greenhouse Gasses and Animal Agriculture conference. UCD, Dublin.
- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. (2013). Nitrous oxide emissions from white clover based grassland used for dairy production. Paper presented at the proceedings of the 17th European Grassland Federation Symposium 2013. pp 79-81 Akureyri, Iceland.
- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. (2013). Nitrous oxide emissions from white clover based grassland used for dairy production. Paper presented at the proceedings of the Agricultural Research Forum, pp. 22 Tullamore, Ireland.
- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. (2012) Nitrous oxide emissions from white clover based grassland used for dairy production under moist maritime climatic conditions. Paper presented at the proceedings of the 12th Congress of the European Society of Agronomy. pp 214-215. Helsinki, Finland.
- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. (2012). Accounting for N losses in dairy production at Solohead Research Farm. Paper presented at the proceedings of the Agri-Environment Conference 'Sustainable Pathways to Food Harvest 2020'. pp 23-24. Dublin, Ireland.

International project meetings

- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. Comparisons of biological N fixation of white clover under four fertiliser nitrogen inputs measured

using two ^{15}N techniques. Presentation at the fourth annual European project meeting of Legume Futures. Bucharest, Romania. 16-18th April 2013.

- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. Nitrous oxide emissions from white clover based grassland used for dairy production under moist maritime climatic conditions. Poster at the third annual European project meeting of Legume Futures. Athens, Greece. 26-29th March 2012.
- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. Teagasc Moorepark-Solohead Research Farm, Ireland. Presentation at the second annual European project meeting of Legume Futures. Cordoba, Spain. 22nd March 2011.
- Burchill, W., Boland, A., Mihailescu, E., and Humphreys, J. Teagasc Solohead Research Farm and commercial case study farms. Presentation at work package three meeting of the European project 'CANTOGETHER'. 10th May 2012. Reggio Emilia, Italy.
- Burchill, W., Lanigan, G., Li, D., Williams, M. and Humphreys, J. Environmental impact of dairy systems at Solohead. Presentation at the fourth annual general meeting of the European project 'Dairyman'. 17th April 2011. Tipperary, Ireland.

Farm open-day:

- Burchill, W., Hennessy, D., and Humphreys, J. (2011) Role of white clover in grass based milk production at Solohead. In Solohead open day handbook, pp 42-46. Cork, Ireland: Teagasc.

Biological N₂ fixation and N loss as N₂O and N₂ in a white clover-based system of dairy production

By William Burchill

Abstract

Pasture-based livestock production systems have a relatively large requirement for nitrogen (N) input compared to other food production systems. These inputs may consist of organic N, mineral N or biologically-fixed N. Of all N that enters these systems, only a small proportion (15-40%) is converted to saleable agricultural products. The remaining N (surplus N) is largely unaccounted for and can be either lost from the system through a range of pathways including gaseous N losses to the atmosphere as nitrous oxide (N₂O), ammonia (NH₃), and dinitrogen (N₂), N losses to groundwater or N may be immobilised in soil organic matter, all of which have differing environmental consequences. Thus the quantification of N losses is critical for assessing the environmental impacts of ecosystem N dynamics and accordingly for improving our ability of N management. This study carried out detailed N flow measurements on a dairy production system in order to account for all N entering into, and leaving from, the system and to determine the fate of farm surplus N which has remained an area of high uncertainty for the research community. This study also allowed for a much more holistic view of N movements on a farm system compared to most previous studies which have primarily focused on either individual N inputs or losses. Encompassed in this holistic study was a comprehensive comparison of three techniques used to measure biological N₂ fixation under varying fertiliser N inputs over a sustained period of two years and a long term study of N₂O from white clover based pastures to assess inter-annual variation in emissions, both of which are lacking in the literature.

The experiments were carried out on a perennial ryegrass (*Lolium perenne* L.)/white clover (*Trifolium repens* L.; 18.2%; grass/clover) based system of dairy production at Solohead Research Farm, Tipperary, Ireland (52° 51' N, 08° 21' W) under moist maritime climatic conditions. Biological N₂ fixation (BNF) associated with white clover was measured using three techniques (¹⁵N natural abundance, ¹⁵N isotope dilution and nitrogen difference) under four fertiliser N inputs (0, 86, 140 and 280 kg N ha⁻¹ yr⁻¹). Nitrous oxide emissions were measured over a four-year period (October 2008 to November 2012) using a static chamber technique. These N flow measurements were combined with measurements of N₂ gas, N losses to water, N exported in animal products, N deposition in rainfall and estimates of NH₃ volatilisation to complete a farm N balance for the dairy production system over two production years: 2011 and 2012.

Biological N₂ fixation in above-ground herbage ranged from 14 to 128 kg N ha⁻¹ yr⁻¹, as measured by both ¹⁵N techniques, across the range of fertiliser N inputs and over the two years of the study. Fertiliser N input reduced BNF and there was also significant inter annual variation in BNF due to differences in clover herbage yield between years. Both ¹⁵N based techniques produced similar estimates of BNF while the nitrogen difference technique gave different results. Nitrous oxide emissions displayed large intra and inter-annual variation with annual estimates ranging from 4.4 to 34.4 kg N ha⁻¹. Weather driven variables including monthly soil temperature (December) and annual rainfall accounted for a large proportion of

the variation in annual N_2O emissions. Nitrogen inputs accounted for N outputs (N exported in products and N losses) within experimental error in the farm N balance for the system. Total N losses of 180 kg N ha^{-1} , average for both years, were in the following proportions: 6.5% as losses to groundwater, 38.5% as NH_3 , 8.5% as N_2O and 46% as N_2 gas. The heavy textured soils with high soil organic carbon and impeded drainage, and moist maritime climate at this site created conditions conducive to denitrification which may explain the relatively high rates of N losses as N_2 gas and at the same time the relatively low N losses to groundwater.

Although N surpluses on pasture based livestock production systems can be substantial, the results of this study suggest that a high proportion of surplus N may be lost as environmentally benign dinitrogen gas when the system is on heavy textured soils with impeded drainage and under moist maritime climatic conditions. Such soil types are representative of around 23% of Irish soils. This study also highlights where the emphasis should be (NH_3 and N_2O) in terms of minimising environmentally damaging N losses and improving the N use efficiency of such systems of dairy production.