The effect of dietary nitrogen on nitrogen partitioning and milk production in grazing dairy cows

---

A thesis presented in partial fulfilment of the
requirements for the
Master of Animal Science
at Massey University
Palmerston North, New Zealand

Stacey Hendriks

2016

ABSTRACT

Two experiments were conducted during spring (8th October to 12th November 2009) as part of a larger study, to study the effects of increasing levels of crude protein (CP) in pasture on milk production, dry matter intake (DMI) and nitrogen (N) partitioning in dairy cows.

The first experiment was undertaken over 25 days (8th October to 1st November 2009), where fifteen multiparous, rumen fistulated, early lactation Holstein-Friesian cows (505 ± 10.4 kg liveweight; 4.1 body condition score ± 0.044, mean ± standard deviation) were assigned to one of three urea supplementation treatments: Control (0 g/day urea; ~20% CP), Medium (350 g/day urea; ~25% CP) and High (690 g/day urea: ~30% CP). Urea was supplemented to the pasture-based diet to increase CP content while maintaining similar concentrations of all other nutrients across treatments. All cows were offered ~20 kg dry matter (DM)/day perennial ryegrass-based pasture (CP = 20.6 ± 0.56% DM; metabolisable energy (ME) = 11.8 ± 0.06 MJ/kg DM). Cows were acclimated to their urea treatment over a 25 day experimental period. The objective of this study was to determine the effect of increased dietary CP in grazing cows on DMI and milk yield.

Dry matter intake was estimated using a back calculation method from the energy requirements of the cows. The results indicate a complex interaction between DMI, milk yield and urea intake. As dietary CP increased, the milk yield increased; however, as urea's contribution to total dietary CP concentration increased, the increase in both DMI and milk yield was less. Milk yield decreased when urea supplementation increased beyond 350 g/day, and the interaction evident in milk yield was mirrored in yields of fat, CP and lactose ($P < 0.001$). The addition of urea had no effect on milk fat, protein and lactose percentages.

The second experiment was conducted over 22 days (22nd October to 12th November 2009), involving ten multiparous, rumen fistulated, early lactation Holstein-Friesian cows (520 ± 5.6 kg liveweight; 4.15 body condition score ± 0.078, mean ± standard deviation). This experiment was undertaken to study N partitioning in pasture-fed grazing dairy cows using urea supplementation as a non-protein N (NPN) model to ensure all other nutritional characteristics of the forage remained the same. All cows were offered ~19 kg DM/day of perennial ryegrass-based pasture (CP = 18.4 ± 0.64% DM; ME = 11.4 ± 0.06 MJ/kg DM). Cows were assigned to one of two experimental groups: Control (0 g/day urea; ~18% CP), and a Urea supplemented group (350 g/day urea; ~23% CP). Cows were acclimated to the diets and metabolism stalls for 14 days, and a further 7 days were used for total collection of urine, faeces and milk.

Increasing dietary CP content had no effect on DMI, milk yield, milk composition, and faecal N. Urinary urea N (UUN) and urine N yield and concentrations increased as dietary CP content increased however, urinary creatinine, ammonia (NH_3), calcium and magnesium were not affected. Rumen urea and NH_3 concentrations were increased as CP content increased. Milk urea N showed trends for linear responses to increasing N intake ($P < 0.001$, $R^2 = 0.47$). A 16.5% increase in N intake resulted in a 42.5% increase in milk urea nitrogen (MUN) concentration; however, the relationship was restricted to low MUN concentrations. Urinary N increased linearly as a result of N intake, although the relationship was restricted due to the underestimation of urinary N and the limited range of N intake values. The 28% increase in urinary N excretion resulted from a sharp 3.6% decline in N efficiency as dietary N content increased.

The main conclusions of this thesis were the ability for excessive urea intake to reduce milk yield in grazing dairy cows. Further research is needed to determine if high soluble NPN concentrations in fresh pasture would affect DMI and milk yield in the same way. Increasing N intake results in linear increases in MUN, urinary N and UUN. These relationships could provide useful tools to predict urinary N excretion due to the strong relationships between these variables. Further research is needed to develop robust prediction equations for the relationships between these variables in grazing dairy cows before they could be used as regulatory tools.

INTRODUCTION

Farming has played an important role in New Zealand's economy for over 100 years (PCE, 2004). Dairy and meat products are the single biggest export earners and currently comprise ~50% of New Zealand's export income (Statistics NZ, 2012). Overall, farming contributes over 5% of gross domestic product in New Zealand and is significantly important to the economy (Statistics NZ, 2012).

There are, currently, around 60,000 farms in New Zealand, with more than half of New Zealand's land area used for farming including production forestry (PCE, 2004; Statistics NZ, 2012). As illustrated in Figure 1, the dominant land use is sheep farming; however, dairy farming also makes up a significant portion of the land use and is the largest industry (~$15.8 billion) in New Zealand, accounting for 25% of export income (Statistics NZ, 2012). The New Zealand dairy industry is the largest single contributor to internationally traded dairy products, with a trade value of nearly US$ 8 billion (United Nations, 2014).

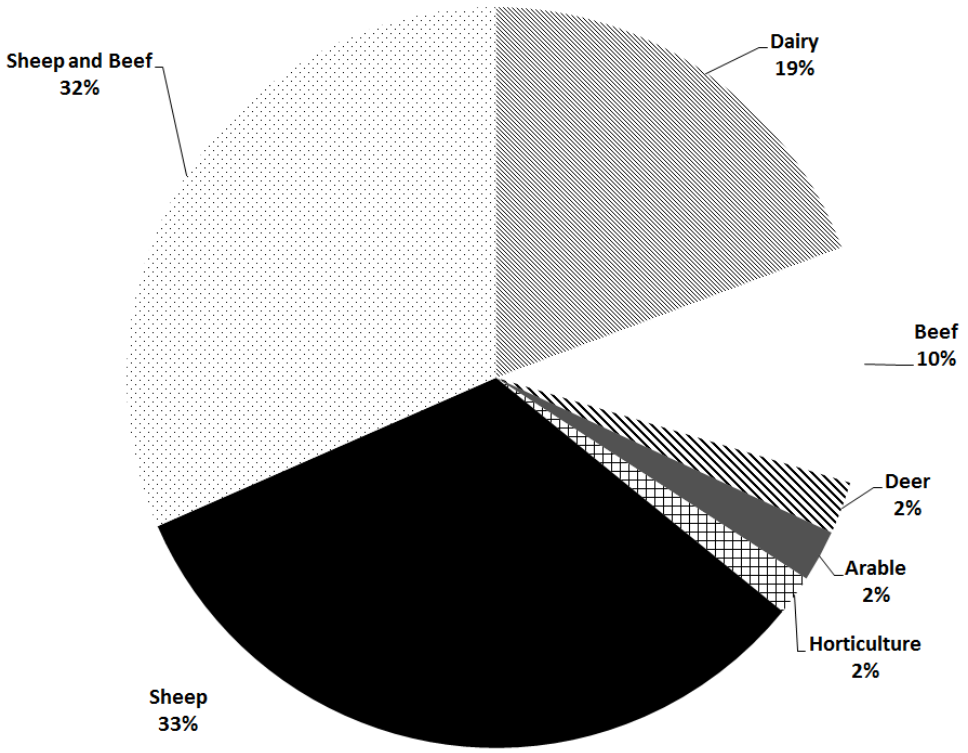


Figure 1: New Zealand land area distribution for different farming types in 2012; excluding forestry and 'other' usage, adapted from (Statistics NZ, 2012).*Viticulture is included within horticulture, as vineyards make up only about 0.4% of the total land area farmed in New Zealand. The total area of land used for farming is approximately 14 million hectares.

New Zealand's dairy farming has traditionally centred on a seasonal, low-cost pasture-based system, where cows calve in late winter/early spring and are subsequently milked during spring, summer and

autumn, but 'dried off' (a management technique to cease lactation) during late autumn/winter when pasture growth is minimal (Ulyatt, 1997; PCE, 2004). This results in a large volume of milk available during spring/early summer. As a result, the global demand for milk products is a key driver of production in New Zealand due to the domestic market being too small to utilise the milk product available (Scarsbrook and Melland, 2015). The pasture-based system allows New Zealand to remain competitive on the international market due to the associated low cost of production (Ulyatt, 1997). Dairy farmers in New Zealand are paid in relation to the fat and protein supplied and the price paid is consistent throughout New Zealand. However, this price is dependent on world market prices, and is therefore difficult to predict (Penno and Kolver, 2000). Combinations of increasing global demand for milk products, along with ongoing financial pressure to increase efficiency both on farm and throughout the industry, have driven the intensification of dairying in New Zealand (PCE, 2004; Scarsbrook and Melland, 2015).

To illustrate this intensification, in 1993/94 there were 2.7 million cows on 1.1 million hectares in New Zealand and 20 years later in 2014/15 there were 5.0 million cows on 1.7 million hectares (LIC and DairyNZ, 2015). Along with this substantial increase in cow numbers and area under dairy farming in New Zealand, the milk yield per hectare increased due to both an increase in stocking rate and an increase in milk yield per cow (Table 1) (LIC and DairyNZ, 2015). As a result, the total milksolids yield increased 157% between 1994 and 2015 (Table 1).

Table 1: Comparative dairy industry figures highlighting potential drivers of environmental impact. Information is sourced from LIC (1994) and LIC and DairyNZ (2015).

Total	**Industry estimate 2014-2015**	**% Change over time 1994-2015**
Dairy cows, million	5.0	82%
Area under dairy, million ha	1.7	55%
Milk yield, million kg milksolids	1890	157%
Average		
Stocking rate, cows/ha	2.87	18%
Herd size, cows/farm	419	123%
Milk yield, kg milksolids/cow	377	36%
Milk yield, kg/ha	1082	53%

ha = hectares

This intensification is due to a large area of sheep, and beef farms and some forestry in the South Island being converted to dairy, due to the availability of water resources for irrigation (PCE, 2013). In 1994, the South Island contained 12% of the total dairy cattle population in New Zealand (LIC, 1994). This increased by 28% from 1994-2015, resulting in a total dairy cow population in the South Island of 2.0 million (LIC, 1994; LIC and DairyNZ, 2015). The current distribution of the New Zealand dairy herd by region is presented in Figure 2 (LIC and DairyNZ, 2015).

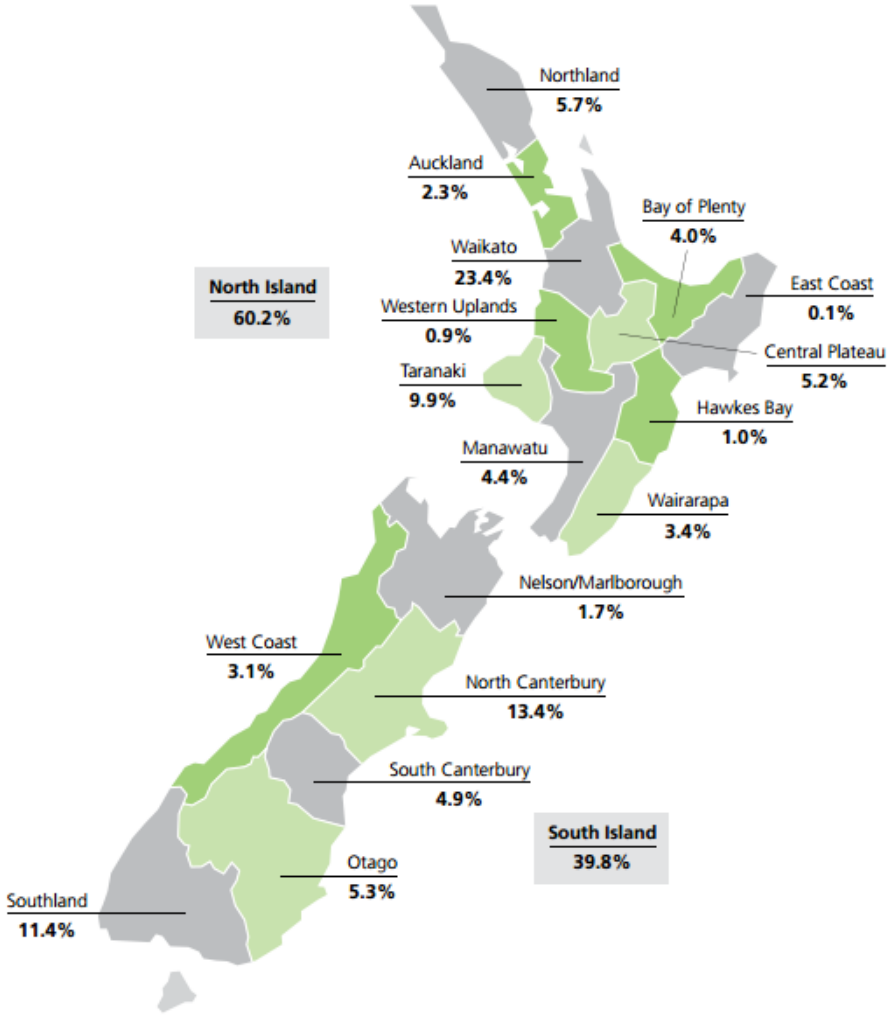


Figure 2: Regional distribution of dairy cows (percentage of total herd) in New Zealand (2014/15) (LIC and DairyNZ, 2015).

New Zealand's pasture-based system results in the feeding of pasture that often contains high concentrations of crude protein (CP) (Ulyatt, 1997), which, in excess, is not utilised efficiently by the dairy cow. These high concentrations of CP are a result of the timing of grazing and are further excacerbated by nitrogen (N) fertiliser inputs (Van Vuuren *et al.*, 1991; Lambert *et al.,* 2004). The growth and intensification of dairying in the past 20 years has resulted in a 17% increase in stocking

rate (Table 1) and an almost 7-fold increase in the use of N fertiliser (from 75,800 t in 1993 to 511,074 t in 2013) (Statistics NZ, 2012).

As a result of intensification of dairy farming in New Zealand, water quality has declined due to increased nutrient loading and subsequent eutrophication (Ballantine and Davies-Colley, 2014). Urine from farm animals is the major source of N in New Zealand's waterways draining agricultural catchments (PCE, 2013;). Over a 22 year time series (1990-2011), excreted N loads from dairy cows has more than doubled (102% increase) and nitrate (NO_3^-) concentrations in waterways have also increased (Figure 3) (Scarsbrook and Melland, 2015). Increased N fertiliser use has also resulted in increased dietary N intake and subsequent deposition of urinary N on pasture, due to inefficient N use by the dairy cow as well as smaller losses of N fertiliser (Monaghan *et al.,* 2005).

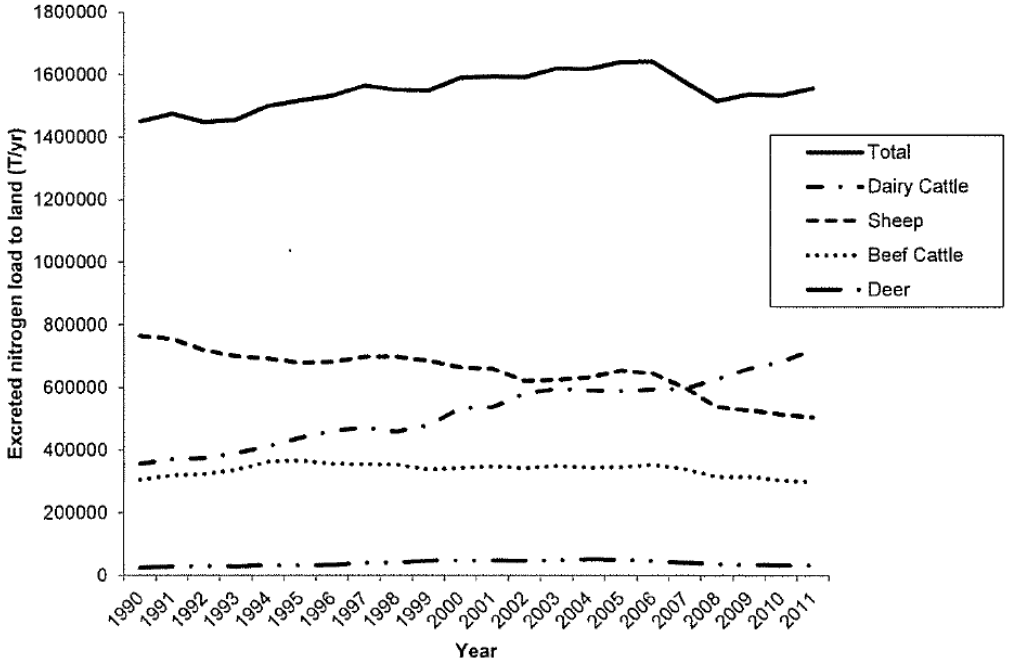


Figure 3: National trends in nitrogen excreted load to land (t/year) from all stock types for the period 1990-2011 in New Zealand (Scarsbrook and Melland, 2015).

The loss of NO_3^- due to its susceptibility to leaching from soil into groundwater is of major concern to freshwater ecosystems. Urinary N enters groundwater via drainage through soil and N fertiliser enters surface waterways via runoff or direct input (Clark, 1997). Together these have adverse effects on water quality due to accelerated eutrophication of surface water, resulting in the increased growth of algae and nuisance weeds, causing a shortage of oxygen for aquatic life (Gregg *et al.,* 1993). This can result in death of aquatic organisms, can have adverse effects on tourism, and increase treatment costs, for potable water (Di and Cameron, 2005; MFE, 2014).

The New Zealand dairy industry is striving for increased productivity, whilst maintaining or reducing the environmental footprint, with particular emphasis on reducing N leaching to waterways (Pacheco *et al.,* 2007). Consequently, research into the reduction of N losses from dairy farm systems is a high priority for the dairy industry to comply with environmental standards (DairyNZ, 2014). Animal nutrition is a major management tool to reduce the N lost through urine; however, a reduction in dietary N can also negatively affect animal production (Fanchone *et al.,* 2013). Therefore, it is important to understand N partitioning in the cow to provide guidelines and models to mitigate N losses through managing nutrition (Tamminga, 1992).

ABBREVIATIONS

ADF	Acid detergent fibre
ATP	Adenosine triphosphate
CP	Crude protein
DM	Dry matter
DMD	Dry matter digestibility
DMI	Dry matter intake
H^+	Hydrogen ion
ME	Metabolisable energy
MJ ME	Megajoules metabolisable energy
MP	Microbial protein
MUN	Milk urea nitrogen
N	Nitrogen
NDF	Neutral detergent fibre
NIRS	Near-infrared spectroscopy
NPS-FM	National Policy Statement for Freshwater Management
N_2O	Nitrous oxide
NO_3^-	Nitrate
NH_3	Ammonia
NH_4^+	Ammonium
NPN	Non-protein nitrogen
OM	Organic matter
RDP	Rumen degradable protein
RUP	Rumen undegradable protein
SP	Soluble protein
TLI	Trophic level index
UUN	Urinary urea nitrogen
WSC	Water-soluble carbohydrates

ACKNOWLEDGEMENTS

Firstly, I would like to thank my two supervisors, Prof. Danny Donaghy and Dr. John Roche. I am incredibly grateful that I have had two incredibly helpful and supportive supervisors to guide me through my Master's degree. I could not have completed my thesis to a high standard without your input.

Danny, thank you for your mentoring during my university journey as both an undergraduate and post-graduate student. Thank you for always having a listening ear; that I could always come and discuss my ideas, progress and when needed, general life matters. Thank you for always accommodating my study within your busy work schedule. Thank you for reading countless drafts and giving me invaluable and occasionally comical feedback with the writing of this thesis.

John, your enthusiasm about research science and particularly animal nutrition was a major catalyst that encouraged me to enrol as a post-graduate student. Thank you for your prompt replies to my countless emails no matter where you were in the world, and taking time out of your busy schedule to fit in meetings. Thank you for your invaluable knowledge and feedback that contributed to the completion of my thesis.

In addition, I would like to thank the rest of my thesis committee. Dr. Angela Sheahan thanks for your insightful comments and help with the collection of my data. Prof. Nicolas Lopez-Villalobos thanks for your statistical advice and encouragement.

Thank you DairyNZ Inc and MPI for funding the experimental programme through the Sustainable Farming Fund. Thank you Massey University for assisting me in the completion of my Master's thesis. I also want to thank my fellow IVABS post-graduate friends for the stimulating discussions, and the much needed laughs during stressful times throughout completing my Master's degree.

Finally, I would like to thank my family, friends and partner. Thank you for your love and support. Particular thanks to my parents who have supported and encouraged me throughout my university endeavours and my life. A big thanks and lots of love go to my partner, Jack, for all his love, wise words and support during the last 2 years.

LIST OF FIGURES

Figure 1: New Zealand land area distribution for different farming types in 2012; excluding forestry and 'other' usage, adapted from (Statistics NZ, 2012).*Viticulture is included within horticulture, as vineyards make up only about 0.4% of the total land area farmed in New Zealand. The total area of land used for farming is approximately 14 million hectares. iii

Figure 2: Regional distribution of dairy cows (percentage of total herd) in New Zealand (2014/15) (LIC and DairyNZ, 2015). v

Figure 3: National trends in nitrogen excreted load to land (t/year) from all stock types for the period 1990-2011 in New Zealand (Scarsbrook and Melland, 2015)..... vi

Figure 4: Cow density at district scale. Stars indicate areas where existing dairy farmers are operating under regulated nitrogen limits (Scarsbrook and Melland, 2015). 5

Figure 5: Transformations of nitrogen in grazed legume based pasture (R; complex organic molecule, N_2; nitrogen gas, N_2O; nitrous oxide, NO; nitric oxide, NH_3; ammonia gas, NH_4^+; ammonium ion, NO_2^-; nitrite and NO_3^-; nitrate)..... 6

Figure 6: Digestion and metabolism of nitrogenous compounds in the rumen (McDonald *et al.,* 2011). 8

Figure 7: Dry matter intake (DMI) response (kg/day) to dietary crude protein (% CP) and urea (U, % of CP) in the diet (Polan *et al*., 1976)..... 15

Figure 8: Milk yield response (kg/day) to dietary crude protein (% CP) and urea (U, % of CP) in the diet (Polan *et al.,* 1976). 15

Figure 9: Milk yield (kg/day) versus dry matter intake (DMI) (kg/day) with varying dietary crude protein (% CP) and urea (U) (% of CP) in the diet (Polan *et al.,* 1976)..... 16

Figure 10: Total daily urea production (diamonds) and gut urea entry rate (squares) relative to nitrogen (N) intake in growing cattle (solid symbols) and lactating dairy cows (open symbols), as measured using dual-labeled urea infusions (adapted from Reynolds and Kristensen, 2008). 18

Figure 11: Dietary crude protein (CP) concentration and the fraction of total urea production (UER) that is returned to the gut via blood and saliva (A) or excreted in urine and milk (B) in cattle, as measured using dual-labeled urea infusions (adapted from Reynolds and Kristensen, 2008).. 19

Figure 12: Interplay of dietary, ruminal, and extraruminal sources of nitrogen and amino acids (AA). CH_2O = carbohydrates; Export - milk, conceptus, scurf, secretions. Gut or tissue compartments are labelled in bold, italic print (Hall and Huntington, 2008). 21

Figure 13: Relationship between total nitrogen (N) intake (g/day) and faecal, milk and urinary N outputs (g/day). The fitted lines were given by equations 1 (-----), 2 (—·—) and 3 (——) (Castillo *et al.,* 2000). 24

Figure 14: Relationship between total nitrogen intake (g/day) and faecal, milk and urinary nitrogen outputs (g/day). The fitted lines were given by equations 4, 5 and 6 in Table 5 (Kebreab *et al*., 2001). 24

Figure 15: Relationship between total nitrogen (N) intake (g/day) and faecal (circles), milk (triangles) and urinary (squares) N outputs (g/day). The fitted lines were according to equations 7, 8 and 9 in Table 6 (Kebreab *et al.,* 2001). 25

Figure 16: Effect of type of energy source on faecal (solid), urinary (unshaded) and milk (grey) nitrogen (g/day) (Kebreab *et al.,* 2001). 26

Figure 17: Effect of protein degradability on faecal (solid), urinary (unshaded) and milk (grey) nitrogen (g/day) (Kebreab *et al.,* 2001). 27

Figure 18: Perennial ryegrass herbage changes in crude protein (g/kg dry matter (DM)), non-structural carbohydrates (g/kg DM), metabolisable energy (MJ ME/kg DM) and neutral detergent fibre (g/kg DM) with regrowth (Chaves *et al*., 2006). 31

Figure 19: Mean urea intake (g/day) in the High (circles) and Medium (squares) treatments during the 5 periods over the course of the experiment. 39

Figure 20: Diurnal profile of cows grazing during peak lactation. Shading represents milking time and vertical dashed lines represent sunrise and sunset. Green arrows represent additions of urea. Modified from Sheahan *et al.* (2011). 40

Figure 21: Interaction between urea treatment and period in estimated dry matter intake (DMI), dietary crude protein content (CP %), milk yield and the yield of milk components ($P < 0.001$); Control (open circles), Medium (squares) and High (circles). 47

Figure 22: Relationship between urea intake (g/day) and milk yield (kg/day) for all cows ($y = 20.5 + 0.0029x$, $x \leq c$; $y = 20.5 + c\,(350\,(0.0029 - {}^{-}0.0235)) + {}^{-}0.0235x$, $x > c$; $c = 350$ g urea/day; $P < 0.001$). 49

Figure 23: Relationship between crude protein content (CP %) and milk yield (kg/day) for all cows ($y = 36.175 - 0.612x$; $P < 0.001$; $R^2 = 0.45$). 50

Figure 24: Relationship between milk yield (kg/day), dry matter intake (DMI) (kg/day) and urea intake (g/day) ($y = -21.05 + 2.69d + 0.028x - 0.0012d^{1} - 0.00000115d^{2}$; $R^2 = 0.75$; $P < 0.001$) ... 51

Figure 25: Relationship between total urine urea nitrogen (mmol/d) and total nitrogen intake (g/d) for all cows. 65

Figure 26: Relationships between total urine nitrogen (g/day), estimated urine nitrogen (g/day) and total nitrogen intake (g/day) for all cows. 66

Figure 27: Relationships between milk urea nitrogen (mmol/L) and total nitrogen intake (g/day) and total urine nitrogen (g/day) for all cows. 67

Figure 28: Relationship between milk urea nitrogen (mmol/L) and rumen ammonia (NH_3-N) concentrations (mg/dL) for all cows. 68

Figure 29: Comparison of the predictions of urinary nitrogen excretion (g/day) based on milk urea nitrogen (mg/dL) according to the current study (y = 75.6 + 17.8x; R^2 = 0.32), along with studies by Jonker *et al.* (1998), Kauffman and St-Pierre (2001) and Nousiainen *et al.* (2004). . 76

LIST OF TABLES

Table 1: Comparative dairy industry figures highlighting potential drivers of environmental impact. Information is sourced from LIC (1994) and LIC and DairyNZ (2013).. iv

Table 2: Crude protein concentration (CP %), nitrogen (N) fractions (g/kg dry matter (DM), degradation rate of true protein (k_d) and rumen undegradable protein digestibility (RUP %) of grass – legume mix and total mixed ration (TMR) diets (adapted from NRC, 2001). 9

Table 3: Effects of supplementing different carbohydrate types to grazing dairy cows in early lactation on productive nitrogen (N) output, milk urea nitrogen (MUN), blood urea nitrogen (BUN) and urinary N to creatinine (N:creatinine) (Higgs *et al.,* 2013)..... 20

Table 4: Intake, nitrogen (N) partitioning and milk yield by cows fed the synchronous diet (SYND) or the asynchronous diet (ASYND) (Kolver *et al.,* 1998). 22

Table 5: Relationship between nitrogen intake (N_i), faecal nitrogen (N_f) , milk nitrogen (N_m) and urinary nitrogen (N_u) across two studies (Castillo *et al.,* 2000; Kebreab *et al.,* 2001). 23

Table 6: Relationship between nitrogen intake (N_i), faecal nitrogen (N_f) , milk nitrogen (N_m) and urinary nitrogen (N_u) for diets of similar composition (Kebreab *et al*., 2001)..... 25

Table 7: Nutrient composition of experimental diets and means for nitrogen intake (NI), net energy (NE), milk urea nitrogen (MUN), blood urea nitrogen (BUN) and urine urea nitrogen (UUN) excretion (Burgos *et al.,* 2007)..... 28

Table 8: Dietary crude protein (CP %), dry matter intake (DMI) and nitrogen (N) intake of dairy cows offered increasing amounts of urea as a supplement to pasture during early lactation in the Control, Medium and High treatments. 46

Table 9: Yield of milk (kg/day) and milk components of dairy cows offered increasing amounts of urea as a supplement to pasture during early lactation in the Control, Medium and High treatments. 48

Table 10: Sequence of equations used to determine final models of milk yield (kg/day). 50

Table 11: Chemical composition of pasture fed to all treatments (mean ± standard error). Percentage crude protein (% CP), available protein (% AP), soluble protein (% SP), water soluble carbohydrates (% WSC), acid detergent fibre (% ADF), neutral detergent fibre (% NDF), lignin,

dry matter (% DM), organic matter (% OM), organic matter digestibility (% OMD), dry matter digestibility (% DMD), and metabolisable energy (ME). Wet chemistry analysis (Dairy One, Analytical Services, New York, USA) and NIRS (NIRS; Feed Tech, Palmerston North, New Zealand). 57

Table 12: Minerals and trace element composition of pasture fed to all treatments (mean ± standard error). Percentage calcium (% Ca), phosphorus (% P), magnesium (% Mg), potassium (% K), sulphur (% S), sodium (% Na), chloride ion (% Cl) and ash (% Ash). Parts per million iron (PPM Fe), zinc (PPM Zn), copper (PPM Cu), manganese (PPM Mn), and molybdenum (PPM Mo). Wet chemistry analysis (Dairy One, Analytical Services, New York, USA). 58

Table 13: Intake, liveweight, digestibility of feed and faecal weight in the Control and Urea supplement treatments. Dry matter intake (DMI), nitrogen (N), crude protein (CP) dry matter (DM), and dry matter digestibility (DMD). 62

Table 14: Water balance in the Control and Urea supplement treatments..... 63

Table 15: Yield of milk and milk components of the Control and Urea supplement treatments during the 7 day nitrogen balance period. 63

Table 16: Yield of urine (mmol/d) and urine components of the Control and Urea supplement treatments during the 7 day nitrogen balance period. 64

Table 17: Nitrogen parameters pertaining to partitioning in urine, faeces, milk and rumen fluid of cows grazing pasture in the Control and Urea supplement treatments. 66

Table 18: Urea dosing schedule for the Medium and High treatments during the experimental period..... 88

Table 19: Urea dosing schedule for the Urea supplement treatment during the acclimation period. 89

Table 20: Urea dosing schedule for the Urea supplement treatment during the metabolism stall and collection period. 89

Table 21: Yield of milk (kg/day), milk components, pasture intake and pasture N content from dairy cows offered increasing amount of urea as a supplement to pasture during early lactation. .. 90

TABLE OF CONTENTS

ABSTRACT ..... **i**

INTRODUCTION ..... **iii**

ABBREVIATIONS ..... **viii**

ACKNOWLEDGEMENTS ..... **ix**

LIST OF FIGURES ..... **x**

LIST OF TABLES ..... **xiii**

Chapter 1: Review of literature; Nitrogen-use efficiency in dairy cows and its adverse effects on the environment **3**

1.1 Introduction **3**

1.2 Water quality in New Zealand **3**

1.3 The nitrogen cycle **6**

1.4 Nitrogen metabolism **7**

1.4.1 Protein degradation 8

1.5 Ammonia toxicity **11**

1.6 Supplementing feed grade urea **13**

1.7 Urea recycling **17**

1.8 Nutrient synchrony **19**

1.9 Relationships between dietary nitrogen and urinary nitrogen **23**

1.9.1 Other factors influencing nitrogen-use efficiency 25

1.10 Dietary nitrogen supply **29**

1.11 Sources of error in N balance studies **32**

1.12 Conclusion **34**

Chapter 2: Effect of high-urea supplementation on pasture dry matter intake and milk production in grazing dairy cows **37**

2.1 Introduction **37**

2.2 Materials and methods **39**

2.2.1 Experimental design 39

2.2.2 Pasture offered 40

2.2.3 Milking and milk sampling 41
2.2.4 Liveweight and body condition score 41
2.2.5 Calculations and statistical analysis 42
2.2.5.1 Calculations 42
2.2.5.2 Statistical analysis 44
2.3 Results 46
2.3.1 Dry matter intake and milk production 46
2.4 Discussion 52
Conclusion 53
Chapter 3: Effect of urea supplementation on N partitioning in dairy cows 54
3.1 Introduction 54
3.2 Materials and methods 56
3.2.1 Experimental design 56
3.2.2 Pasture offered 56
3.2.3 Milking and milk samples 58
3.2.4 Liveweight and body condition score 58
3.2.5 Urine and faecal sampling 59
3.2.6 Ruminal samples 60
3.2.7 Calculations and statistical analysis 60
3.2.7.1 Calculations 60
3.2.7.2 Statistical analysis 61
Chapter 3.3: Results 62
3.3.1 Intake, digestibility of feed, liveweights and faecal weight 62
3.3.2 Water balance 62
3.3.3 Milk production 63
3.3.4 Urine excretion 63
3.3.5 Nitrogen balance 64
Chapter 3.4: Discussion 69
Nitrogen intake 69
Faecal nitrogen 70
Milk production 70
Nitrogen balance 72

Urinary nitrogen 73
Milk urea nitrogen 74
Ruminal nitrogen 77
Conclusion 79
General discussion and conclusions 80
APPENDICES 84
Appendix 1: Analytical procedures adapted from (Dairy One, 2014) 84
Dry matter 84
Protein 84
Crude protein 84
Soluble protein 84
Fibre 85
Acid detergent fibre (ADF) 85
Neutral detergent fibre (NDF) 85
Lignin 86
Minerals 86
Supplemental services 87
Ash 87
Starch 87
Appendix 2: Urea dosing schedule for Chapter 2 88
Appendix 3: Urea dosing schedules for Chapter 3 89
Appendix 4: Additional data for Chapter 2 90
BIBLIOGRAPHY 91

Chapter 1: Review of literature; Nitrogen-use efficiency in dairy cows and its adverse effects on the environment

1.1 Introduction

The New Zealand dairy industry is striving for increased productivity while concomitantly decreasing nutrient losses to waterways (Bryant *et al.,* 2010; Christensen *et al.,* 2011). Nitrogen is the major nutrient that is lost to waterways in New Zealand and is of major concern to freshwater ecosystems. Due to New Zealand's predominantly pasture-based system, where cows are grazed outdoors, urination on pasture and races by grazing animals is the major cause of N losses, through subsequent NO_3^- leaching and nitrous oxide (N_2O) emissions (Pakrou and Dillon, 2000; Di and Cameron, 2005). Urination onto pasture results in highly variable concentrations of N being deposited in a urine patch (Haynes and Williams, 1993). A *meta*-analysis undertaken by Selbie *et al.* (2015) calculated an average urine N loading rate of 613 kg N/ha for dairy cattle, which is much higher than the plants' requirements (Selbie *et al.,* 2015). Nitrogen that is not utilised is prone to leaching into groundwater, as it is not adsorbed to any extent by the soil surface (Selbie *et al.,* 2015). The loss of NO_3^-, due its susceptibility to leaching from soil into groundwater, is of major concern to freshwater ecosystems (Gregg *et al.,* 1993; Wilcock *et al.,* 2013). Nitrate in waterways causes increased growth of algae and nuisance weeds, which affect aquatic organisms and recreational users of water bodies (Gregg *et al.,* 1993; MFE, 2014). This has led to research focused on mitigation strategies that reduce the quantity of N deposited on paddocks and, subsequently, the quantity of N lost to ground and surface water through NO_3^- leaching and runoff, respectively. Several methods involving animal nutrition have been researched to reduce N losses with little success, due to the complex N dynamics in ruminants which will be discussed from Chapter 1.4 onward.

1.2 Water quality in New Zealand

Water quality has declined due to increased nutrient loading and subsequent eutrophication (Pacheco *et al.,* 2007; Ballantine and Davies-Colley, 2014). Over a 22 year time series (1990-2011), excreted N loads from dairy cows has more than doubled (102% increase) and NO_3^- concentrations in waterways have also increased (Figure 3) (Scarsbrook and Melland, 2015). Trends in levels of N and NO_3^- are among several nationally measured variables to assess water quality at river sites. A study by Ballantine and Davies-Colley (2014) assessed the water quality trends in New Zealand from 1989-2009 by analysing data collected at 77 National Rivers Water Quality Network sites on 35 major river systems that, together, drain about 50% of New Zealand's land area. A significant

increasing trend was identified at the national scale for total and oxidised N in water, which indicates deteriorating water quality (Ballantine and Davies-Colley, 2014). The relationships between median values for total N, oxidised N and pastoral land cover were mainly positive, indicating that the observed trends for N levels in rivers are consistent with observed changes in the scale and intensity of agricultral land use across New Zealand (Ballantine and Davies-Colley, 2014; Scarsbrook and Melland, 2015).

As well as degradation of water quality in rivers, the water quality in lakes has also declined. Verburg *et al.* (2010) assessed lake water quality for 112 lakes for the period 2005-2009. Trophic state was assessed using a trophic level index (TLI) calculated from total N, total phosphorus and chlorophyll *a* (Burns *et al.,* 1999). Verburg *et al.* (2010) reported that 44% of currently monitored lakes were eutrophic or worse (e.g. TLI >4, rich in nutrients) and 33% were oligotrophic or better (e.g. TLI <3, poor in nutrients). Water quality trends indicated that 19 lakes (28% of the total measured) had deteriorated (increased in TLI) since 2005 and eight lakes (12%) had improved (Verburg *et al.,* 2010). Although there were more lakes where TLI had declined than lakes where TLI had improved, there was a slight but significant decrease in mean TLI, indicating improved conditions during the period. It was suggested that this was because the lakes in which water quality declined were on average oligotrophic while the lakes in which water quality improved were on average eutrophic (Verburg *et al.,* 2010). This indicates that some industry-led practice change and current operative regional N leaching plans have already contributed to improving the quality of high value lakes. However, due to the reduction in lake and river water quality with increasing percentage of pastoral land cover in their catchments (Verburg *et al.,* 2010; Ballantine and Davies-Colley, 2014), it is a priority for local and national governments to continue to improve the management of freshwater resources by setting freshwater objectives for freshwater bodies (MFE, 2013). This should focus on preventing further degradation of lakes, rivers and groundwater resources, as well as rehabilitating water bodies that have been degraded (Scarsbrook and Melland, 2015).

In New Zealand, the National Policy Statement for Freshwater Management (NPS-FM) requires all regional councils to establish objectives and set limits for freshwater quality and quantity in their regional plans, with these limits being fully implemented by 2025 (NPS-FM, 2014). These freshwater objectives and limits must ensure that the national 'bottom lines' for water quality (ecosystem health and human health for recreation) are achieved and water quality cannot be allowed to degrade across a region (NPS-FM, 2014). This will require regional councils to incorporate policies and methods, such as property-scale nutrient discharge allowances (NPS-FM, 2014). Policies and methods will need to be managed and addressed within a specified timeframe (NPS-FM, 2014).

The NPS-FM took effect on 1st August 2014 and regional councils must implement the NPS-FM by the end of 2015. Throughout New Zealand, regional councils have developed policies to set limits, particularly for N leaching into groundwater from agricultural land (Enviroment Canterbury, 2014). For example, catchments in the central North Island (around lakes Taupo and Rotorua), Taranaki, Manawatu-Wanganui (e.g. upper Manawatu river), Canterbury (Hurunui-Waiau zone) and Otago all have N leaching limits set in operative regional plans (Figure 4) (Scarsbrook and Melland, 2015).

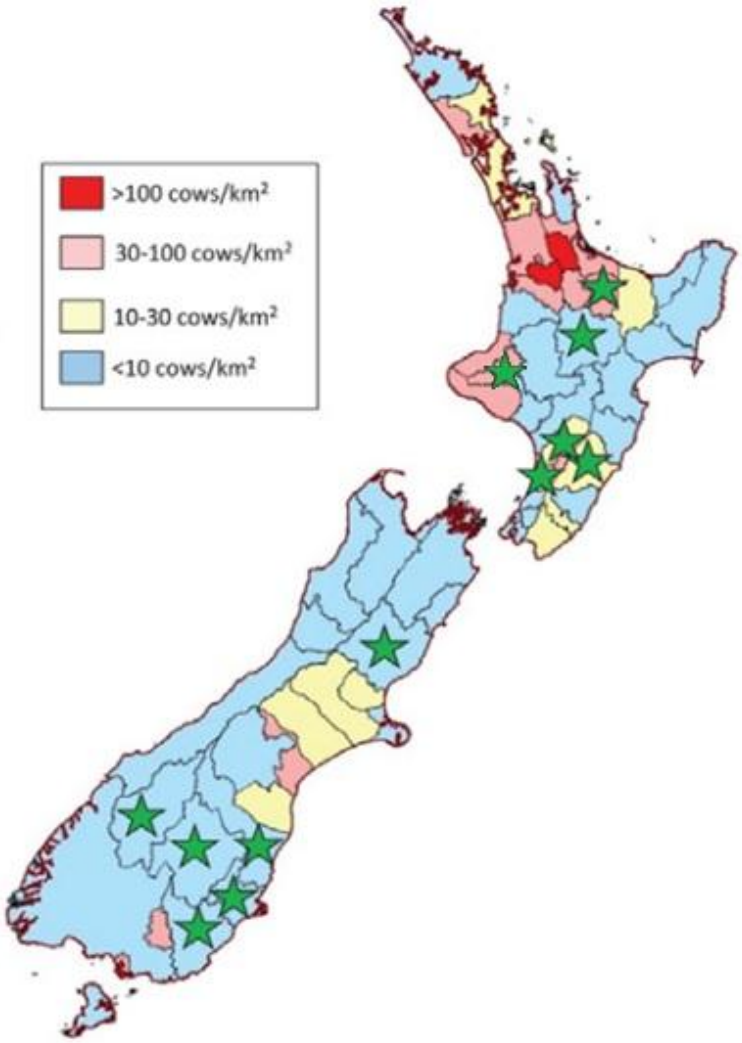


Figure 4: Cow density at district scale. Stars indicate areas where existing dairy farmers are operating under regulated nitrogen limits (modified from Scarsbrook and Melland, 2015).

Although these limits will help local government achieve freshwater objectives and reduce N losses to the environment, the limits will also constrain dairy land use intensity and productivity, unless research can identify how dairying can operate within these limits (Scarsbrook and Melland, 2015).

1.3 The nitrogen cycle

Nitrogen is essential for plant growth and undergoes several transformations in the soil (Figure 5). Plants absorb their N from the soil water in the form of NO_3^- and ammonium (NH_4^+) ions. Nitrogen enters the N cycle in soil through decomposition of soil organic matter (OM), and through application of N fertiliser, faeces and urine as shown in Figure 5 (Tamminga, 1992).

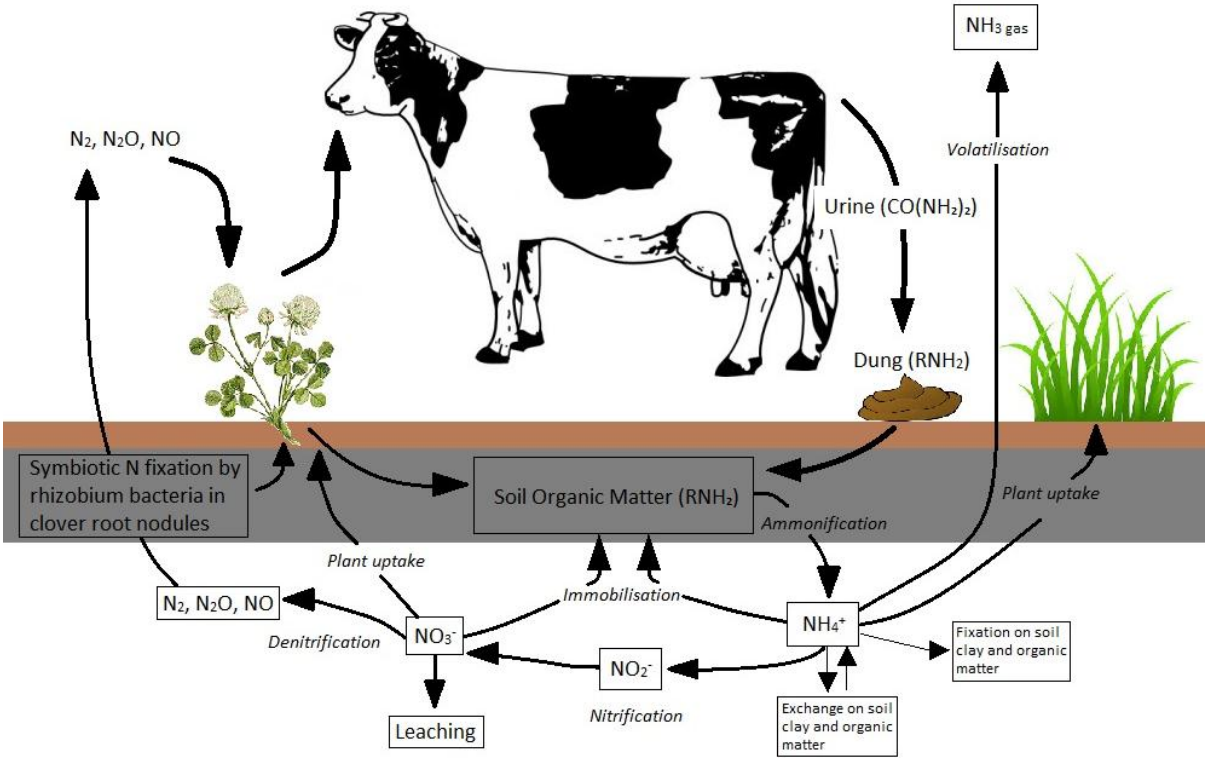


Figure 5: Transformations of nitrogen in grazed legume based pasture (R; complex organic molecule, N_2; nitrogen gas, N_2O; nitrous oxide, NO; nitric oxide, NH_3; ammonia gas, NH_4^+; ammonium ion, NO_2^-; nitrite and NO_3^-; nitrate).

These N inputs from OM, fertiliser, faeces and urine are converted to NH_4^+ by a process of ammonification, undertaken by soil micro-organisms in an aerobic environment. Ammonia (NH_3) can also be directly applied to soil from NH_3 fertiliser application (Whitehead, 1995). Ammonium ions are positively charged and can be either directly absorbed by the plant, fixed onto the soil surface, or undergo nitrification, where NH_4^+ is converted to NO_3^-, also by soil micro-organisms (Tamminga, 1992). This also occurs in an aerobic environment and this NO_3^- can be directly absorbed by the plant. Nitrate is negatively charged and due to the negatively-charged clay and organic colloids, it is not adsorbed by the soil surface; if it is not taken up by the plant, it is lost into surface and groundwater through NO_3^- runoff and leaching, respectively (Di and Cameron, 2005; Qiu *et al.,* 2010). If not leached or taken up by the plant, NO_3^- may undergo denitrification by soil micro-

organisms under anaerobic conditions, resulting in the formation of N_2O which is emitted into the atmosphere(Selbie *et al.,* 2015).

Nitrogen leaching from New Zealand's pasture-based systems increases exponentially with increased N inputs. Nitrogen fertiliser and urine deposited on pastures and races by grazing animals contributes 70 to 90% of N leaching (Ledgard *et al.,* 2009). Deposition of urine by grazing animals results in a non-uniform return of large quantities of N to the soil. Deposition of N at rates of up to 1000 kg/ha, predominantly urinary urea, is added to a small volume of soil, 'the urine patch' (Haynes and Williams, 1993). This quantity of N in the urine patch exceeds the plants' immediate requirements (Haynes and Williams, 1993; Whitehead and Raistrick, 1993; Dijkstra *et al.,* 2013). Consequently, this surplus of N in urine patches results in mineral N accumulating in the soil profile (Haynes and Williams, 1993). This mineral N is available to soil micro-organisms and will undergo nitrification. Because NO_3^- is poorly held by the soil surface, when excess precipitation occurs and water moves down the soil profile, the NO_3^- will move downwards with the water (Haynes and Williams, 1993; Di and Cameron, 2005; Ledgard *et al.,* 2009).

The primary source of NO_3^- in drainage waters from grazed pastures is animal urine (Haynes and Williams, 1993). Studies have reported total N losses in urine and faeces ranging from 70-80% of dietary N and as a result, have been the major focus of a number of studies (Whitehead, 1995; Castillo *et al.,* 2000; Kebreab *et al.,* 2000; Burgos *et al.,* 2007). These studies, mainly undertaken using housed animals fed total mixed rations (TMR), have investigated strategies to improve N-use efficiency in animals to reduce the N concentration in urine (Castillo *et al.,* 2000; Kebreab *et al.,* 2001; Burgos *et al.,* 2007).

1.4 Nitrogen metabolism

In ruminants, dietary protein has five main routes of metabolism; these include microbial degradation in the rumen, absorption in the small intestine, deposition in tissue, secretion in milk, and catabolism and excretion of excess protein in urine and faeces (McDonald *et al.,* 2011). A large proportion of dietary protein is degraded in the rumen in pasture-fed cows (Tamminga, 1979). When dairy cattle are fed all forage diets, measurements of rumen degradable protein (RDP) are often >70% of N intake (Van Vuuren *et al.,* 1992; NRC, 2001). Dietary protein and non-protein N (NPN) entering the rumen are subjected to microbial degradation (McDonald *et al*., 2011). Non-protein N consists of amides, amines, free amino acids, urea and NO_3^- and can come from the diet as well as from endogenous sources (e.g. saliva) (Leng and Nolan, 1984; Bach *et al.,* 2005; Breves and

Wolffram, 2006; McDonald *et al.,* 2011), while dietary protein consists of both undegradable and degradable protein, as represented in Figure 6 (Bach *et al.,* 2005; McDonald *et al.,* 2011).

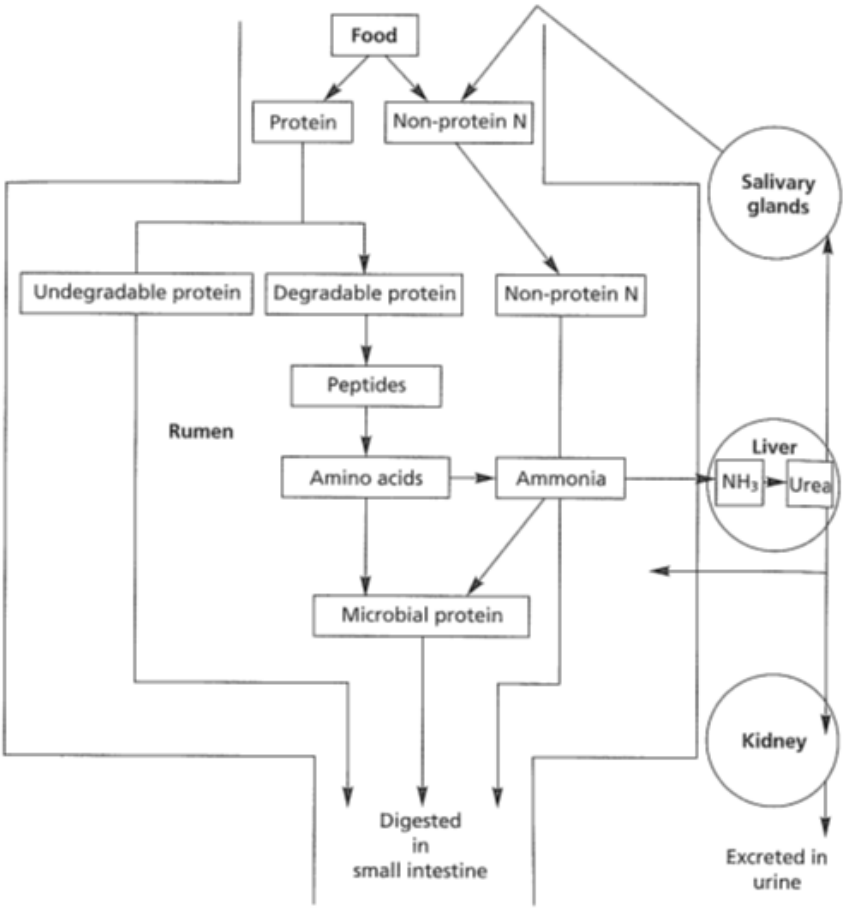


Figure 6: Digestion and metabolism of nitrogenous compounds in the rumen (McDonald *et al.,* 2011).

1.4.1 Protein degradation

The degradation rate of protein entering the rumen is affected by the type of protein (proportions of NPN and true protein), chemical and physical characteristics of the protein, rumen passage rate and rumen pH (Leng and Nolan, 1984). The Cornell Net Carbohydrate Protein System model used to calculate RDP and rumen undegradable protein (RUP) divides protein into three different fractions based on the characteristics of the protein and their chemical determination (NRC, 2001). Fraction A is the percentage of CP that is instantaneously solubilised at time zero. Data from literature studying *in vitro* protein degradation have estimated that NPN compounds are degraded at a rate of 200-300%/hour (Sniffen *et al.,* 1992), but according to the NRC (2001), it is assumed that NPN has a degradation rate (k_d) of infinity. Due to this rapid degradation in the rumen, feed types that are high in NPN are assumed to be 100% degraded in the rumen (NRC, 2001). However, this is not an entirely correct assumption, as protein degradability is closely related to rate of passage and, as a result, a

small amount of NPN can bypass degradation in the rumen (NRC, 2001). Fraction B represents the amount of potentially degradable true protein which is dependent on the fractional rate of degradation and the passage rate. In contrast to NPN, the rates of degradation of true protein are highly variable and result in variable amounts of protein being degraded in the rumen (NRC, 2001). Fraction C is the bound true protein that makes up the portion of CP in the diet that is undegradable in the rumen (NRC, 2001). Fresh pasture often contains high levels of NPN (Fraction A; Table 2), and dairy cattle fed forage diets often have low levels of RUP (Fraction C and sometimes B; <30% of total N intake from forage) passing through the rumen into the small intestine (Van Vuuren *et al.,* 1992; NRC, 2001).

Table 2: Crude protein concentration (CP %), nitrogen (N) fractions (g/kg dry matter (DM), degradation rate of true protein (k_d) and rumen undegradable protein digestibility (RUP %) of grass/legume mix and total mixed ration (TMR) diets (adapted from NRC, 2001).

		N Fractions (g/kg DM)				
Item	**CP %**	**A**	**B**	**C**	**k_d, %/h of B**	**RUP digestibility %**
Grass/Legume mix	26.5	82.4	163.2	19.4	12.3	75
TMR	17.3	75.5	82.2	15.2	7.5	75.6

A, rapidly rumen degraded protein; B, slowly rumen degraded protein and C, rumen undegradable protein. Grass – Legume mix, intensively managed ryegrass (*Lolium perenne,* L.) pasture; TMR, alfalfa silage (36.5%), corn silage (25.5%), rolled high moisture shelled corn (27.2%), solvent extracted soybean meal, 48% CP (7.3%) and roasted soybeans (2.5%). Ration formulation based on medium neutral detergent fibre (NDF) and medium CP diet formulation in Broderick (2003).

Undegradable protein moves to the small intestine, where it is digested and absorbed by the animal; however, both RDP and NPN are subject to degradation by rumen microbes. Although RDP (Fraction B) can be subjected to ruminal degradation, whether it will undergo ruminal degradation is not entirely determined by chemical characteristics of the feedstuff (Tamminga, 1979). Degradation of protein is also dependent on passage rate of digesta through the rumen (Tamminga, 1979; Hall and Huntington, 2008). Passage rate is dependent on the physical characteristics of the feed and the voluntary intake of the animal (Cronje, 2000). As particle size decreases and/or if feed is low in dry matter (DM), the level of feed intake increases. Increased levels of voluntary feed intake results in increased rate of passage and, as a result, the degradation of dietary protein in the rumen decreases due to a decrease in rumen retention time (Tamminga, 1979). Therefore, when voluntary feed intake is high, the amount of protein that is actually degraded in the rumen will be lower than the total amount of RDP available from the diet (Cronje, 2000). The protein in fresh pasture plants is approximately 75-80% degradable; however the RUP is usually higher than the feed analysis would suggest (Van Vuuren *et al.,* 1992; Ulyatt, 1997; NRC, 2001). The rate of passage of pasture-based feed tends to be lower in comparison to TMR diets (NRC, 2001). As a result, the degradation rate of

true protein (Fraction B) tends to be higher in pasture-based diets, with less reaching the small intestine (Table 2; NRC, 2001).

Chemical factors, such as the presence of bonds within and between protein chains (tertiary and quaternary structure), affect protein degradability. These bonds involve cross-linking, the presence of disulphide bonds, and decreased number of accessible hydrolysable sites in the protein molecules, resulting in decreased degradability (Romagnolo *et al.,* 1994; Bach *et al.,* 2005). Solubility of feed protein is partly determined by the relative amounts of soluble albumins, highly soluble globulins and the less soluble prolamins and glutelins (Tamminga, 1979; Romagnolo *et al.,* 1994). Feeds whose major protein fractions are albumins and globulins (e.g. leaves and stems) have higher protein solubility than feeds whose major protein fractions are prolamins and gutelins (e.g. cereal grains and by-product feeds) (Blethen *et al.,* 1990).

Protein degradation is also affected by ruminal pH, as this establishes the type of bacteria that will predominate in the rumen (Bach *et al.,* 2005). The optimal rumen pH for microbes to function ranges from 5.5 to 7.0; however, protein degradation is reduced at the lower end of this spectrum (Bach *et al.,* 2005). The rumen pH is often related to the substrate fed, with high forage diets degrading faster than high concentrate rations regardless of the ruminal pH (Cardozo *et al.,* 2000, cited in Bach *et al.,* 2005).

The degradation of protein is undertaken by the rumen microbes and involves a series of enzymatic reactions. The main microbes involved in protein degradation are protozoa and bacteria (Tamminga, 1979), with bacteria being the major contributors (Bach *et al.,* 2005). Bacteria and protozoa both have proteolytic and peptidase functions to hydrolyse protein to form peptides (Bonhomme, 1990), which can be further hydrolysed to form amino acids (Tamminga, 1979). Amino acids and NPN can be further degraded by deaminase and decarboxylase activity in bacteria to produce NH_3 (Bonhomme, 1990; McDonald *et al.,* 2011). At any stage of degradation, peptides, amino acids or NH_3 can be utilised by rumen microbes to synthesise microbial protein (MP) (Leng and Nolan, 1984; Bach *et al.,* 2005). Synthesis of nitrogenous compounds into MP requires energy from carbohydrates and includes extensive recycling of N between the body and gut lumen pools (Leng and Nolan, 1984; Reynolds and Kristensen, 2008).

Ammonia is toxic in high concentrations (blood NH_3 0.80-1.04 mg/100 ml) and must be removed from the rumen if provided in excess of the microbial population requirements (Bartley *et al.,* 1976; Nocek and Russell, 1988). Maximum rumen MP synthesis is achieved when the concentration of NH_3 in the rumen reaches >6 mM (Pisulewski *et al.,* 1981). Therefore, if energy is not limiting, then

increasing dietary N, resulting in ruminal NH_3 concentrations >6mM, will have no further effect on MP synthesis and can reduce microbial efficiency (Pisulewski *et al.,* 1981). Ammonia that is not utilised by rumen microbes can be absorbed across the rumen wall into the portal vein and is primarily converted to urea by the liver (Leng and Nolan, 1984; Tamminga, 1996; Bach *et al.,* 2005; Marini *et al.,* 2006; Reynolds and Kristensen, 2008; Bryant *et al.,* 2010).

Urea is often considered a waste product of protein degradation and can either be excreted in urine or salvaged in the kidney and recycled back into the rumen through direct transfer from blood across the gut wall or via saliva (Figure 6) (Leng and Nolan, 1984; Marini *et al.,* 2006; Reynolds and Kristensen, 2008; Calsamiglia *et al.,* 2010; McDonald *et al*., 2011). The urea recycled back into the rumen is degraded to NH_3 through the action of microbial urease (Bonhomme, 1990), and the urea recycled can be used for MP or again it can be absorbed as NH_3 into the portal blood as shown in Figure 6 (McDonald *et al.,* 2011). Microbial protein synthesised from the products of protein degradation and urea recycling is eventually carried through to the abomasum and small intestine, where it is then subjected to digestion and absorption by the animal (McDonald *et al.,* 2011).

1.5 Ammonia toxicity

Ammonia is toxic in high concentrations, as mentioned previously, and must be removed from the rumen if provided in excess of microbial requirements (Nocek and Russell, 1988). The ability of the cow to absorb NH_3 across the rumen wall and convert it to urea in the liver allows excess NH_3 to be excreted to avoid toxicity. The liver has a key role in NH_3 homeostasis, where NH_3 can be detoxified through two pathways: ureagenesis in the periportal region of the liver and glutamine synthesis in the perivenous region of the liver (Haussinger, 1990). The two pathways work together and are anatomically aligned behind each other so that NH_3 that escapes from periportal ureagenesis can be removed by perivenous glutamine synthetase (Haussinger, 1990). Plasma NH_3 will increase only if the two systems approach saturation (Zhu *et al*., 2000). Situations that result in levels of NH_3 reaching the liver that exceed the capacity for these systems to detoxify NH_3, results in NH_3 toxicity (Visek, 1984). This is often associated with high dietary NPN levels, such as when feeding high levels of feed grade urea, typically containing 46% N (Zhu *et al.,* 2000).

Ammonia toxicity can be identified from visual symptoms as well as from plasma NH_3 levels and from rumen pH. Symptoms reported in literature include uneasiness, dullness, muscle and skin tremors, excessive salivation, frequent urination and defecation, rapid respiration, incoordination, stiffening of the front legs, prostration, tetany and death (Helmer and Bartley, 1971). Muscle tetany is a common symptom of NH_3 toxicity and was the most frequently observed symptom in an

experiment by Barley *et al.* (1976), where the toxicity of several cooked mixtures of grain and urea were tested using 26 rumen-fistulated, mature fasted cattle. Urea was provided through the rumen fistula at a dosage rate of 0.5 g urea/kg liveweight.

Plasma NH_3-N concentration is a good indicator of NH_3 toxicity, due to the strong correlation between plasma NH_3-N and toxicity (R^2 = 0.71) (Bartley *et al.,* 1976). In the study conducted by Bartley *et al.* (1976), the chance of toxicity occurring was extremely high when blood NH_3-N concentration exceeded 0.8 mg/100 ml in 60 minutes. Rumen pH can also be used as an indicator of NH_3 toxicity. Bartley *et al.* (1976) reported that in toxic cases, rumen pH was elevated to 7.41 in 60 minutes, which was significantly higher than 7.16 for the nontoxic cases. Rumen pH was correlated with toxicity (R^2 = 0.32); however, rumen NH_3-N was not correlated with toxicity and the average rumen NH_3-N concentration was the same for the toxic as for the nontoxic samples (R^2 = 0.04) (Bartley *et al.,* 1976).

Ammonia exists in equilibrium between two forms; its ionic (NH_4^+) and non-ionic (NH_3) forms, and the relative concentrations of these two forms is largely dependent on pH (Visek, 1984). At neutral pH, protonated NH_4^+ is most prevalent. Since the concentration of NH_3 is inversely proportional to the hydrogen ion (H^+) concentration, as pH increases, this results in a higher concentration of NH_3 because the concentration of H^+ ions decreases (Reid, 1953; Visek, 1984). As urea is hydrolysed to NH_3 it often results in an increase in rumen pH or prevents depression of pH due to the addition of H^+ to NH_3 to form NH_4^+ (Kertz *et al.,* 1982; Kertz *et al*., 1983). The increased rumen pH causes a shift in the equilibrium and increases the concentration of NH_3. Most mammalian membranes are permeable to NH_3 and impermeable to NH_4^+ and, as a result, the absorption of rumen NH_3 into the blood increases when high concentrations of NH_3 are present and pH is elevated (pH ≥6.0) in the rumen, further exacerbating the NH_3 toxicity issue (Hogan, 1961; Bartley *et al.,* 1976; Visek, 1984). This was reported by Kertz *et al.* (1982) with cows fed a 2.5% urea-containing ration having a significantly higher rumen pH compared with the 1.0% urea and non-urea rations. Due to rumen pH determining the absorption rate of NH_3, and the absorption rate of NH_3 determining the plasma NH_3-N concentration, it is expected that the rumen pH and plasma NH_3-N are major indicators of NH_3 toxicity (Bartley *et al.,* 1976; Kertz *et al.,* 1982; Kertz *et al.,* 1983). In contrast, a high concentration of rumen NH_3 does not necessarily indicate NH_3 toxicity and therefore, rumen NH_3-N is not correlated with toxicity. Plasma NH_3-N concentration and rumen pH are better indicators of a toxicity (Bartley *et al.,* 1976).

When urea feeding trials are undertaken on fisulated cows, it is easier to treat toxicity to prevent death than on cows without cannulas. Bartley *et al.* (1976) reported that emptying the

ruminoreticulum when muscle tetany was observed, rapidly cleared NH_3 from the blood and all animals survived when this treatment method was used. The method suggested by Word *et al.* (1969), where 5% v/v acetic acid solution was administered to cattle exhibiting symptoms of NH_3 toxicity, was unpredictable, as several animals died using this method (Bartley *et al.,* 1976). In field conditions it is suggested that the animal is stabbed in the paralumbar fossa area and an opening of sufficient size be made to evacuate the rumen contents (Bartley *et al.,* 1976). This is a common procedure for the treatment of foamy bloat.

1.6 Supplementing feed grade urea

Feed grade urea has been fed in ruminant rations for more than 100 years to replace a portion of dietary protein (Kertz, 2010). Due to the low cost of urea in comparison to high protein feed stuffs the use of urea as a replacement for dietary protein has economic advantages (Reid, 1953; Helmer and Bartley, 1971; Kertz, 2010). The ruminant has the ability to convert NPN into MP (Kertz, 2010), and therefore, in North America during the 1970's, urea usage increased as a cheap protein supplement in TMR (Kertz, 2010). However, the rapid release of NH_3 as a result of feeding urea results in inefficient utilisation of NH_3 by rumen microbes, which can lead to NH_3 toxicity when high concentrations are absorbed across the rumen wall and into the blood stream (Visek, 1968). In the study by Polan *et al.* (1976), this occurred when urea was fed at levels >40% of dietary CP in addition to a dietary CP content >12.8% (Helmer and Bartley, 1971; Polan *et al.,* 1976). The feeding of urea in TMR diets during the 1970's resulted in poor results for several dairy farms, likely as a result of excessive urea use and subsequent NH_3 toxicity (Kertz, 2010). Due to the interest in using urea as a dietary protein supplement during the 1970's, a number of studies have investigated the relationship between different levels of urea feeding and NH_3 toxicity (Wilson *et al.,* 1975; Polan *et al.,* 1976; Visek, 1984).

The adverse effects of feeding urea have been demonstrated in several studies (Reid, 1953; Wilson *et al.,* 1975; Polan *et al.,* 1976; Kertz *et al.,* 1982). It appears that excessive urea feeding (>1% urea in a TMR) can lead to a reduction in intake, and subsequently, a reduction in milk yield, as demonstrated in the studies by Wilson *et al.* (1975) and Kertz *et al.* (1982). In the early to mid-1970's, several studies were undertaken to decipher whether the reduced intake with dietary urea was due to taste, odour, or metabolism (Wilson *et al.,* 1975; Kertz *et al.,* 1982; Visek, 1984). A no-choice and two-choice experimental design undertaken by Kertz *et al.* (1982) indicated that when urea was isolated physically by pellets coated with ground corn, cows selected against urea-containing pellets on a two-choice basis and reduced intake on a no-choice basis. When the choice

was between two urea-containing rations, cows preferred the pellets in which urea flavour and odour should have been most evident (Kertz *et al.,* 1982). Therefore, urea odour in the feedbox or urea in the drinking water did not reduce feed intake and is unlikely to be the factor causing rejection of rations containing urea (Wilson *et al.,* 1975; Kertz *et al.,* 1982).

Kertz *et al.* (1982) also reported that when urea was administered into the rumen prior to feeding a non-urea ration, the intake was reduced where rumen pH and plasma NH_3-N levels indicated NH_3 toxicity. Wilson *et al.* (1975) also reported this; however, they went a step further and reported that urea administered into the rumen and urea diets fed orally, resulted in decreased intakes for both treatments. Wilson *et al.* (1975) and Chalupa *et al.* (1979) concluded that there are other physiological parameters affecting intake of urea diets other than odour and taste. Wilson *et al.* (1975) concluded that the metabolic intermediates of urea catabolism accounted for the intake depression, when increasing levels of urea were fed. Therefore, it has been suggested that NH_3 toxicity is the major factor limiting intake in high urea-containing rations (Wilson *et al.,* 1975; Kertz *et al.,* 1982).

When high urea feeding results in decreased intake, the decreases in energy intake result in lower milk yield as reported by Van Horn *et al.* (1967) and Polan *et al.* (1976). Van Horn *et al.* (1967) fed concentrates containing no urea, 2.2% urea, or 2.7% urea. The urea-containing rations resulted in significant decreases in intake (Van Horn *et al.,* 1967). Polan *et al.* (1976) fed rations containing 9.4, 11.1, 12.8, 14.5 or 16.2% CP, with urea supplying 0, 10, 20, 30 or 40% of CP. The subsequent decreased intake with increasing urea intake was in agreement with results from Van Horn *et al.* (1976), but only when the level of dietary CP was above 12.8% (Polan *et al.,* 1976). As presented in Figure 7, intake was optimal in the mid-protein range of 12.8% and declined thereafter.

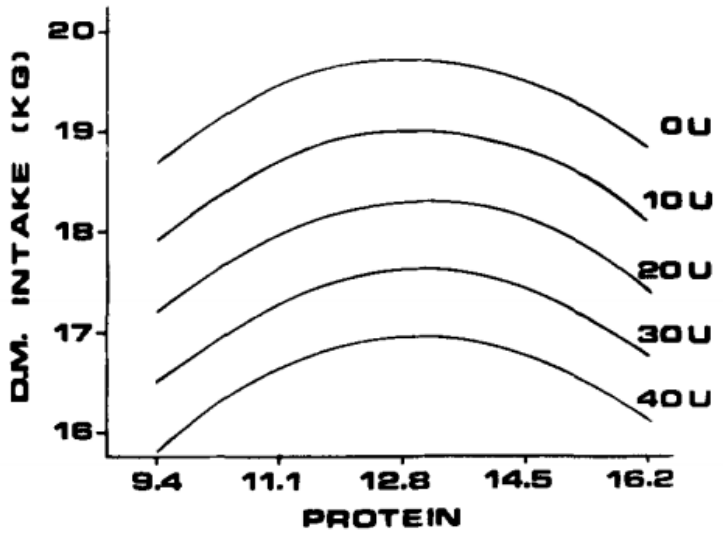


Figure 7: Dry matter intake (DMI) response (kg/day) to dietary crude protein (% CP) and urea (U, % of CP) in the diet (Polan *et al*., 1976).

Van Horn *et al.* (1967) stated that milk yield appeared to be directly related to concentrate intake, with the milk yield being lower in the diets containing urea. Polan *et al.* (1976) showed that urea has an adverse effect on milk yield when the dietary CP content was >11%, with milk yield further decreasing as both dietary CP content and urea content increased, as presented in Figure 8.

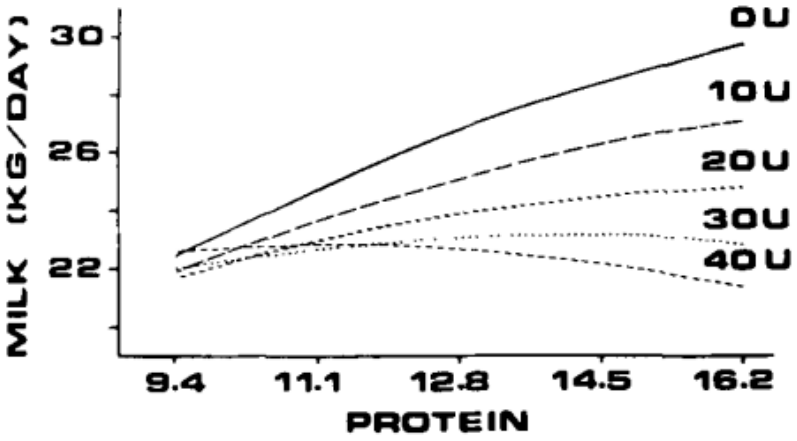


Figure 8: Milk yield response (kg/day) to dietary crude protein (% CP) and urea (U, % of CP) in the diet (Polan *et al.,* 1976).

Polan *et al*. (1976) went further to plot the relationship between dry matter intake (DMI) and milk yield (Figure 9). Within CP level, at greater urea levels the DMI and milk yield were depressed. The correlation of milk yield with DMI was r = 0.68. This indicates that where conventional sources of N are substituted for urea, the feed use efficiency decreases, particularly when dietary CP levels are high (Polan *et al.,* 1976).

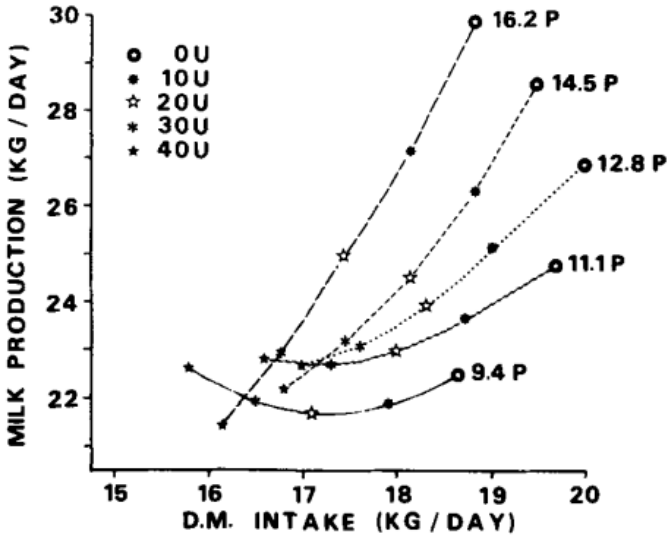


Figure 9: Milk yield (kg/day) versus dry matter intake (DMI) (kg/day) with varying dietary crude protein (% CP) and urea (U) (% of CP) in the diet (Polan *et al.,* 1976).

In a study by Kertz *et al.* (1982), where rations contained 2.5% urea, 1% urea or no urea, intake was depressed in the 2.5% urea-containing ration compared with the non-urea and 1% urea-containing rations. The 2.5% urea-containing ration had significantly higher reticulum pH compared with the other treatments, likely indicating NH_3 toxicity ($P < 0.05$) (Kertz *et al.,* 1982). These cows had not previously been exposed to urea-containing rations. During the last 5 minutes of the feeding period, a non-urea ration was substituted for the 2.5% urea-containing ration and, as a result, intake increased more than twice the 0.77 kg consumed during the first 5 minutes of the feeding period (Kertz *et al.,* 1982). This indicated that cows recognised that the ration did not contain urea and rapidly resumed consumption.

Following this discovery, an experiment was undertaken to evaluate the effect of preconditioning to urea-containing rations. When cows were preconditioned to urea rations, intake reduction was greater than that by cows that had not previously been exposed to urea-containing rations (Kertz *et al.,* 1982). Upon their third exposure to 2.5% urea in the ration, the cows reduced and ceased intake but readily consumed a non-urea ration (Kertz *et al.,* 1982). This indicated that one or more exposures to high dietary levels of urea are required for the cows to become conditioned to the diet and this is supported by the findings of Chalupa *et al,* (1979). This conditioning resulted in the cows establishing a negative physiological feedback, where the cows could identify the urea-containing rations to prevent reoccurrence of toxicity (Chalupa *et al.,* 1979; Kertz *et al.,* 1982). This phenomenon has been termed 'conditioned negative aversion' (Kertz *et al.,* 1982; Kertz *et al.,* 1983; Kertz, 2010).

1.7 Urea recycling

Cattle evolved to be efficient in using dietary N when consuming low protein diets by recycling urea back to the rumen (Reynolds and Kristensen, 2008; Calsamiglia *et al.,* 2010; McDonald *et al.,* 2011). Urea provides a source of N for MP synthesis, when N from the diet is insufficient (Reynolds and Kristensen, 2008). Urea is important in the N efficiency of ruminants as they are able to recycle urea into the rumen rather than excreting it in the urine especially when their diets are deficient in protein, as it allows N to be salvaged (Russell *et al.,* 1992; Marini and Van Amburgh, 2003; Marini *et al.,* 2006). An experiment conducted by Wickersham (2006) demonstrated this urea recycling in growing cattle fed prairie hay with a protein concentration <12%; virtually all (98%) urea entering the blood pool was returned to the gut, and little (2%) was excreted in urine (Wickersham, 2006, cited in Reynolds and Kristensen, 2008).

Amounts and degradability of carbohydrates as well as amounts and degradability of dietary and endogenous sources of N are known to influence relative amounts of urea recycled to the rumen (Reynolds and Kristensen, 2008). Because there is no endogenous system to ensure a supply of energy to the rumen, as there is for N with urea recycling, it has been suggested that the animal may be adapted to recycle urea back to the rumen to match carbohydrate fermentation (Cocimano and Leng, 1967; Hall and Huntington, 2008). When a diet is supplemented with a rapidly fermentable energy source, the ruminal urea entry rate can increase (Kennedy and Milligan, 1980). This allows the animal to capitalise on this nutrient when it is available, thus maintaining optimal microbial growth (Abdoun *et al.,* 2007).

The level of protein in the diet is also an important factor influencing urea recycling (Ruiz *et al.,* 2002). Grazing dairy cows consume diets high in protein (>18.5% CP) (Corson *et al.,* 1999), and this compromises N-use efficiency. Nitrogen-use efficiency is defined as the N output in milk relative to the N input through diet (Phuong *et al.,* 2013). As the dietary N content increases above the animals' requirements the urea recycled to the rumen is decreased and the urea excreted in urine increases resulting in a decrease in N-use efficiency (Reynolds and Christensen, 2008). This is demonstrated in the study by Marini and Van Amburgh (2003) where heifers were fed increasing amounts of soybean meal and urea, which linearly increased N intake and UUN output. As shown in Figure 10, increasing N intake resulted in linear increases in daily urea production; however, the effect of N intake on gut urea entry rate was small (Marini and Van Amburgh, 2003). This is in agreement with the studies conducted by Ruiz *et al.* (2002).

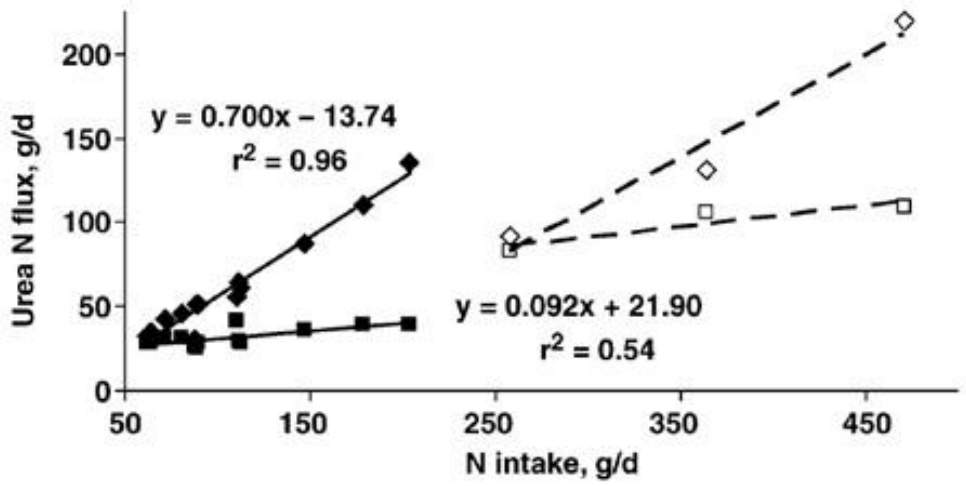


Figure 10: Total daily urea production (diamonds) and gut urea entry rate (squares) relative to nitrogen (N) intake in growing cattle (solid symbols) and lactating dairy cows (open symbols), as measured using dual-labeled urea infusions (adapted from Reynolds and Kristensen, 2008).

Increasing dietary N intake and subsequent ruminal urea production will result in increased absorption of NH_3 and urea production in the liver (Marini and Van Amburgh, 2003; Reynolds and Kristensen, 2008). Consequently, this has a negative effect on urea transfer to the rumen and incorporation of urea N into MP (Kennedy and Milligan, 1980; Recktenwald *et al.,* 2014). If the diet is in excess of CP, as the New Zealand pasture-based diet frequently is (>18.5% CP) (Corson *et al.,* 1999), the additional dietary N accumulates as NH_3 in the rumen and is absorbed across the rumen wall into the blood (Tamminga, 1992). This NH_3 is then converted to urea and is excreted in the urine and milk, with a reduction in the urea recycled to the rumen via blood and saliva due to the high dietary CP content as shown in Figure 11 (Leng and Nolan, 1984; Bach *et al*., 2005; Reynolds and Kristensen, 2008; Calsamiglia *et al*., 2010; McDonald *et al*., 2011).

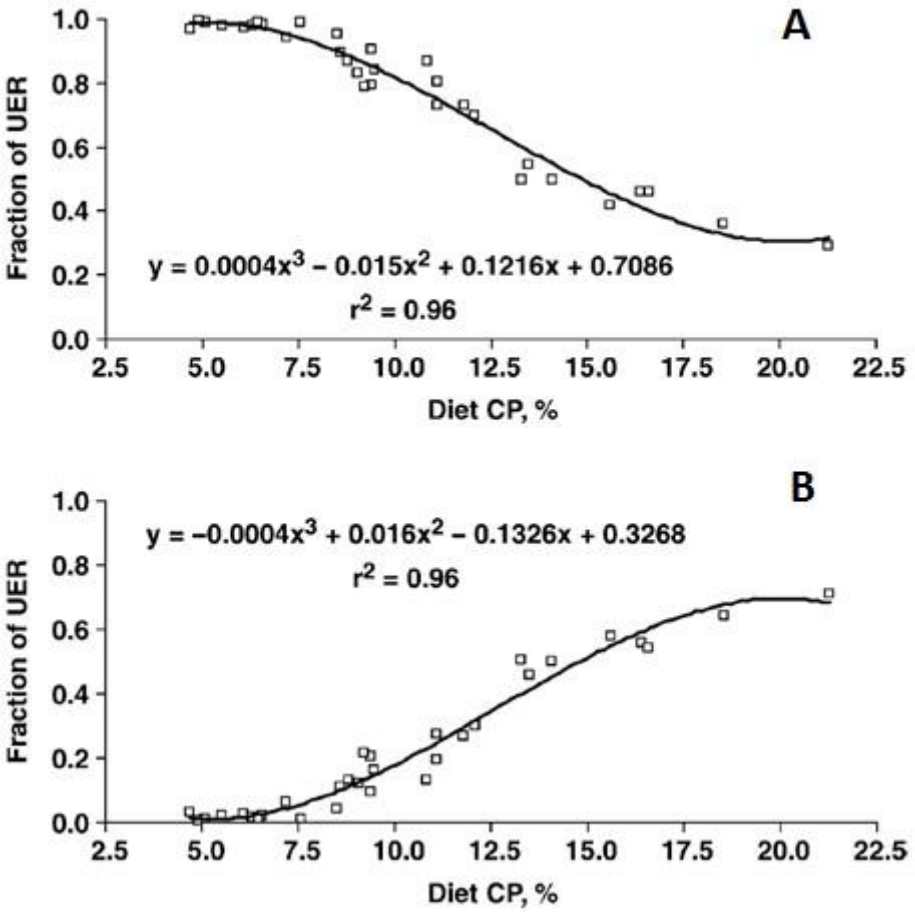


Figure 11: Dietary crude protein (CP) concentration and the fraction of total urea production (UER) that is returned to the gut via blood and saliva (A) or excreted in urine and milk (B) in cattle, as measured using dual-labeled urea infusions (adapted from Reynolds and Kristensen, 2008).

These high levels of N input result in reduced urea recycling (Ulyatt, 1997), and contribute to low feed N-use efficiency (~25%) (Calsamiglia *et al.,* 2010), with highly variable levels of dietary N (75-80%) lost in urine (Whitehead, 1995; Ulyatt, 1997; Castillo *et al.,* 2000; Kebreab *et al.,* 2001). This has implications for the productive performance of dairy cattle and has become a serious environmental issue in New Zealand, contributing to N losses to waterways and N_2O emissions to the atmosphere (Lantinga and Groot, 1996; Huhtanen and Hristov, 2009; Agle *et al.,* 2010).

1.8 Nutrient synchrony

Dairy cows consume CP to supply N in the rumen and the microbes require adenosine triphosphate (ATP) generated from carbohydrate fermentation to synthesise MP (Dijkstra *et al.,* 1993). It has been suggested that synchronising the supply of carbohydrate and N in the diet would increase NH_3 incorporated into MP and increase the amount of protein reaching the small intestine (Kolver *et al.,* 1998; Hall and Huntington, 2008; Reynolds and Kristensen, 2008). Provided that the additional

protein was partitioned into milk yield, this would result in improved N-use efficiency and a subsequent reduction in N excretion (Bach *et al.,* 2005; Hoekstra *et al.,* 2007; Reynolds and Kristensen, 2008). However, the effects of synchrony reported in literature have generally not generated this expected response (Kolver *et al.,* 1998; Cabrita *et al.,* 2006; Reynolds and Kristensen, 2008).

Two types of synchrony experiments have been undertaken. Firstly, the synchrony of diets is altered by altering the energy supply in the diet. Higgs *et al.* (2013) studied the effect of starch, fibre or sugar-based supplements on N-use efficiency. Manipulating synchrony by changing dietary ingredients presents some problems, since it is possible that some of the apparent effects of synchrony are associated with the manipulation of the ingredients used (Cabrita *et al.,* 2006).

In an experiment by Higgs *et al.* (2013), 85 cows were randomly assigned to 1 of 5 treatments at parturition. Treatments consisted of a pasture-only control along with pasture with starch, starch and N, fibre and N or a sugar-based supplement as shown in Table 3. Cows fed the starch treatment had lower urinary N excretion and the highest productive N output (149 g/day) (Higgs *et al.,* 2013). Cows fed the fibre and N treatment had similar productive N output (137 g/day) and greater urinary N, but also consumed 100 g/day more dietary N (Table 3) (Higgs *et al.,* 2013).

Table 3: Effects of supplementing different carbohydrate types to grazing dairy cows in early lactation on productive nitrogen (N) output, milk urea nitrogen (MUN), blood urea nitrogen (BUN) and urinary N to creatinine (N:creatinine) (Higgs *et al.,* 2013).

	Diet					
Item	**Control**	**Starch**	**Starch + N**	**Fibre + N**	**Sugar**	**P-value**
N intake, g/d	527	507	607	604	504	-
Productive N output, g/d	116	149	134	137	115	-
MUN, mmol/L	7.24	5.10	7.09	6.40	6.60	<0.001
BUN, mmol/L	7.05	5.10	6.72	6.07	6.60	<0.001
N:creatinine, mmol/L	0.23	0.16	0.19	0.21	0.20	0.004

These results indicate that including starch supplements in the diets of dairy cows fed pasture did not greatly improve the synchronisation of the carbohydrate and nitrogen supply (Higgs *et al.,* 2013); approximately 90% of the difference in N excretion is attributable to the reduction in N intake, whereas only 10% could be attributed to more productive N output.

A plausible explanation for the nutrient synchrony theory not working in practice when a diet high in protein is fed is that the protein supplied by the diet may not only exceed the requirements of the microbial population in the rumen, but may also exceed the requirements of the animal. Additional protein captured as MP in the rumen due to nutrient synchrony enters the small intestine

(McDonald *et al.,* 2011). Microbial protein entering the small intestine may be absorbed as amino acids and partitioned into milk, deposited in tissue or excreted in urine. If the MP results in an excess supply of amino acids to the animal, this will result in amino acids from MP being converted to urea and excreted in urine as shown in Figure 12 (Hall and Huntington, 2008).

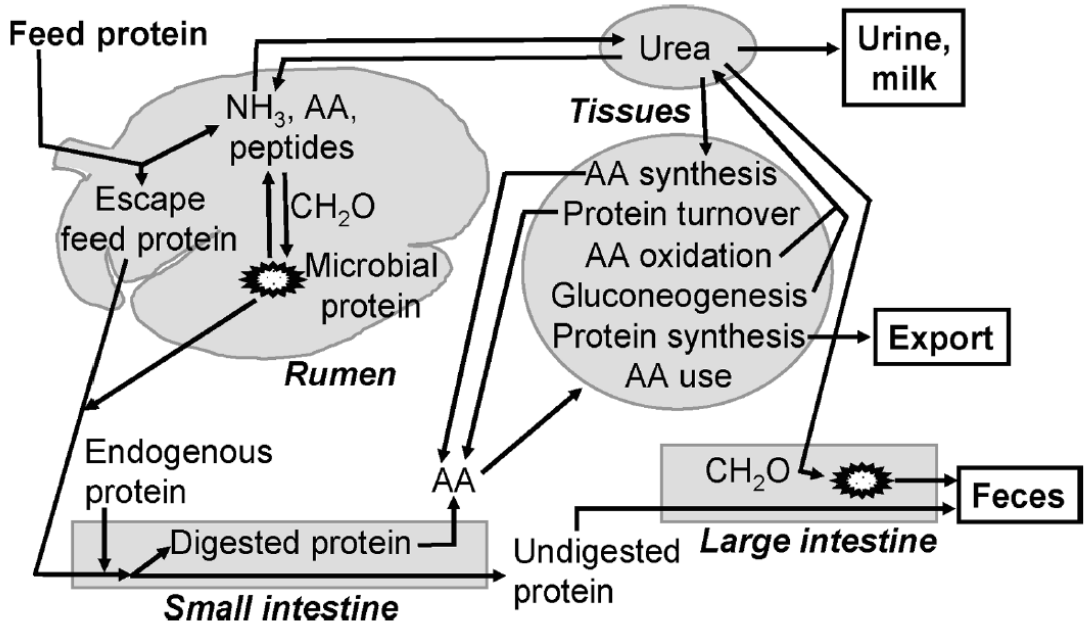


Figure 12: Interplay of dietary, ruminal, and extraruminal sources of nitrogen and amino acids (AA). CH_2O = carbohydrates; Export - milk, conceptus, scurf, secretions. Gut or tissue compartments are labelled in bold, italic print (Hall and Huntington, 2008).

This experimental outcome of Higgs *et al.* (2013) emphasises the importance of dietary ingredients altering the outcome of synchrony experiments due to differences in the composition of the diet, particularly the amount of N in the diet. It also indicates that N intake is the most important factor affecting N utilisation, with nutrient synchrony having little or no effect on N utilisation (Huhtanen and Hristov, 2009; Higgs *et al.,* 2013). Therefore, the theoretical benefits of rumen synchrony in improving N-use efficiency are not usually observed in practice (Kolver *et al.,* 1998; Gehman *et al.,* 2004; Reynolds and Kristensen, 2008).

The second type of synchrony experiment involves altering the synchrony of diets by altering the feeding frequency or the feeding pattern, such as that undertaken by Kolver *et al.* (1998), where the effect of synchronous and asynchronous diets on N-use efficiency was studied. This method allows synchrony to be altered without changing the ingredients in the diet (Cabrita *et al.,* 2006). In an experiment by Kolver *et al.* (1998), 12 Holstein cows in early to mid-lactation (8 fitted with ruminal cannulae) were used to investigate if synchronisation of the rate of ruminal degradation of supplemental carbohydrate and N from fresh pasture would increase the amount of N retained for

growth and milk yield. A concentrate based on ground shelled corn was fed either at the time that pasture was fed at 0900 and 1700 h (synchronous) or 4 h after pasture was fed at 1300 and 2100 h (asynchronous) (Kolver *et al.,* 1998). Nitrogen partitioning to faeces, urine and milk was not influenced by diet, and there were no differences in milk yield (Table 4).

Table 4: Intake, nitrogen (N) partitioning and milk yield by cows fed the synchronous diet (SYND) or the asynchronous diet (ASYND) (Kolver *et al.,* 1998).

Variable	SYND	ASYND	P-value
Dry matter, kg/d			
Pasture intake	9.65	10.2	0.20
Concentrate intake	9.17	9.12	0.34
Total intake	18.8	19.3	0.26
Nitrogen, kg/d			
Total intake	0.55	0.57	0.23
% of nitrogen intake			
Faecal N	23.3	24.7	0.32
Urine N	30.3	29.5	0.30
Milk N	24.7	24.1	0.60
Milk, kg/d	29.1	29.9	0.42

Kolver *et al.* (1998) demonstrates the inability for synchronisation to improve N-use efficiency in pasture-fed cows and the ability of a cow to utilise N even when an asynchronous diet is fed. It is likely that recycling of urea to the rumen contributes to the stabilisation of microbial growth, even when N supply is not well synchronised (Cabrita *et al.,* 2006; Hoekstra *et al.,* 2007; Reynolds and Kristensen, 2008; Kertz, 2010). "The deposition of N in nitrogenous compounds absorbed in excess of requirements on days when protein is provided, into pools other than urea and NH_3, which are subsequently catabolised to generate N for urea synthesis, may also buffer the effects of infrequent protein supply" (Reynolds and Kristensen, 2008).

The conflicting results mentioned above are supported by a review published by Cabrita *et al,* (2006). It was reported that across 10 studies in lactating dairy cows, there was no consistent effect of attempts to synchronise carbohydrate fermentation and N availability on ruminal NH_3 concentration or microbial N supply in lactating dairy cows (Carbrita *et al.,* 2006). Similarly, in these studies, there was no indication of a positive effect of synchronising rumen energy and N supply on DMI or milk yield (Cabrita *et al.,* 2006). It appears that N intake is a more important factor in improving N utilisation, with nutrient synchrony having only small or no effect on N utilisation (Huhtanen and Hristov, 2009; Higgs *et al.,* 2013).

1.9 Relationships between dietary nitrogen and urinary nitrogen

Nitrogen-use efficiency is typically low in dairy cows, averaging around 25%, but can range from 10 to 40% (Calsamiglia *et al.,* 2010). Nitrogen that is not partitioned toward milk yield ends up in body tissue or urine. Kebreab *et al.* (2001) reported that while there is a large variation of N excretion in urine, due to an exponential correlation with N intake, there is little variation of N in milk and faeces, with typical increases by less than 20% per unit increase in N intake. Generally speaking, 75-80% of dietary N is excreted and for N-use efficiency to be increased, less dietary N must be excreted and more partitioned into products (Kebreab *et al.,* 2001; Marini and Van Amburgh, 2003). Overseas research has examined the relationships between N intake and some N fractions in blood, milk and urine. The relationships between dietary N, urinary N, faecal N and milk N in dairy cows fed TMR and forages have been studied (Burgos *et al*., 2007; Spek *et al*., 2013). However, these relationships are not representative of the New Zealand pasture-based system.

Castillo *et al*. (2000) investigated the relationship between N intake and output in milk, faeces and urine using data from 580 dairy cows and 90 treatments published in the literature. Kebreab *et al.* (2001) also investigated the same relationship using data from Holstein-Friesian cows fed 30 different diet types consisting of 10 grass silages and 6 concentrates. Both papers used regression analysis to describe the relationships between N intake and output in faeces, urine and milk. There was a positive linear relationship between N intake and N output in faeces and milk (Castillo *et al.,* 2000; Kebreab *et al.,* 2001). However, excretion in urine increased exponentially, as N intake increased with the point of inflection being around 400 g N/day (Figures 13 and 14) (Castillo *et al.,* 2000; Kebreab *et al.,* 2001). The regressions to describe the relationships between N intake and N output in faeces, milk and urine are expressed in Table 5 and are presented in Figures 13 and 14.

Table 5: Relationship between nitrogen intake (N_i), faecal nitrogen (N_f), milk nitrogen (N_m) and urinary nitrogen (N_u) across two studies (Castillo *et al.,* 2000; Kebreab *et al.,* 2001).

Castillo *et al.,* 2000			**Kebreab *et al*., 2001**		
1.	$N_f = 0.21 (N_i) + 52.3$	$R^2 = 0.48$	4.	$N_f = 0.16 (N_i) + 76.7$	$R^2 = 0.30$
2.	$N_m = 0.17 (N_i) + 41$	$R^2 = 0.42$	5.	$N_m = 0.19 (N_i) + 38.2$	$R^2 = 0.30$
3.	$N_u = 30.4 (e^{0.0036Ni})$	$R^2 = 0.76$	6.	$N_u = 0.003 (N_i^{1.8})$	$R^2 = 0.67$

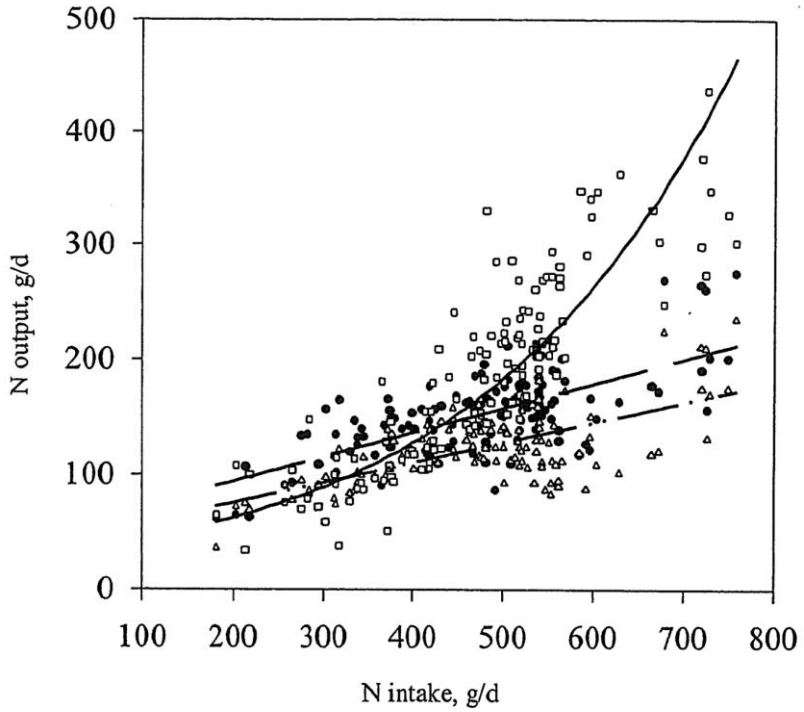


Figure 13: Relationship between total nitrogen (N) intake (g/day) and faecal, milk and urinary N outputs (g/day). The fitted lines were given by equations 1 (-----), 2 (— · —) and 3 (——) (Castillo *et al.,* 2000).

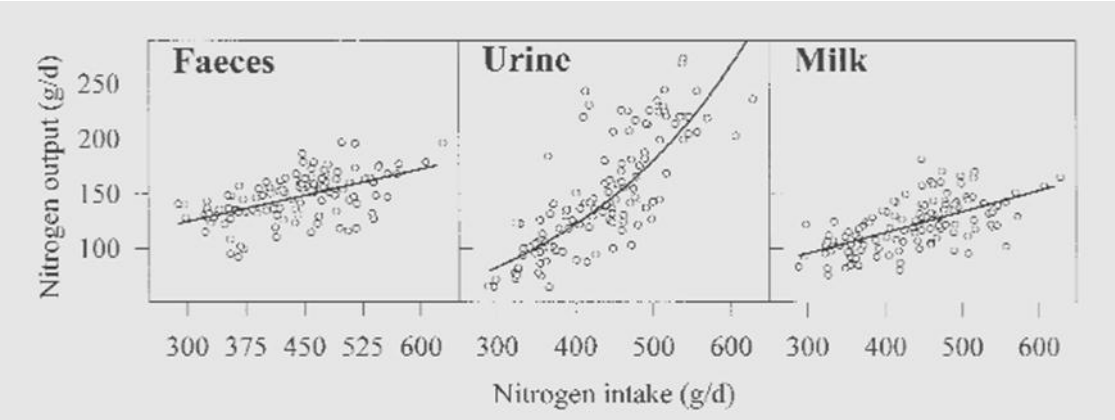


Figure 14: Relationship between total nitrogen intake (g/day) and faecal, milk and urinary nitrogen outputs (g/day). The fitted lines were given by equations 4, 5 and 6 in Table 5 (Kebreab *et al*., 2001).

The regression analysis performed by Castillo *et al.* (2000) and Kebreab *et al.* (2001) used experimental datasets from studies using various supplementary feeds and fertiliser levels to manipulate the levels of N intake ranging from approximately 200 to 750 g N/day. The data regressed in Figures 13 and 14 failed to account for differences in composition across diets. The use of differing levels of N fertiliser and different levels of supplements resulted in slight differences in

diet compositions across the diets fed and this probably affects the accuracy of the relationship predicted, as demonstrated by Kebreab *et al.* (2001).

1.9.1 Other factors influencing nitrogen-use efficiency

To estimate the underlying relationships between N intake and urine, faecal and milk N, experiments containing similar diets that only differed in their levels of protein were analysed by Kebreab *et al.,* (2001). As presented in Table 6 and Figure 15 there was a strong exponential relationship between N intake and urinary N output and a positive linear relationship between N intake and N output in milk and faeces; however, accounting for dietary differences resulted in higher correlated regressions (Kebreab *et al.,* 2001). Due to the limited data range analysed, the accuracy of these relationships is questionable and prompts the need for further research.

Table 6: Relationship between nitrogen intake (N_i), faecal nitrogen (N_f) , milk nitrogen (N_m) and urinary nitrogen (N_u) for diets of similar composition (Kebreab *et al*., 2001).

Kebreab *et al.,* (2001)		
7.	$N_f = 0.15\ (N_i) + 78.0$	$R^2 = 0.53$
8.	$N_m = 0.17\ (N_i) + 34.8$	$R^2 = 0.90$
9.	$N_u = 0.0052\ (N_i^{1.7})$	$R^2 = 0.95$

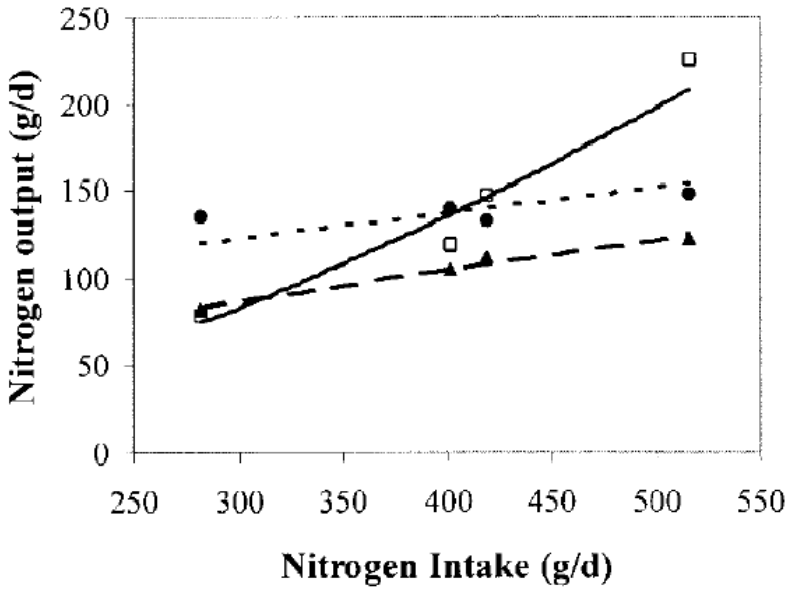


Figure 15: Relationship between total nitrogen (N) intake (g/day) and faecal (circles), milk (triangles) and urinary (squares) N outputs (g/day). The fitted lines were according to equations 7, 8 and 9 in Table 6 (Kebreab *et al.,* 2001).

The exponential relationship for urinary N output emphasises that urinary N is the principal route for excess N output (Susmel *et al*., 1995; Castillo *et al.,* 2000; Kebreab *et al.,* 2001). This emphasises the importance of reducing N intake to reduce the accumulation of NH_3 in the rumen and subsequent

excretion as urea, or improving N utilisation by increasing productive N output (N partitioned to milk) (Kebreab *et al.,* 2001; Marini and Van Amburgh, 2003; Higgs *et al.,* 2013).

The differences between the relationships reported by both Castillo *et al.* (2000) and Kebreab *et al.* (2001) indicate that the major influencing factor in the relationship between N intake and output is the N intake; however, the discrepancy between the relationships due to differences in composition across diets fed indicates the need for further research. These discrepancies are further supported by the significant effect of energy type and degradability and protein degradability on N utilisation reported by Kebreab *et al.* (2001). Kebreab *et al.* (2001) compared four types of energy sources, including fibre, barley, maize and sugar, with similar levels of CP, to determine the effect that differing dietary energy source would have on urine, faecal and milk N output (Figure 16). Perennial ryegrass (*Lolium perenne* L.) silage was offered with four different concentrates. The concentrates were based on high amounts of NDF (neutral detergent fibre), low degradability starch (maize), high degradability starch (barley) and molasses (sugar) (Kebreab *et al.,* 2001). Compared with the fibre, maize, and sugar treatments, the high degradable starch-based supplement (barley) had higher urinary N output, as presented in Figure 16 (Kebreab *et al.,* 2001). This confirms that energy type and degradability can affect N utilisation.

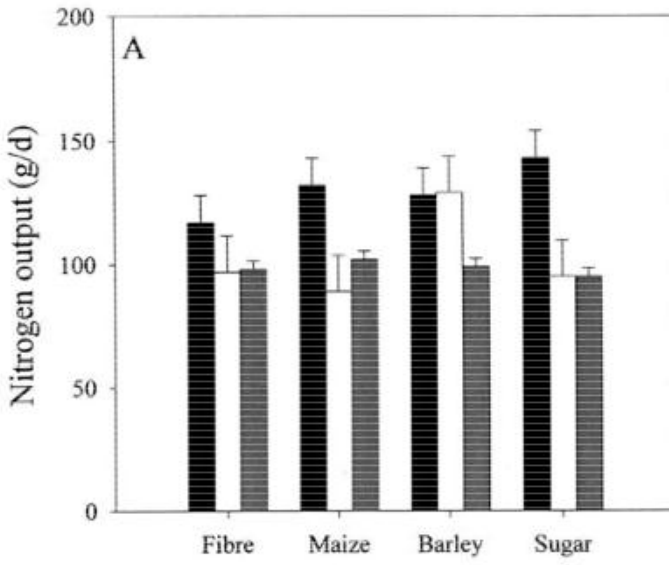


Figure 16: Effect of type of energy source on faecal (solid), urinary (unshaded) and milk (grey) nitrogen (g/day) (Kebreab *et al.,* 2001).

Kebreab *et al.* (2001) also compared protein degradability in the concentrate and reported that for approximate N intakes of 470 g/day, there were significant effects of protein degradability ($P < 0.05$) on faecal and urinary N output. For example, cows receiving low degradable protein had lower levels of N in total excreta compared to cows receiving medium and high degradable protein (average N in

total excreta; High, 338; Medium, 328; Low, 314 g N/day) as presented in Figure 17 (Kebreab *et al.,* 2001). A 24% decline in urinary N excretion was reported in low compared with high levels of protein degradability, with no compromise in milk output being most notable (Kebreab *et al.,* 2001). This confirms the ability for protein degradability to affect N utilisation.

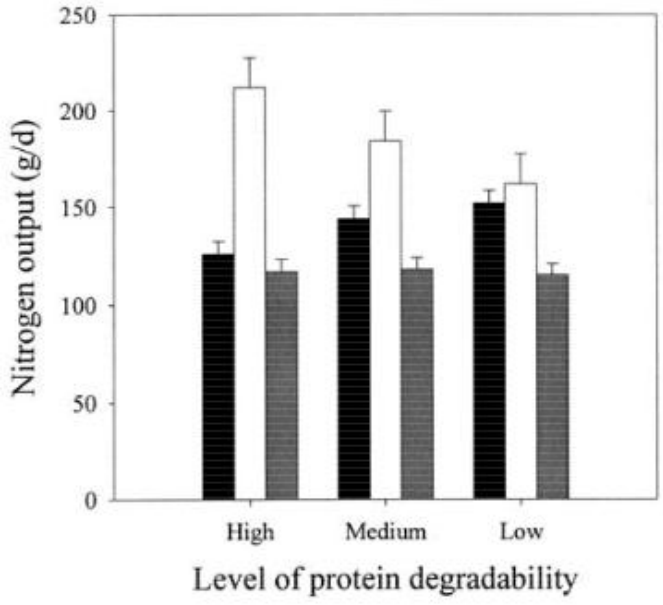


Figure 17: Effect of protein degradability on faecal (solid), urinary (unshaded) and milk (grey) nitrogen (g/day) (Kebreab *et al.,* 2001).

Finally, the effect of fertiliser level on silage, applied prior to harvesting, on N output was studied by fertilising grass with either 75 (medium) or 150 kg N/ha (high) and harvesting the grass as silage two weeks later before feeding the silage to multiparous Holstein-Friesian dairy cows. It was reported the urinary N output per unit N intake was significantly reduced in medium N-fertilised grass, compared with high N-fertilised grass (Kebreab *et al.,* 2001). Increasing levels of N fertiliser result in changes in composition of the sward, likely altering the energy type and degradability of the sward (Van Vuuren *et al.,* 1991). This can affect N utilisation, as previously discussed, and therefore, affect the relationships generated from diets where N levels have been manipulated through fertilisation of the sward.

These experiments demonstrate that differences in diet composition can affect N-balances, although the primary factor affecting urinary N output is N intake (Higgs *et al.*, 2013). It is important to minimise external variables that could influence the experimental outcome of future N-balance studies. If the protein level is manipulated across treatments without affecting the other components of the diet, it can be assumed that the results of the research are not going to be confounded by the ingredients of the diet. This will allow relationships to be studied in future that

indicate effects of changes in the CP content of the diet on N partitioning without external factors influencing the outcome of these relationships.

Burgos *et al.* (2007) demonstrated the effects of changes in the CP content of the diet on N partitioning using TMR diets, while maintaining similar concentrations of all other nutrients across treatments. Burgos *et al.* (2007) conducted an experiment to assess the relationship between urinary urea N (UUN) excretion and milk urea N (MUN) and to test whether the dietary CP content affected this relationship (Burgos *et al.,* 2007). Twelve lactating multiparous Holstein cows at three stages of lactation (early; 123 ± 26 (mean ± standard deviation), mid; 175 ± 3; and late; 221 ± 12 days in milk) were assigned to dietary CP levels of 15, 17, 19 and 21% of DM. These levels of increasing dietary CP were achieved through the addition of graded amounts of urea to the basal TMR. The intention was to linearly increase dietary CP content while maintaining similar concentrations of all other nutrients among treatments, to minimise external variable effects as presented in Table 7 (Burgos *et al.,* 2007).

Table 7: Nutrient composition of experimental diets and means for nitrogen intake (NI), net energy (NE), milk urea nitrogen (MUN), blood urea nitrogen (BUN) and urine urea nitrogen (UUN) excretion (Burgos *et al.,* 2007).

Dietary CP % of DM	15% DM	17% DM	19% DM	21% DM	P-value
Nutrient % of DM					
Urea	0	0.7	1.5	2.2	-
CP	15.1	16.6	18.6	20.7	-
NDF	31.9	32.1	32.3	32.3	-
NE, Mcal/kg	1.56	1.56	1.55	1.55	-
NI, g/d	500.6	600.0	688.6	758.0	<0.001
MUN, mg/dL	7.90	11.96	17.28	24.53	<0.001
BUN, mg/dL	8.23	12.96	18.68	25.85	<0.001
UUN, g/d	85.6	149.4	227.5	320.6	<0.001

CP = crude protein; DM = dry matter and NDF = neutral detergent fibre

Milk urea N increased quadratically as N intake increased, and this is in agreement with the study conducted by Broderick (2003). Broderick (2003) also reported a quadratic relationship between MUN and N intake in cows fed diets ranging in CP concentrations from 15.1-18.4% of DM. Blood urea N response was quadratic with increasing dietary CP content. This finding differs from the findings reported by Olmos Colmenero and Broderick (2006), who reported a linear response in blood urea N for cows fed diets ranging in CP concentrations from 13.5-19.4% of DM (Burgos *et al.,* 2007). The disagreement between results could have been due to the high levels of CP in the diet (>400 g N/day for all treatments), along with the highly rumen degradable source of N (urea) used in the study by Burgos *et al.* (2007). As the dietary CP concentration increased above 400 g N/day, the

UUN increased exponentially, in concordance with the analysis by Castillo *et al.* (2000) and Kebreab *et al.* (2001). A major limitation in the experiment by Burgos *et al*. (2007) is that the animals were allowed only 6 days to adjust to the various levels of urea, followed by a 1 day collection period. Therefore, a large dataset was not generated and this could decrease the accuracy of the relationships analysed.

1.10 Dietary nitrogen supply

Dietary N supply has been identified as the primary driver of N-use efficiency and has been extensively studied (Dijkstra *et al.,* 2013). Nitrogen-use efficiency is decreased and urinary N excretion is increased with the increase of N in the diet (Marini and Van Amburgh, 2003). High N intake favours absorption of NH_3 due to the accumulation of NH_3 in the rumen and subsequent urea production in the liver (Marini and Van Amburgh, 2003; Reynolds and Kristensen, 2008). Consequently, this has a negative effect on urea transfer to the rumen and incorporation of urea N into MP (Kennedy and Milligan, 1980; Recktenwald *et al.,* 2014), and results in low N-use efficiencies.

An *in vitro* study conducted by Satter and Slyter (1974), where the effect of ammonia concentration on microbial protein production in the rumen was investigated, indicated that dietary N levels equivalent to 11-14% CP/kg DM correspond with the point of NH_3 accumulation in the rumen (Satter and Slyter, 1974). This corresponds to a ruminal NH_3 concentration >5 mg/dL according to Satter and Slyter (1974), however reported optimal ruminal NH_3 concentrations *in vivo* and *in vitro* to support maximum MP synthesis range from 5-13 mg/dL (Reynal *et al.,* 2003; Boucher *et al.,* 2007). *In vitro*, urea recycling does not occur, indicating that the point of accumulation may correspond to a slightly higher dietary N level. There are also differences in literature due to differences in dietary composition, the proportions of RDP and RUP, the experimental methods and the manner in which NH_3-N concentrations were altered (Satter and Slyter, 1974; Boucher *et al.,* 2007).

Pasture provides a nutritional and inexpensive source of feed for dairy farms in New Zealand. It often provides N levels in excess of 18.5% dietary CP (Corson *et al.,* 1999). A study conducted by Roche *et al.* (2009) analysed the temporal trends in herbage quality characteristics for perennial ryegrass. The annual variation in CP concentration was between approximately 20-25% with an average concentration of 22.3% CP (Roche *et al.,* 2009). The capacity of grass swards to take up N is high in comparison with other crops and, under favourable conditions, may be more than 500 kg N/ha/year (Whitehead, 1995). Dietary protein concentration is an important on farm variable that is difficult to control practically in a pasture-based system such as that used in New Zealand (Agle *et*

al., 2010) and as a result, urinary N losses are an issue when feeding pasture-based diets (Tamminga, 1992).

Crude protein content and N fractions of pastures are variable and influenced by factors such as plant species and varieties, season of the year, physiological growth stage and fertiliser application (Roche *et al.,* 2009; Keim and Anrique, 2011). Nitrogen is an essential component of proteins, nucleic acids and chlorophyll, and therefore, when plant growth is not limited, the plant can take up on average 1-3 kg N/ha/day resulting in pasture herbage N concentrations within the range 1-5% (Whitehead, 1995). Protein levels throughout the year generally remain above 18.5% of DM, with levels of NO_3^- absorbed by the plant being the limiting nutrient for growth (Whitehead, 1995; Castillo, 1999; Corson *et al.,* 1999). When the temperatures are above 5°C, soil mineralisation of N occurs and if the supply of N is not limiting and rainfall/irrigation is adequate, growth is not limited (Whitehead, 1995). Therefore, the CP content of intensively managed pasture is often ≥18.5% CP year round as a result of intensive vegetative growth and feeding management (Corson *et al.,* 1999).

For best practice pasture management it is recommended that pasture is offered when the grass is young and leafy to provide high quality herbage. This coincides with the 2-3 leaf stage where the CP content is high (≥14%) (Fulkerson and Donaghy, 2001) and the cell wall content is low, resulting in a high feeding value (Van Vuuren *et al.,* 1991; Tamminga, 1996; Pacheco *et al.,* 2007). Chaves *et al.* (2006) studied the effects of herbage maturation on the chemical characteristics of perennial ryegrass herbage. As the sward matured, the non-structural carbohydrate levels increased reaching a peak before declining (Figure 18). The metabolisable energy (ME) and CP content both decreased quadratically, and the NDF increased as the sward matured (Chaves *et al.,* 2006).

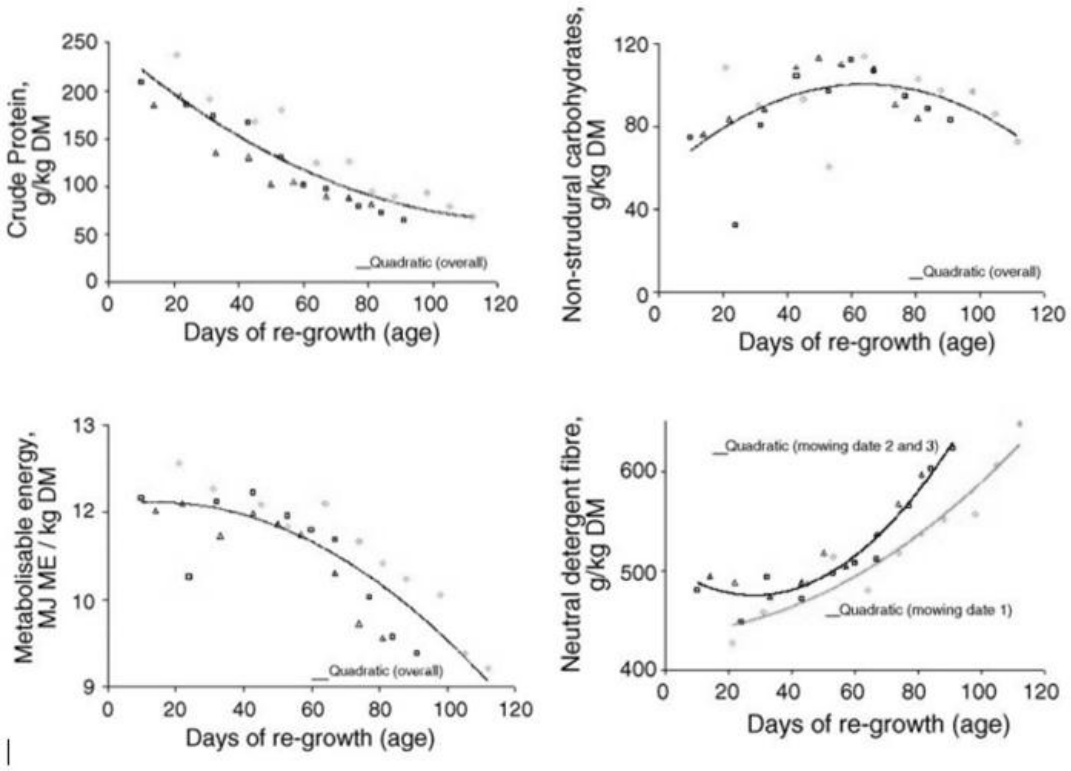


Figure 18: Perennial ryegrass herbage changes in crude protein (g/kg dry matter (DM)), non-structural carbohydrates (g/kg DM), metabolisable energy (MJ ME/kg DM) and neutral detergent fibre (g/kg DM) with regrowth (Chaves *et al*., 2006).

It is important that the pasture is fed before ME decreases and the NDF increases substantially (Chaves *et al.,* 2006). This requires feeding pasture at a regrowth age ≤44 days to prevent a significant reduction in quality and digestibility of the feed (Chaves *et al.,* 2006; Heeren *et al.,* 2014). Feeding of pasture depends on grazing management and rotation length, which is closely related to pasture growth and season (Matthews *et al.,* 1999). Grazing typically coincides with regrowth intervals of 18-44 days (Chapman *et al.,* 2014). However, this results in feeding pasture when the CP content is higher than animal requirements (≥18.5% CP) (Corson *et al.,* 1999), leading to a surplus of N consumed by the animal (Ledgard *et al.,* 2009).

In addition to feeding pasture when CP content is in excess of 14%, during the period from the late 1970's to the mid-1980's, the rate of N fertiliser increased alongside increasing dairy farm intensification (Whitehead, 1995). Increasing rates of N fertiliser application have increased the productivity of grass, promoting increased DM yields and stocking rates (LIC and DairyNZ, 2013). However, this has also further increased the CP content of pasture throughout the year along with the proportions of NPN in the sward (Lantinga and Groot, 1996; Tamminga, 1996; Hoekstra *et al.,* 2007). It has been reported that, on average, as the rate of fertilisation increases, the CP content of

the sward also increases, with an increase of 50-90 g CP/kg DM/ha per year per 100 kg N applied (Peyraud and Astigarraga, 1998).

Nitrogen fertilisation and the selection of genetically improved cultivars of grass and legume species have been successfully used to increase pasture production (Lambert *et al.,* 2004). As well as increasing pasture production, fertiliser N can also affect the concentration of N in the pasture (Whitehead, 1995) and, in particular, the proportion of CP that is NPN (Whitehead, 1995; Tamminga, 1996; Hoekstra *et al.,* 2007). Pasture usually has about 70-90% of the total N present as true protein and 10-30% present as NPN (Brady, 1960, cited in Waldo, 1968; Lexander *et al.,* 1970, cited in Tamminga, 1986; NRC, 2001). Increased amounts of N applied can result in NPN concentrations exceeding 30% of the total N present in the pasture (Whitehead, 1995; Peyraud and Astigarraga, 1998). Non-protein N increases the RDP content of the diet and is degraded rapidly in the rumen (Sniffen *et al.,* 1992). Higher proportions of NPN in the sward result in rapid release of NH_3 (NRC, 2001), and consequently accumulation of NH_3 in the rumen due to surplus NH_3 levels. As a result NH_3 is absorbed across the rumen wall (Satter and Slyter, 1974), converted to urea in the liver and excreted in urine (Tamminga, 1996).

The CP content of pasture at a maturity stage optimal for grazing often exceeds 14% CP (Chaves *et al.,* 2006). The CP content at which NH_3 begins to accumulate in the rumen due to NH_3 levels exceeding microbial requirements occurs at ~11-14% CP/kg DM depending on the feed characteristics (Satter and Slyter, 1974). The use of N fertiliser further exacerbates the CP content of pasture, as the addition of N fertiliser further increases the CP content of pasture throughout the year (Tamminga, 1996).

The high N levels in pasture result in surplus N consumed by the animal (Ledgard *et al.,* 2009), and this leads to high levels of N excreted in urine (75-80% of dietary N) (Whitehead, 1995; Castillo, 1999). The high CP content of pasture and low efficiency of protein utilisation in dairy cows is an environmental concern.

1.11 Sources of error in N balance studies

To study the partitioning of N in dairy cows accurately, the total N intake in relation to total N output must be considered. The N output includes the N in milk, urine, faeces, foetal tissue and body tissue. Various methodological aspects of N-balance studies that cause errors in N intake and output measurements, as well as overestimation of retained N, have been studied (Spanghero and Kowalski, 1997).

An animal in late gestation will experience N retention by the foetus and associated structures (~2 g N/day at the 20th week of pregnancy) (NRC, 1985). The N retention becomes quantitatively more important during late gestation and as a result it is recommended to use dairy cows that are not more than 6 months pregnant when conducting N-balance studies (Castillo, 1999).

Spek *et al.* (2013) conducted a meta-analysis from 47 N-balance studies carried out in North America and Europe, to test the accuracy and precision of urinary N and UUN prediction equations and whether they were affected by the method of measuring urinary N and UUN. A distinction was made between urinary excretions based on either urine spot samples or calculated assuming a zero N-balance, and excretions that were determined by a total collection of urine only. Spek *et al.* (2013) demonstrated the importance of analysing urinary N and UUN based on the total collection of urine, instead of determining urinary N and UUN from urine spot samples or by assuming zero N-balance. Although from a practical and animal welfare point of view (when using indwelling catheters), it might be preferred to derive urinary N by assuming a zero N-balance and calculate urinary N as the difference between N intake and N excreted in faeces and milk (Spek *et al.,* 2013).

Spanghero & Kowalski (1997) used data from 35 published papers (125 different diets) to analyse the methodologies of N-balance studies. They identified that published values of N retention in N-balance studies are often higher than expected. An average N-balance of 38.8 g/day was calculated, which, converted into CP (coefficient = 6.25), reflects a protein gain of about 250 g/day. Based on a protein to water ratio of 1:3 (Blaxter, 1989), this latter value allows a lean tissue gain of approximately 1 kg/day (Spanghero & Kowalski, 1997). Based on the genetic potential of the cows used in the N-balance studies (high yielding cows in early lactation), it was concluded that this estimate of lean tissue gain was overestimated. This indicates a potential underestimation of faecal, urinary and/or milk N as a source of error in these N-balance studies (Spanghero & Kowalski, 1997).

Faecal N could be underestimated due to incomplete collection of faeces, volatile losses of NH_3 from faeces in stalls and/or during the drying of samples. As a result, it is important to collect faeces every 24 hours and to analyse N content of wet samples to minimise the NH_3 losses (Spanghero & Kowalski, 1997; Castillo, 1999). Urinary N could be underestimated due to the volatile NH_3 losses from the urine collection containers as a result of enzyme-catalysed urea hydrolysis. A strong acid such as HCl or H_2SO_4 can be added to the urine sample to minimise N losses due to NH_3 volatilisation (Spanghero & Kowalski, 1997). Petersen *et al.* (1998) measured NH_3 volatilisation losses from simulated dung pats and urine patches applied to soil. Their study indicated that NH_3 losses from faeces was insignificant, while NH_3 losses from urine ranged from 3-52% of urinary N (Petersen *et al.,*

1998). This emphasises the importance of minimising NH_3 losses in particular from urine during N-balance studies.

Milk N is another important N component in N-balance studies that could be underestimated. In the studies analysed by Spanghero & Kowalski (1997), the milk samples were most commonly collected at regular milking intervals and analysed using the Kjedahl method. Collection and analysis of milk samples doesn't have the issues experienced with the collection and analysis of faecal and urine samples. Burgos *et al.* (2007) reported that there are differences between lactation stages and dietary CP concentrations in relation to the partitioning of N. The use of animals in the same stage of lactation would further reduce errors associated with estimating milk N (Castillo, 1999).

Scurf and dermal losses are the additional source of N output; however, these are difficult to measure and are often considered insignificant (<1.5 g N/day for a 600 kg cow) (Spanghero & Kowalski, 1997; Castillo, 1999). Therefore, none of the trials examined by Spanghero & Kowalski (1997) considered these losses in the N-balance experiments. These factors influencing the estimation of N output in relation to dietary N should be taken into account when deisgning an N-balance experiment. It is important to minimise the causes of error when designing a methodology to improve methodological accuracy. It is also important to exert caution when interpreting differences between N-balance studies due to potential overestimations of N retention.

1.12 Conclusion

Nitrogen leaching is an important issue that is currently receiving much attention in New Zealand. New Zealand's pasture-based system results in the feeding of pasture containing high concentrations of CP (Ulyatt, 1997), which is not utilised efficiently by the dairy cow; these high concentrations of CP are further exacerbated by N fertiliser inputs, grazing management and use of improved cultivars (Van Vuuren *et al.,* 1991). Crude protein, in particular NPN, provided in excess of microbial requirements, is degraded in the rumen to form NH_3. Ammonia accumulates in the rumen and as a result is absorbed across the rumen wall (Reynolds and Kristensen, 2008). Ammonia reaching the liver is converted to urea and excreted in urine (McDonald *et al.,* 2011). Deposition of urine on pastures and races by grazing animals results in rates of up to 1000 kg N/ha added to a small area of soil, resulting in surplus N in urine patches (Haynes and Williams, 1993). Because NO_3^- is poorly held by the soil surface it is susceptible to leaching (Ledgard *et al.,* 2009). The primary source of NO_3^- in drainage waters from grazed pastures is animal urine (Haynes and Williams, 1993). Regional councils have begun to produce strict regulations with regards to the quantities of N that

can be leached from dairy farms into particular river catchments. As a result, N losses in urine have been the major focus of a number of studies.

High CP concentrations result in the accumulation of NH_3 in the rumen and if levels exceed what can be utilised by rumen microbes this can have negative effects on animal production (Van Vuuren *et al.,* 1991). It has been reported that excessive CP concentrations, in particular, NPN, could lead to a reduction in DMI, due to negative physiological feedback mechanisms reported to prevent reoccurrence of NH_3 toxicity (Kertz *et al.,* 1982).

The studies undertaken in this thesis involved the use of multiparous Holstein-Friesian cows in peak lactation. Animals were at the same stage of lactation and were not more than 6 months pregnant, to minimise sources of error in the N-balance experiment. The animals were fed a low CP pasture as a basal diet. This allowed the effect of increasing dietary CP and urea intake on DMI, milk production (Chapter 2) and N partitioning (Chapter 3) to be investigated while maintaining similar concentrations of all other nutrients. This ensured that differences in pasture composition between treatments were minimised. It was anticipated that this would result in more reliable data and allow relationships to be explored that have not previously been investigated for a pasture-based diet. It can be argued that urea does not adequately represent dietary protein, due to its rapid degradation to NH_3 in the rumen (Choung *et al.,* 1990); however, grazed pasture has CP levels of ≥18.5% (Corson *et al.,* 1999), a large proportion of which can be present as NPN (≥30%) (Van Vuuren *et al.,* 1991; NRC, 2001).

One of the major problems with pasture diets is the excessive CP content; a reduction in dietary N or excessive dietary N can negatively affect milk production, efficiency of nutrient utilisation, reproduction, the environment and the overall profit ability of the dairy operation (Polan *et al.,* 1976; Ipharraguerre and Clark, 2005; Fanchone *et al.,* 2013). The potential of dietary N to have an intake depressing effect in pasture-fed dairy cows could be important if pasture-based production systems are adopted by more farmers worldwide (Ramsbottom *et al.,* 2015). The objective of the study reported in Chapter 2 is to investigate the effect of increased dietary CP and urea intake in grazing dairy cows on DMI and milk production; the specific objective of the experiment is to assess the relationship between urea intake and milk yield.

Animal nutrition is a major management tool to reduce the N lost through urine; therefore, it is important to understand N partitioning in the cow to provide guidelines and models for controlling N pollution through managing nutrition (Tamminga, 1992). The potential of dietary N and MUN, as a predictor of N components, is of particular interest, as this can be directly related to the potential of

a diet to contribute to NO_3^- leaching (Pacheco *et al.,* 2007). Understanding the relationships between N inputs and N outputs in dairy cows may allow quantification of urinary N excretion using a mathematical model rather than directly measuring urinary N on farm. The objective of the study reported in Chapter 3 study is to study N partitioning in pasture-fed grazing dairy cows, using urea supplementation as a NPN model. The specific objectives are to assess the relationships between dietary N, urinary N, faecal N and milk N in grazing dairy cows consuming pasture with increasing concentrations of NPN.

Chapter 2: Effect of high-urea supplementation on pasture dry matter intake and milk production in grazing dairy cows

2.1 Introduction

There has been a recent rejuvenated interested in pasture-based production systems due to the associated lower production costs (Ramsbottom *et al.,* 2015) and perceived animal welfare benefits (Dillon *et al.,* 2005; Macdonald *et al.,* 2008). Profitability of pasture-based dairy farms is dependent on high pasture utilisation (Dillon *et al.,* 2005; Ramsbottom *et al.,* 2015). To facilitate this, the system is generally designed to match the profiles of feed supply and demand (Dillon *et al.,* 2005), through a compact spring-calving profile. To achieve this, pasture-based dairy production is centred on a seasonal calving system and the feed is harvested directly by the cow (Crosse *et al.,* 1994).

Several management strategies have been adopted by farmers to increase pasture growth (Lambert *et al.,* 2004). For example, in conjunction with dairy farm intensification, the application of N fertiliser and the selection of genetically improved cultivars of grass and legume species have been successfully used to increase pasture production (Lambert *et al.,* 2004). As well as increasing pasture production, fertiliser N can also affect the concentration of N in the pasture (Whitehead, 1995), and in particular, the proportions of CP that is NPN (Whitehead, 1995; Tamminga, 1996; Hoekstra *et al.,* 2007). Differences in NO_3^- accumulation have been found between plant species and varieties, and studies suggest that annual ryegrasses have higher NO_3^- concentrations than perennial ryegrasses (Darwinkel, 1975). The concentrations of NPN in the pasture are influenced by the time interval between fertiliser N application and grazing and the regrowth stage at application (Van Vuuren *et al.,* 1991; Whitehead, 1995). The highest levels of NPN are present in the sward directly following fertiliser application and the concentration decreases with time post-application (Wilman, 1965; Peyraud and Astigarraga, 1998).

For cows to consume pasture low in N content; fertiliser application must occur immediately after the previous grazing and the rotation length must allow a sufficient time interval between grazings for the N content to decrease (Moller *et al.,* 1996; Chaves *et al.,* 2006). With increasing dairy farm scale and the outsourcing of jobs to contractors, it is often more practical for fertiliser to be applied to the entire farm at once and, as a result, pastures are fertilised at different stages of regrowth. This results in differences in the time interval between N application and subsequent grazing, and pastures high in CP, and specifically NPN, at the time of grazing, even though this is not recommended best practice (Whitehead, 1995; Moller *et al.,* 1996).

Dairy cows grazing these pastures are likely to consume levels of NPN exceeding what can be utilised by rumen microbes (Van Vuuren *et al.,* 1991). Consequently, accumulation of NH_3 in the rumen and subsequently in the blood could lead to a reduction in DMI, due to negative physiological feedback mechanisms reported to prevent reoccurrence of NH_3 toxicity i.e., conditioned negative aversion (Kertz *et al.,* 1982). Despite the high concentrations of CP (Roche *et al.,* 2009), and in particular, NPN (Van Vuuren *et al.,* 1991) in fresh pasture and the effects of excess NPN on DMI in TMR systems; there have been no studies to investigate the effect of increasing concentrations of soluble NPN in fresh pasture on DMI and milk production of grazing dairy cows.

The objective of the current study was to investigate the effect of increased dietary CP and RDP in grazing dairy cows on DMI and milk production, using urea supplementation as a NPN model to ensure that all other nutritional characteristics of the forage remained the same. The specific objective of the experiment was to assess the relationship between urea intake and milk yield.

2.2 Materials and methods

2.2.1 Experimental design

The Ruakura Animal Ethics Committee (Hamilton, New Zealand) approved all animal manipulations (Approval No. 11896) in accordance with the New Zealand Animal Welfare Act (1999). As part of a larger experiment (Cheng *et al.,* 2013) conducted during spring (8th October to 12th November 2009), fifteen multiparous, rumen fistulated, early lactation Holstein-Friesian cows (505 ± 10.4 kg liveweight; 4.1 body condition score ± 0.04, mean ± standard deviation) were assigned to one of three urea supplementation treatments: Control (0 g/day urea; ~20% CP), Medium (350 g/day urea; ~25% CP) and High (690 g/day urea: ~30% CP). To minimise the risk of urea toxicity, cows were gradually acclimated to their urea treatment over a 25 day experimental period (8th October to 1st November 2009), which was broken up into 5 day periods (Figure 19; Appendix 2). The acclimation period offered a unique opportunity to determine what effect, if any, urea dose had on DMI and milk yield.

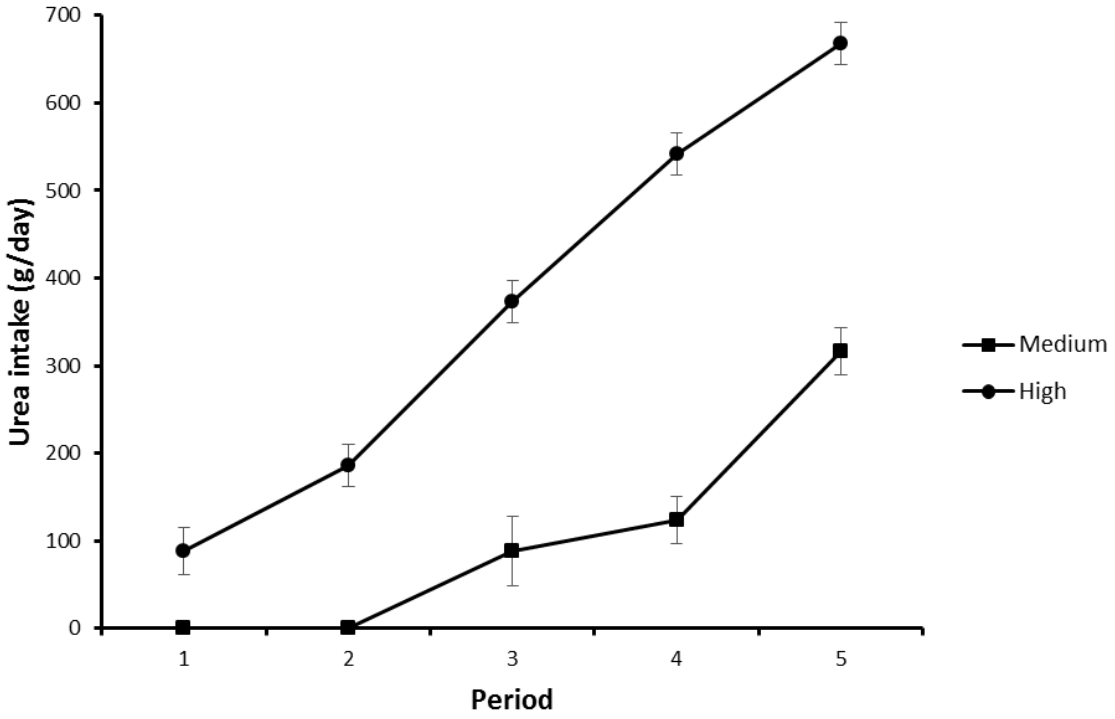
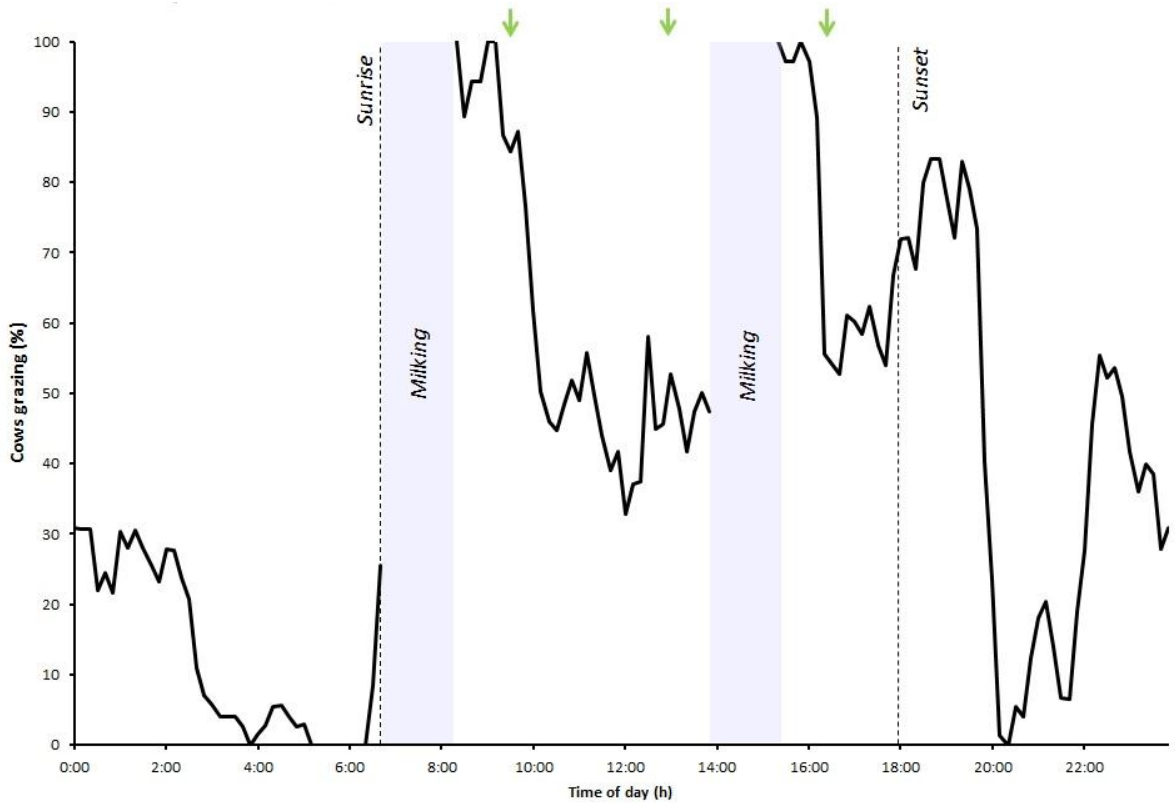


Figure 19: Mean urea intake (g/day) in the High (circles) and Medium (squares) treatments during the 5 periods over the course of the experiment.

Sheahan *et al.* (2011) reported three significant grazing bouts during the day; sunrise, late morning, and early afternoon (Figure 20). Using these behaviour profiles, the timing of urea supplementation was designed to coincide with the availability of soluble protein (SP) following ingestion. Urea was

provided through the rumen fistula in three equal doses 30 minutes after offering the fresh pasture (i.e. 0930 and 1630) and again at 1300 h.

Figure 20: Diurnal profile of cows grazing during peak lactation. Shading represents milking time and vertical dashed lines represent sunrise and sunset. Green arrows represent additions of urea. Modified from Sheahan *et al.* (2011).



2.2.2 Pasture offered

To ensure a low base level of N in the pasture offered, N fertiliser was not applied to the sward for at least 6 weeks before the experiment. The cows had *ad libitum* access to fresh water at pasture. The three treatment groups were grazed in the same paddock. The cows grazed a sward consisting of predominantly perennial ryegrass, with small percentages (<5%) of white clover (*Trifolium repens* L.), weeds and other grasses (*Dactylus glomerata* L.*, Holcus lanatus* and some *Poa* species). The pre-grazing residuals were ~2800 kg DM/ha and post-grazing residuals were ~1500 kg DM/ha. Cows were offered a herbage allowance of 20 kg DM/cow/day. Back-grazing beyond the day's allocation was prevented using electric fences.

Pasture was sampled on three days each week, bulked weekly and analysed for chemical composition by near-infrared spectroscopy (NIRS) (Feed Tech, Palmerston North, New Zealand) as explained by Corson *et al.* (1991). Samples were freeze-dried and ground to pass through a 1.0-mm sieve (Christy Lab Mill, Suffolk, UK) and analysed for ME, CP, NDF, DM, OM, acid detergent fibre

(ADF), DM digestibility, and water-soluble carbohydrates (WSC). Chemical composition reflected a high quality vegetative pasture (CP = 20.6 ± 0.56% DM; NDF = 39.5 ± 0.52% DM; ADF = 21.8 ± 0.36% DM; WSC = 19.6 ± 0.69% DM; DM digestibility = 83.3 ± 0.45% DM and ME = 11.8 ± 0.06 MJ/kg DM).

2.2.3 Milking and milk sampling

Cows were milked twice daily and milk yield was recorded morning (a.m.) and afternoon (p.m.) (Westfalia Surge, GEA, New Zealand). A representative milk sample was collected at a.m. and p.m. milking on one day each week and analysed as an individual sample and then combined for a composite analysis. The samples were preserved with bronopol (2-bromo-2-nitro-propane-1,3-diol) and then refrigerated at 4°C until analysis. All milk samples were analysed for milk composition: fat, CP, true protein, casein, and lactose using Fourier-transform infrared spectroscopy (FT120; Hillerød, Denmark). Total CP yield was calculated using the measured daily milk CP % and measured milk yield:

$$\text{Total CP (kg/day)} = \frac{\text{milk yield (kg) x daily milk CP \%}}{100}$$

Total fat yield was calculated in the same manner using the measured milk fat %, milk solids % and milk yield.

Milk CP % was calculated using the measured milk N %:

$$\text{Milk N \%} = \frac{\text{milk CP \%}}{6.38}$$

Total N in milk was calculated using milk N % and milk yield:

$$\text{Total N (g/day)} = \frac{\text{milk yield (g) x daily milk N \%}}{100}$$

2.2.4 Liveweight and body condition score

All cows were weighed and body condition assessed before, during and at the conclusion of the experiment. Body condition score for all cows was assessed by one assessor, by palpating individual body parts and an average score recorded on a 10-point scale, in which 1 is emaciated and 10 is obese (Roche *et al.,* 2004).

2.2.5 Calculations and statistical analysis

2.2.5.1 Calculations

Dry matter intake per day was estimated by back calculation from the energy requirements of the cows (Nicol and Brookes, 2007), using the liveweight of each cow, the calculated liveweight change of each cow per day, the measured milk yield and milk composition and the estimated ME content of pasture.

Liveweight change per day was calculated using the liveweight recorded and the difference in liveweight over time. The liveweight change was calculated as:

$$\text{Liveweight change (kg/day)} = \frac{\text{Liveweight}_{\text{Final}} - \text{Liveweight}_{\text{Initial}}}{\text{Time (days)}}$$

Changes in liveweight for individual cows ranged from -3.93 kg/day to 3.46 kg/day which is not possible when feeding an adequate pasture-based diet (Roche *et al.,* 2006). Liveweight changes of grazing animals measured over short periods are relatively inaccurate (Thomson and Barnes, 1993). Liveweights were regressed over time in order to predict the average liveweight change. The liveweight change was 0.38 kg/day which was similar to results presented by Roche *et al.* (2006), where average liveweight change for cows post-peak lactation ranged from 0.27-0.41 kg/day. The experiment by Roche *et al.* (2006) analysed liveweight change in New Zealand Holstein-Friesians fed three increasing levels of concentrate in addition to a basal pasture diet (Roche *et al.,* 2006).

Maintenance requirements were calculated on a daily basis using liveweight. Maintenance requirement was calculated as:

$$\text{Maintenance (MJ/day)} = 0.56 \text{ MJ ME/kg} \times \text{liveweight}^{0.75} \text{ (kg)} + \text{Activity (MJ ME/day)}$$

As recommended by Nicol and Brookes (2007), 5% per MJ ME was subtracted from the maintenance requirements for the days that the diet exceeded 11 MJ ME/kg DM.

Activity requirements associated with grazing and additional costs of walking were calculated on a daily basis using the approximate horizontal distance walked per day of 2 km. Activity requirement was added to the total maintenance requirement (Nicol and Brookes, 2007) and was calculated as:

$$\text{Activity (MJ/day)} = 0.0037 \text{ MJ ME/kg liveweight/horizontal km walked}$$

Energy requirements for liveweight change were calculated on a daily basis using the assumed liveweight change (0.38 kg/day) for each day. Liveweight gain requirement was calculated as:

Liveweight gain (MJ/day) = 38 MJ ME/kg gain x 0.3 kg/day / 0.65

This equation includes an efficiency of use of feed ME for liveweight gain, based on a lactating dairy cow fed grass silage made from spring pasture (Holmes *et al.,* 1981). As recommended by Nicol and Brookes (2007), 5% per MJ ME was subtracted from the liveweight gain requirement for the days that the diet exceeded 11 MJ ME/kg DM.

Milk energy requirement was calculated on a daily basis, using milk yield and composition. Milk energy requirement is the ME required for milk production based on fat, protein, lactose and milk yield. Based on the formula presented by NRC (2001) the milk energy requirement was calculated as:

Milk energy requirement (MJ/day) = (0.0929 x Fat % + 0.0547 x CP % + 0.0395 x Lactose %) / 0.64 x 4.184 MJ/Mcal

This equation includes an efficiency of use of feed ME for milk production based on a lactating dairy cow fed grass silage (Holmes *et al.,* 1981).

Total energy requirements per cow per day were then calculated as the sum of the energy required for maintenance, liveweight change and milk production.

Dry matter intake was estimated for each cow using the total energy requirements per day, divided by the estimated ME concentration of the pasture. Dry matter intake was calculated as:

$$\text{DMI (kg DM/day)} = \frac{\text{total energy requirements (MJ ME/day)}}{\text{ME content of pasture (MJ ME/kg DM)}}$$

Total N intake was calculated using N intake from pasture and N intake from urea supplementation. Nitrogen intake from pasture was calculated from pasture DMI and pasture N concentration. Nitrogen intake from pasture was calculated as:

$$\text{Nitrogen intake}_{\text{pasture}}\text{ (g N/day)} = \frac{\text{DMI (kg/day) x pasture CP content (g CP/kg) x 1000}}{6.25}$$

Nitrogen intake from urea was calculated as:

$$\text{Nitrogen intake}_{\text{urea}}\text{ (g N/day)} = \text{Urea supplemented (g urea/day) x 0.466}$$

Total N intake was calculated as:

$$\text{Total N intake (g N/day)} = \text{N intake}_{\text{pasture}}\text{ (g N/day)} + \text{N intake}_{\text{urea}}\text{ (g N/day)}$$

Crude protein content of the diet was calculated using total N intake and DMI. Crude protein content was calculated as:

$$CP\ (\%) = \frac{\text{total N intake (g N/day)} \times 6.25}{\text{DMI (kg/day)} \times 1000} \times 100$$

The N content of the diet was calculated using the total N intake and DMI.

$$\text{Nitrogen content of the diet (g/kg DM)} = \frac{\text{Total N intake (g)}}{\text{DMI (kg DM)}}$$

2.2.5.2 Statistical analysis

Data were analysed using the statistical package SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). Means and standard errors for each of the different variables describing chemical composition of the pasture offered across all treatments over the 5 experimental periods were obtained using the MEAN procedure.

Intake measures and production traits were analysed using the MIXED procedure, with a mixed model for repeated measures. The model included the fixed effect of treatment, period, and interaction between treatment and period, and the random effect of cow to account for repeated measures on the same cow. Based on the Akaike Information Criterion, a homogenous residual variance was determined as the most appropriate structure for the repeated measures across periods. Least square means and standard errors were obtained and used for multiple mean comparisons using the Least Significant Difference test.

A piecewise regression analysis was undertaken using the NLIN procedure to determine the effect of urea intake on milk yield. The following piecewise regression model was used to examine the relationship between the independent variable (urea intake) and the dependent variable (milk yield):

$y = a + b1x$ $(x \leq c)$ **$y = a1 + c\,(b1 - b2) + b2x$ $(x > c)$**

y = dependent variable (milk yield) a = intercept of the equation

b1 = regression coefficient when $x \leq c$ b2 = regression coefficient slope when $x > c$

x = independent variable (urea intake) c = estimated break point

Multiple regression analyses were undertaken using the GLM procedure to determine the effect of DMI, urea intake and dietary CP content on milk yield. Regression equations for the dependent variable (milk yield) were estimated for each of the treatments. Linear and quadratic regression models were used to examine the effect of urea intake, DMI and dietary CP content on milk yield.

Those factors that had a significant linear or quadratic effect on milk yield ($P < 0.05$) were combined in a multiple regression model. Based on the Akaike Information Criterion and criteria of R^2 and P-value, it was decided whether additional factors improved the model fit. Both additive and interactive effects of DMI and urea intake, DMI and CP content, and CP content and urea intake on milk yield were analysed.

2.3 Results

2.3.1 Dry matter intake and milk production

The DMI, CP % and N intake for the Control, Medium, and High treatments are presented in Table 8 and Figure 21. There was a treatment by time interaction (P <0.001) for all three variables. The High treatment had lower mean DMI compared with the Control and Medium treatments across all periods (P <0.05). The DMI declined in the High treatment after period 2, while DMI was stable in the other treatment groups across all periods. From period 2 the High treatment had higher N intakes compared with the Control and Medium treatments (P <0.05). Nitrogen intake varied across periods between the Control and Medium treatments, but was not different until period 5.

Table 8: Dietary crude protein (CP %), dry matter intake (DMI) and nitrogen (N) intake of dairy cows offered increasing amounts of urea as a supplement to pasture during early lactation in the Control, Medium and High treatments.

	Urea treatment	Period 1	2	3	4	5	SE	P - value
DMI, kg/d	**Control**	17.2^{av}	15.8^{aw}	17.1^{av}	16.6^{axy}	16.5^{ay}	0.37	<0.001
	Medium	16.9^{avx}	15.5^{aw}	17.4^{av}	16.4^{axy}	16.1^{ay}	0.42	<0.001
	High	15.5^{bv}	13.7^{bwy}	14.8^{bx}	13.5^{by}	11.2^{bz}	0.37	<0.001
	SE	0.38	0.38	0.38	0.38	0.38		
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001		
N intake, g/d	**Control**	603^{av}	573^{aw}	541^{axz}	517^{ay}	532^{ayz}	19.4	<0.001
	Medium	569^{av}	539^{awx}	536^{ax}	548^{avwx}	648^{by}	21.7	<0.001
	High	564^{bv}	582^{bv}	640^{bw}	671^{bxy}	671^{by}	19.4	<0.001
	SE	20.1	20.1	20.1	20.1	20.1		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
Dietary CP, %	**Control**	21.7^{av}	22.5^{aw}	19.6^{axy}	19.3^{ayz}	20.0^{axz}	0.34	<0.001
	Medium	21.7^{av}	22.5^{awx}	19.9^{ax}	21.6^{by}	25.9^{bz}	0.38	<0.001
	High	22.7^{bvx}	26.4^{bv}	26.9^{bw}	31.2^{cx}	37.4^{cy}	0.34	<0.001
	SE	0.35	0.35	0.35	0.35	0.35		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		

^{a,b,c}Means with different superscripts are significantly different at the 5% confidence level down rows. $^{v, w, x, y, z}$Means with different superscripts are significantly different at the 5% confidence level across columns.

The High treatment had higher dietary CP % compared with the Control and Medium treatments across all periods (P <0.05). The dietary CP % was not different between the Control and Medium treatments until period 4, which coincided with a substantial increase in urea supplementation in the Medium treatment during period 4. The dietary CP % increased with urea supplementation but increased to a greater degree in the High treatment following period 1 which corresponded with the increased consumption of urea and the decline in DMI.

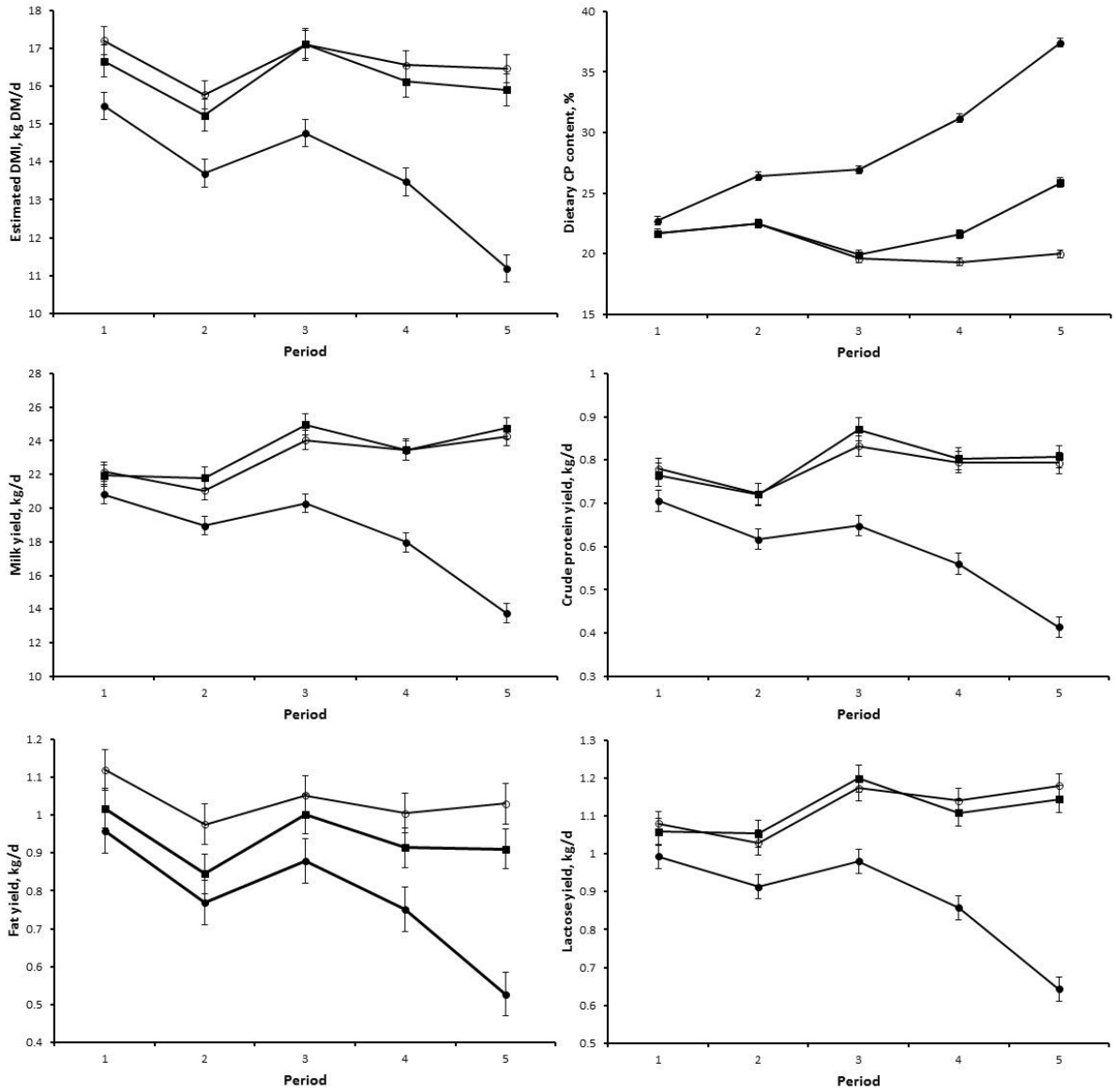


Figure 21: Interaction between urea treatment and period in estimated dry matter intake (DMI), dietary crude protein content (CP %), milk yield and the yield of milk components (P <0.001); Control (open circles), Medium (squares) and High (circles).

The treatment by time interaction for DMI was also evident in milk yield. The mean yields of the three treatments during the 5 periods of the experiment are presented in Table 9 and Figure 22.

Table 9: Yield of milk (kg/day) and milk components of dairy cows offered increasing amounts of urea as a supplement to pasture during early lactation in the Control, Medium and High treatments.

	Urea	Period						
	treatment	**1**	**2**	**3**	**4**	**5**	**SE**	**P- value**
Milk, kg/d	**Control**	22.2^{av}	21.0^{aw}	24.0^{axy}	23.4^{ayz}	24.3^{axz}	0.57	<0.001
	Medium	21.9^{av}	21.8^{avw}	24.9^{awy}	23.5^{ax}	24.8^{ay}	0.63	<0.001
	High	20.8^{av}	19.0^{bwy}	20.3^{bx}	18.0^{by}	13.8^{bz}	0.56	<0.001
	SE	0.59	0.59	0.59	0.59	0.59		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
Fat, kg/d	**Control**	1.12^{au}	0.98^{avwy}	1.05^{acwxz}	1.01^{avx}	1.03^{ayz}	0.05	<0.001
	Medium	1.02^{av}	0.84^{awy}	1.00^{bcv}	0.91^{ax}	0.91^{axy}	0.06	<0.001
	High	0.96^{bv}	0.77^{bwy}	0.88^{bx}	0.75^{by}	0.53^{bz}	0.05	<0.001
	SE	0.05	0.05	0.05	0.05	0.05		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
Protein, kg/d	**Control**	0.78^{avy}	0.72^{aw}	0.83^{ax}	0.79^{axvz}	0.79^{ayz}	0.02	<0.001
	Medium	0.77^{avy}	0.72^{aw}	0.87^{ax}	0.80^{avz}	0.81^{ayz}	0.03	<0.001
	High	0.71^{bv}	0.62^{bwy}	0.65^{bx}	0.56^{by}	0.41^{bz}	0.02	<0.001
	SE	0.03	0.03	0.03	0.03	0.03		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
Lactose, kg/d	**Control**	1.08^{v}	1.03^{av}	1.17^{awx}	1.14^{axy}	1.18^{awy}	0.03	<0.001
	Medium	1.06^{v}	1.05^{av}	1.20^{aw}	1.11^{avx}	1.15^{awx}	0.04	<0.001
	High	0.99^{v}	0.91^{bw}	0.98^{bv}	0.86^{bx}	0.64^{by}	0.03	<0.001
	SE	0.03	0.03	0.03	0.03	0.03		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
Milk composition, %								
Fat	**Control**	4.84^{v}	4.43^{w}	4.24^{xy}	4.15^{y}	4.09^{xz}	0.28	<0.001
	Medium	4.90^{v}	4.08^{wxz}	4.21^{xy}	4.10^{wy}	3.89^{z}	0.32	<0.001
	High	4.73^{v}	4.18^{wy}	4.46^{x}	4.30^{y}	3.96^{z}	0.28	<0.001
	SE	0.29	0.29	0.29	0.29	0.29		
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001		
Protein	**Control**	3.44^{v}	3.34^{wv}	3.39^{v}	3.32^{wx}	3.21^{y}	0.13	<0.001
	Medium	3.64^{v}	3.42^{wy}	3.61^{v}	3.54^{x}	3.38^{y}	0.15	<0.001
	High	3.45^{v}	3.31^{w}	3.25^{x}	3.17^{y}	3.07^{z}	0.13	<0.001
	SE	0.14	0.14	0.14	0.14	0.14		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
Lactose	**Control**	4.87	4.90	4.88	4.86	4.85	0.06	<0.001
	Medium	4.82^{v}	4.83^{v}	4.80^{v}	4.72^{w}	4.61^{x}	0.06	<0.001
	High	4.77^{v}	4.81^{wx}	4.83^{x}	4.76^{wv}	4.65^{y}	0.06	<0.001
	SE	0.06	0.06	0.06	0.06	0.06		
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001		

^{a,b,c}Means with different superscripts are significantly different at the 5% confidence level down rows. $^{v, w, x, y, z}$Means with different superscripts are significantly different at the 5% confidence level across columns. Additional data are presented in Appendix 4.

The mean milk yield was lower in the High treatment across all periods (P <0.05) in comparison to the Control and Medium treatments. The milk yield declined in the High treatment after period 3, while milk yield was stable in the other treatment groups across all periods. The interaction evident in milk yield was mirrored in yields of fat, CP and lactose (P <0.001). Yields of fat, CP and lactose were lower in the High treatment across all periods compared with the Control and Medium treatments (P <0.05), and declined in the High treatment following period 3. This corresponds with the decline in milk yield in this treatment (P <0.001) in comparison to the Control or Medium treatments during these periods. The yields of fat, CP and lactose were stable in the other treatment groups across all periods.

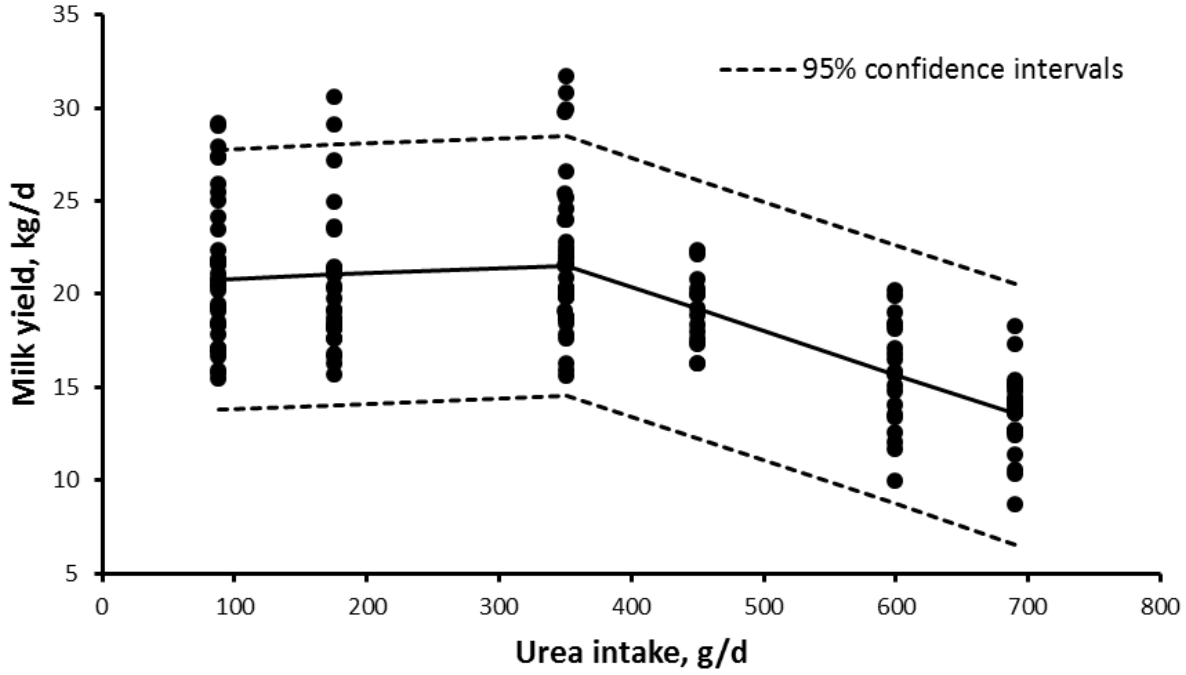


Figure 22: Relationship between urea intake (g/day) and milk yield (kg/day) for all cows (y = 20.5 + 0.0029x, x ≤ c; y = 20.5 + c (350 (0.0029 - ¯0.0235)) + ¯0.0235x, x > c; c = 350 g urea/day; P <0.001).

Urea intake had a significant effect on milk yield (P <0.001). Milk yield plateaued with increasing urea intake, with a point of inflection corresponding to a urea intake of 350 g urea/day as presented in Figure 22. At 350 g urea/day the milk yield decreased at a rate of 2.35 kg per 100 g urea intake, equivalent to a 10.9% decline per 100 g urea intake. Crude protein content had a significant effect on milk yield (P <0.001). Milk yield declined with increasing NPN (and CP) content at a rate of 0.612 kg per 1% increase in CP content (Figure 23).

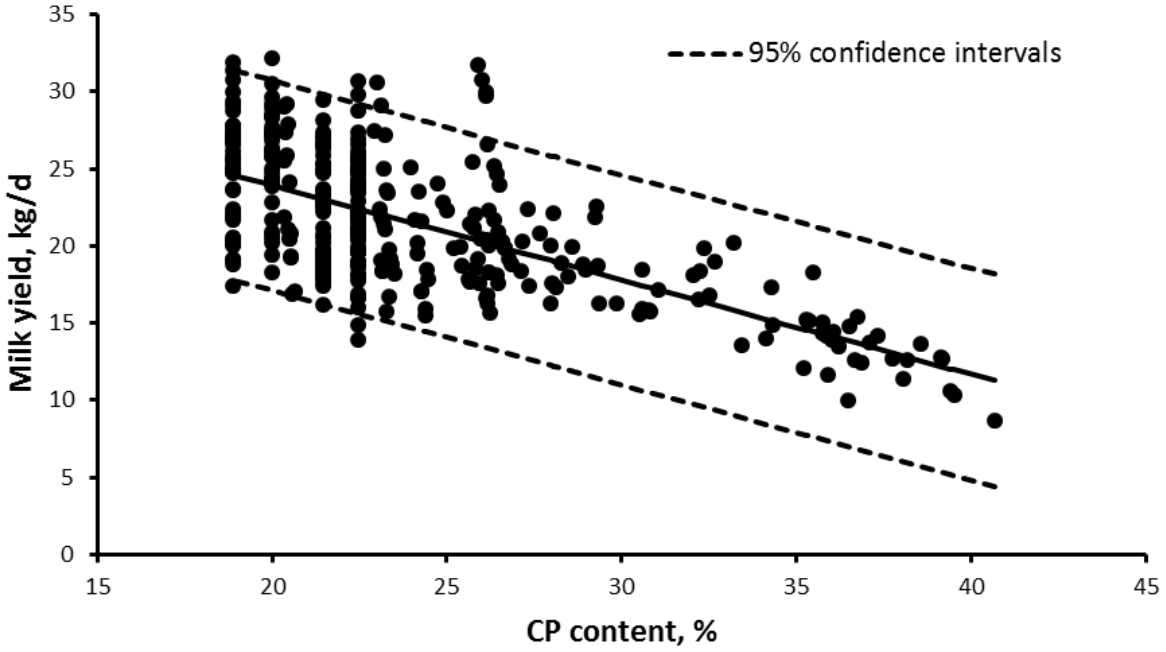


Figure 23: Relationship between crude protein content (CP %) and milk yield (kg/day) for all cows (y= 36.175 - 0.612x; P <0.001; R^2 = 0.45).

Multiple regression analyses were undertaken to determine the effect of DMI, urea intake and dietary CP content on milk yield. Table 10 contains equations and the general format for the sequence of models developed.

Table 10: Sequence of equations used to determine final models of milk yield (kg/day).

Model	Type	R^2 value	P-value
Urea intake, g/d			
y = 23.2 – 0.0116x	Linear	0.31	<0.001
y = 19.4 – 0.000039x^2 + 0.018x	Quadratic	0.41	<0.001
Dry matter intake, kg/d			
y = -11.33 + 2.11d	Linear	0.76	<0.001
y = 1.62d + 0.016d^2 – 7.69	Quadratic	0.76	0.12
Multivariate regression			
y = -11.09 - 0.000058x + 2.10d	Additive linear effect	0.74	<0.001
y = -10.79 + 2.06d + 0.0021x - 0.0000031x^2	Additive quadratic effect	0.74	<0.001
y = -18.11 + 2.57d + 0.0155x – 0.0011d^1	Interactive linear effect	0.74	<0.001
y = -21.05 + 2.69d + 0.028x – 0.0012d^1 - 0.00000115d^2	Interactive quadratic effect	0.75	<0.001
Final model			
y = -21.05 + 2.69d + 0.028x – 0.0012d^1 - 0.00000115d^2	Interactive quadratic effect	0.75	<0.001

y = milk yield (kg/day); x = urea intake (g/day); d = dry matter (DM) intake (kg DM/day); d^1 = dry matter intake x urea intake and d^2 = dry matter intake x urea $intake^2$

Models for the additive and interactive effects of DMI and CP content, and CP content and urea intake did not produce significant effects and are not presented in Table 10. The final model for the interactive quadratic effect of DMI and urea intake on milk yield is presented in Figure 24.

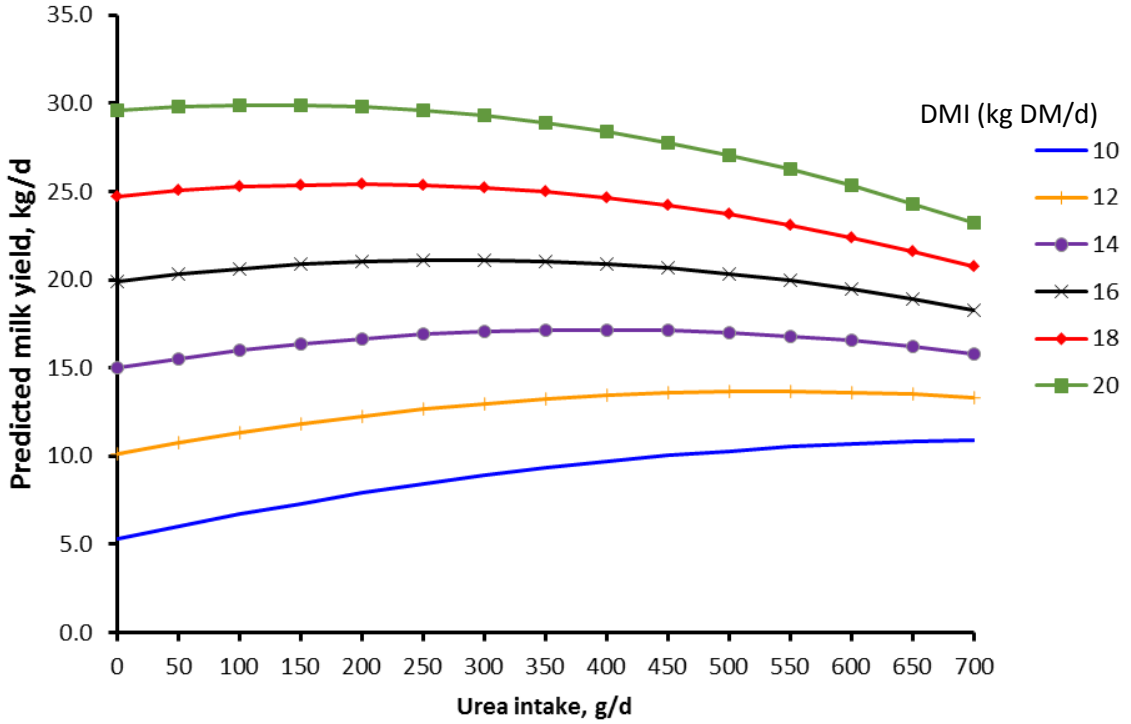


Figure 24: Relationship between milk yield (kg/day), dry matter intake (DMI) (kg/day) and urea intake (g/day) ($y = -21.05 + 2.69d + 0.028x – 0.0012d^1 - 0.00000115d^2$; $R^2 = 0.75$; $P < 0.001$).

While there is potential to over-extrapolate using this model as it predicts milk yield outside of the data range measured, this model was developed to investigate whether the data available could predict milk yield response to increasing DM and urea intake in a similar manner to that reported in literature despite its limitations. From linear and quadratic regression coefficients, equations were developed for predicting milk yield from DMI and urea intake is presented in Figure 24. The response in milk yield to increasing DMI and urea intake is statistically significant ($P < 0.05$) and is strongly correlated with an R-squared value of 0.75. The effect of urea intake on milk yield was dependent on DMI; as DMI increased, an increasing level of urea intake resulted in a declining rate of increase in milk yield and eventually a negative effect on milk yield.

2.4 Discussion

Temperate pastures tend to have high concentrations of CP (Roche *et al.,* 2009), and in particular NPN, exceeding what can be utilised by rumen microbes (Van Vuuren *et al.,* 1991). Increasing dairy farm scale, with N fertiliser applied by contractors on a set calendar date often results in pastures fertilised at different stages of regrowth (Whitehead, 1995). As a result, different CP concentrations, and in particular NPN concentrations are offered to dairy cows at the time of grazing (Whitehead, 1995). In addition, to maximise pasture utilisation, pastures are often grazed at earlier stages of regrowth, despite the high concentrations of CP, particularly in spring and autumn (Roche *et al.,* 2009). There have been no studies to investigate the effect of increasing dietary CP and, in particular, increasing concentrations of NPN in fresh pasture on DMI and milk yield of grazing dairy cows. Excessive urea feeding (> 1% urea in a TMR) can lead to a reduction in DMI and, subsequently, a reduction in milk yield as demonstrated by Polan *et al.* (1976) and Wilson *et al.* (1975). The experiment was designed to ensure that all other nutritional characteristics of the forage remained the same, while urea supplementation increased the concentrations of NPN in the diet. This allowed the effect of dietary CP concentration on DMI and milk yield to be evaluated in grazing systems.

The intake depressing effect of urea has been well documented. Urea odour and taste were eliminated as potential factors causing intake depression in previous studies (Kertz *et al.,* 1982; Kertz, 2010). Wilson *et al.* (1975) concluded that the metabolic intermediates of urea catabolism accounted for the intake depression when increasing levels of urea were fed. The current study supports these findings, as a reduction in intake could not be associated with palatability issues due to the method of urea supplementation via the rumen fistula. This also supports the findings from Kertz *et al.* (1982), where one or more exposures to high dietary levels of urea resulted in cows becoming conditioned to the diet; cows established a negative physiological feedback, termed conditioned negative aversion, resulting in reductions in DMI to prevent toxicity (Kertz, 2010).

The results indicate the existence of complicated interactions between the DMI of the cows and urea supplementation. It could be concluded that milk yield decreased when urea supplementation increased beyond 350 g/day, or approximately 2% DMI (Figure 22). This is in agreement with the findings of Van Horn *et al.* (1967), where inclusion of 2.2 and 2.7% urea in a formulated concentrate feedresulted in depression in milk yield, which appeared to be directly related to concentrate intake. In the current study, DMI's were calculated from milk yield and, as a result, it cannot be confirmed if declining milk yield during periods 4 and 5 was mirrored by a decline in DMI, such as that observed by Van Horn *et al.* (1967). Kertz *et al.* (1982) stated that if administration of urea decreases DMI,

then energy intake and the relative efficiency of energy utilisation for milk yield will both be decreased.

Previous studies have also highlighted a quadratic effect of urea inclusion on DMI and milk yield in TMR-fed cows, where, as dietary CP concentration increased the milk yield increased, however, as urea's contribution to total dietary CP concentration increased, the increase in DMI and milk yield was less (Polan *et al.,* 1976). This has led to recommendations that urea intake should not exceed 1% of total ration DMI (Kertz, 2010), or that urea's contribution to total dietary CP does not exceed 30% (Polan *et al.,* 1976).

However, the results of the current study indicate that these thresholds for urea are different for grazing cows and, arguably more important, that the threshold level of urea is dependent on the total DMI of the cows (Figure 24). These results indicate that the maximum inclusion tolerance for urea as a CP % is affected by the actual DMI of the cows, with higher yielding cows less tolerant of urea than lower yielding cows. The results presented here in grazing cows support the published findings of Polan *et al.* (1976). In their study, cows were producing 20-30 kg milk/day; they deduced that when urea's contribution to dietary CP exceeded 30%, milk yield declined with increasing CP intake. In the current study, the tolerable contribution of urea to CP was 24% and 14%, for cows producing 20 or 30 kg milk/day, respectively. Despite the current study supporting the findings of Polan *et al.* (1976), it is important to note the major limiting factor of the model presented in Figure 24, was the inability to accurately measure DMI, and, as a result, DMI was estimated by back calculation of the energy requirements of the cows.

2.5 Conclusion

The current study measured the effect of urea intake on milk yield and analysed the relationship between the two variables, as well at the relationship between urea intake, DMI and milk yield. This study confirms that excessive urea intake has the potential to reduce milk yield in grazing cows. Further research is needed to determine if high soluble NPN concentrations in fresh pasture would affect DMI and milk yield in the same way.

Chapter 3: Effect of urea supplementation on N partitioning in dairy cows

3.1 Introduction

The New Zealand dairy industry is striving for increased productivity, while maintaining or reducing the environmental footprint, with particular emphasis on reducing N leaching to waterways (Pacheco *et al.,* 2007). Nitrogen is the major nutrient that is lost to waterways in New Zealand. Due to New Zealand's pasture-based system, where cows are grazed outdoors, urination on pasture and races by grazing animals is the major cause of N losses through subsequent NO_3^- leaching and N_2O emissions (Pakrou and Dillon, 2000; Di and Cameron, 2005). Urinary N enters groundwater via drainage through soil and fertiliser N losses, and enters surface waterways via runoff or direct input (Clark, 1997). Nitrogen fertiliser and urine deposition on pastures and races by grazing animals contributes 70 to 90% of N leaching (Ledgard *et al.,* 2009). Therefore, increasing dairy intensification, leading to increased N inputs, through N fertiliser and high CP concentrations in pasture, results in high N losses in urine, and has resulted in a decline in water quality (Scarsbrook and Melland, 2015). Nitrate causes accelerated eutrophication of surface water, which is of major concern to freshwater ecosystems (Gregg *et al.,* 1993).

The primary source of NO_3^- in drainage waters from grazed pastures is animal urine (Haynes and Williams, 1993). Grazing dairy cows consume diets high in protein (>18.5% CP) (Roche *et al.,* 2009), and increased rates of N fertiliser application and short time intervals between N fertiliser application and grazing, in conjunction with dairy farm intensification (Whitehead, 1995; Vibart *et al.,* 2009), have further increased CP content of pasture and, in particular, proportions of NPN in the sward (Lantinga and Groot, 1996; Hoekstra *et al,* 2007). Dietary N in excess of microbial requirements and high proportions of NPN in the sward result in rapid release and accumulation of NH_3 in the rumen (NRC, 2001). Ammonia is absorbed across the rumen wall, converted to urea in the liver and excreted in urine (Tamminga, 1996). As dietary N content increases, the urea recycled to the rumen decreases (Ulyatt, 1997) and urea excreted in urine increases; therefore, N-use efficiency is reduced (Reynolds and Christensen, 2008). This has implications for the productive performance of dairy cattle and has become a serious environmental issue in New Zealand, contributing to N losses to waterways and N_2O emissions to the atmosphere (Huhtanen and Hristov, 2009; Agle *et al.,* 2010).

Regional councils have strict regulations regarding the quantities of N that can be leached from dairy farms into particular river catchments. To comply with environmental standards, robust relationships that quantify N outputs from dairy farms will be required (Higgs *et al.,* 2013).

Consequently, research into the reduction of N losses from dairy farm systems is a high priority for the dairy industry (DairyNZ, 2014). Animal nutrition is a major management tool to reduce the N lost in urine; however, a reduction in dietary N can also affect animal production (Fanchone *et al.,* 2013). Therefore, it is important to understand N partitioning in the cow to provide guidelines for controlling N pollution through managing nutrition (Tamminga, 1992). Overseas research has examined the relationships between dietary N, urinary N, faecal N, and milk N in housed dairy cows fed TMR and forages (Castillo *et al.,* 2000; Kebreab *et al.,* 2001; Burgos *et al.,* 2007; Spek *et al.,* 2013). However, these relationships may not be representative of the New Zealand pasture-based system.

The objective of the current study was to investigate N partitioning in pasture-fed grazing dairy cows, using urea supplementation as a NPN model to ensure all other nutritional characteristics of the forage remained the same. The specific objectives of the experiment were to assess the relationships between dietary N, urinary N, faecal N and milk N in grazing dairy cows consuming pasture with increasing concentrations of NPN.

3.2 Materials and methods

3.2.1 Experimental design

The Ruakura Animal Ethics Committee (Hamilton, New Zealand) approved all animal manipulations (Approval No. 11896) in accordance with the New Zealand Animal Welfare Act (1999). Ten multiparous, rumen fistulated, early lactation Holstein-Friesian cows (520 ± 5.6 kg liveweight; 4.2 body condition score ± 0.08, mean ± standard deviation) were assigned to one of two experimental groups: Control (0 g/day urea; ~18% CP), and a Urea supplement group (350 g/day urea; ~23% CP).

The experiment was conducted over a period of 22 days (22nd October to 12th November 2009). To minimise the risk of urea toxicity, cows in the Urea supplement group were gradually acclimated to their urea treatment over an 11 day acclimation period (Appendix 3). The timing of urea supplementation was identical to that described in Section 2.1 in Chapter 2. Following the acclimation period, animals entered the metabolism stalls for days 12-21 and continued to receive full urea supplementation (350 g/day urea) (Appendix 4). The dose of urea allocated was representative of the urea dose at which milk yield began to decline at higher levels in the Chapter 2 experiment. Days 12-14 allowed animals to adjust to the metabolism stalls. Urea doses were reduced to 250 g urea/day (3x 84 g urea daily) for the Urea supplement treatment during days 16-21 as shown in Appendix 3 due to visual signs of lethargy. While the animals were in the metabolism stalls, the N-balance measurements occurred during the collection period (days 15-21). On the final day, day 22, all cows were removed from the metabolism stalls.

3.2.2 Pasture offered

To ensure a low base level of N in the pasture offered, N fertiliser was not applied to the sward for at least 6 weeks before the experiment. The cows had *ad libitum* access to fresh water. The two treatment groups were grazed in the same paddock during the acclimation period and the cows grazed identical to that described in in Section 2.2 in Chapter 2, with the same grazing residuals and herbage allowances. Following the acclimation period when animals were housed in metabolism stalls, freshly cut pasture was provided. The pasture was offered twice daily at 0900 and 1600 h following milking. All cows were offered 66 ± kg fresh pasture (~11 kg DM) at 0900 h and 45 ± kg fresh pasture (~9 kg DM) at 1600 h. Total fresh pasture offered and pasture refused were weighed. Fresh pasture intake was calculated as the difference between total fresh pasture offered and refused:

Fresh pasture intake weight (kg) = Total pasture offered (kg) – total pasture refused (kg)

The total DMI per cow per day was calculated using the daily DM % of the pasture analysed and the fresh pasture weight:

$$\text{Dry matter intake (kg)} = \frac{\text{fresh pasture weight intake (kg)} \times \text{DM \%}}{100}$$

Pasture was sampled daily, bulked at the end of the collection period and analysed for chemical composition by wet chemistry (Dairy One, Analytical Services, New York, USA) and NIRS (Feed Tech, Palmerston North, New Zealand) as explained by Corson *et al.* (1991) and shown in Appendix 1. Subsamples were collected from the bulked pasture sample, freeze-dried and ground to pass through a 1.0-mm sieve (Christy Lab Mill, Suffolk, UK) and analysed for WSC, OM, OM digestibility, DM digestibility (DMD) and ME by NIRS and for ME, CP, available protein (AP), SP, ADF, NDF, lignin, starch, minerals (calcium, potassium, phosphorus, magnesium, sodium, iron, zinc, copper, manganese, sulphur, chloride, molybdenum), ash and starch using wet chemistry (Table 11 and 12).

Table 11: Chemical composition of pasture fed to all treatments (mean ± standard error). Percentage crude protein (% CP), available protein (% AP), soluble protein (% SP), water soluble carbohydrates (% WSC), acid detergent fibre (% ADF), neutral detergent fibre (% NDF), lignin, dry matter (% DM), organic matter (% OM), organic matter digestibility (% OMD), dry matter digestibility (% DMD), and metabolisable energy (ME). Wet chemistry analysis (Dairy One, Analytical Services, New York, USA) and NIRS (NIRS; Feed Tech, Palmerston North, New Zealand).

Item	Pasture
n	7
% CP	18.4 ± 0.64
% AP	17.8 ± 0.60
% SP	46.4 ± 1.56
% WSC[1]	22.7 ± 0.81
% ADF	25.3 ± 0.34
% NDF	43.1 ± 0.41
% Lignin	2.7 ± 0.23
% Starch	0.96 ± 0.36
% OM[1]	91.3 ± 0.17
% OMD[1]	84.4 ± 0.31
% DMD[1]	79.0 ± 0.27
ME (MJ/kg DM)[1]	11.4 ± 0.06

[1]NIRS pasture analysis

Table 12: Minerals and trace element composition of pasture fed to all treatments (mean ± standard error). Percentage calcium (% Ca), phosphorus (% P), magnesium (% Mg), potassium (% K), sulphur (% S), sodium (% Na), chloride ion (% Cl) and ash (% Ash). Parts per million iron (PPM Fe), zinc (PPM Zn), copper (PPM Cu), manganese (PPM Mn), and molybdenum (PPM Mo). Wet chemistry analysis (Dairy One, Analytical Services, New York, USA).

Item	Pasture
n	7
% Ca	0.53 ± 0.02
% P	0.32 ± 0.01
% Mg	0.18 ± 0.01
% K	3.03 ± 0.07
% S	0.24 ± 0.00
% Na	0.13 ± 0.01
% Cl	1.35 ± 0.14
% Ash	9.95 ± 0.14
PPM Fe	177 ± 24.0
PPM Zn	31.3 ± 1.66
PPM Cu	4.0 ± 0.00
PPM Mn	63.3 ± 3.31
PPM Mo	1.09 ± 0.22

Chemical composition reflected a high quality vegetative pasture as presented in Table 11. The CP content (18.4% ± 0.64) was low, as intended.

3.2.3 Milking and milk samples

Cows were milked twice daily and milk yield was recorded morning and afternoon (Westfalia Surge, GEA, New Zealand; acclimation period) (Tru-Test milk meters, Tru-Test Ltd, New Zealand; metabolism stalls). A representative milk sample was collected at a.m. and p.m. milkings and analysed as an individual sample and then combined for a composite analysis as described in Section 2.3 of Chapter 2. In addition to the analysis described in Chapter 2.2.3, somatic cell count was analysed using Fourier-transform infrared spectroscopy (FT120; Foss, Denmark) and urea N in milk was quantified using a kinetic ultra-violet and colorimetric assay (Modular P800; Germany). Total CP, fat, N and milk N % were calculated in the same manner as described in Section 2.3 of Chapter 2.

Milk urea N was calculated using the measured daily milk urea concentration:

Milk urea N = milk urea (mmol/L) x 0.46

3.2.4 Liveweight and body condition score

All cows were weighed and body condition assessed before, during and at the conclusion of the collection period. Body condition score for all cows was assessed by one assessor, by palpating

individual body parts and an average score recorded on a 10-point scale, in which 1 is emaciated and 10 is obese (Roche *et al.,* 2004).

3.2.5 Urine and faecal sampling

All urine and faeces were collected through the use of urine and faecal separators. Urine and faeces were weighed daily to obtain yields. A representative urine sample was collected at 0900 h daily, from the bulk urine collected during the day and analysed. Each urine sample was treated with acid (H_2SO_4), with the amount of H_2SO_4 adjusted, as necessary, to maintain acidified urine between pH 2.0 and 4.0. The samples were then frozen at -20°C until analysis. The acidification method used to minimise NH_3 losses was applied to the samples collected, however, it was not undertaken with the bulk urine collected, due to the safety concerns with the large volumes of H_2SO_4 required to acidify the bulk urine collected. This raises concerns that the efficacy of the urine preservation was compromised with an issue of potential urea N loss due to enzyme catalysed urea hydrolysis resulting in NH_3 losses. The urine samples were freeze-dried and ground to pass through a 1-mm sieve (Christy Lab Mill, Suffolk, UK) and weighed before analysis. The samples were analysed for ^{15}N and N concentration using an isotope ration mass spectrometer (PDZ Europa Ltd; Rudheath, Northwich, Cheshire, UK). Urea, creatinine, NH_3, calcium and magnesium in urine were quantified using a kinetic UV and colorimetric assay (Modular P800; Germany).

The total urine N excreted was calculated using the measured urine N content and urine weight:

Total urine N (g/day) = urine N (g/L) x urine weight (kg)

The estimated urine N excreted was calculated using the measured N intake, faecal N and milk N (assuming that N retention was negligible):

Estimated urine N (g/day) = Total N intake – (Total faecal N + Total milk N)

A representative faecal sample was collected at 0900 h daily, from the bulk faeces collected during the day and analysed. The samples were freeze-dried and ground to pass through a 1-mm sieve (Christy Lab Mill, Suffolk, UK) and weighed before analysis. Faecal samples were analysed to determine faecal N concentration. Two additional duplicate fresh faecal samples were collected from each cow daily and analysed for composite DM content. The two samples of ~200 g wet weight were oven dried at 60°C for 48 h and re-weighed to determine the percentage DM of the faeces. Faecal analysis to determine DM and faecal N concentration was carried out by DairyNZ analytical services (DairyNZ, Hamilton, New Zealand).

Total faecal N excreted was calculated using the measured faecal N content and faecal weight:

$$\text{Total faecal N (g/day)} = \frac{\text{daily faecal N \%}}{100} \times \text{dry faecal weight (kg)} \times 1000 \text{ g}$$

The dry faecal weight was calculated using the determined faecal DM% and fresh faecal weight:

$$\text{Dry faecal weight (kg)} = \frac{\text{faecal DM \%}}{100} \times \text{fresh faecal weight (kg)}$$

The dry matter digestibility % of the pasture consumed was calculated using the calculated DMI and faecal dry weight:

$$\text{DMD (\%)} = \frac{\text{DMI} - \text{dry faecal weight (kg)}}{\text{DMI}} \times 100$$

The apparent N digestibility % of the pasture was calculated using the calculated N intake from pasture and total faecal N excreted:

$$\text{Apparent N digestibility (\%)} = \frac{\text{pasture N intake (g N/day)} - \text{total faecal N (g N/day)}}{\text{pasture N intake (g N/day)}} \times 100$$

3.2.6 Ruminal samples

Rumen fluid samples were collected for each cow at 0930 h daily by squeezing rumen contents through muslin cloth to collect the fluid. A sample of the rumen fluid was collected and analysed for urea and NH_3 concentrations using enzymatic kinetic assay (Hitachi 902 analyser; Hamilton, New Zealand).

3.2.7 Calculations and statistical analysis

3.2.7.1 Calculations

The water intake from pasture was calculated using the measured fresh pasture intake and the DMI:

1. Water intake from pasture (kg) = fresh pasture intake (kg) – DMI (kg)

The water excreted in faeces was calculated using the measured fresh faecal weight and dry faecal weight:

2. Water excreted in faeces (kg) = fresh faecal weight (kg) – dry faecal weight (kg)

The difference between water intake and water excreted was then calculated using the water intake from feed, water imbibed, water in milk, water in faeces and water in urine. Water excreted in milk and urine was assumed to be equal to the milk and urine weights measured. The difference between water intake and water excreted was calculated as:

Water intake – water excreted (kg) = (water intake from pasture + water imbibed (kg)) – (water in milk + water in faeces + water in urine (kg))

Nitrogen-use efficiency was calculated using the milk N and N intake:

$$\text{Nitrogen-use efficiency (\%)} = \frac{\text{total milk N (g/day)}}{\text{total N intake (g/day)}}$$

Nitrogen balance was calculated using the total N intake – total N ouput:

N balance (g/day) = total N intake – (total urine N + total faecal N + total milk N)

The total N intake from pasture, the total N intake from urea, the total N intake per cow per day, the CP content of the diet, and N content of the diet was calculated as described in Section 2.5.1 of Chapter 2.

3.2.7.2 Statistical analysis

Data were analysed using the statistical package SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). Means and standard errors for each of the different variables describing chemical composition of the pasture offered across all treatments were obtained using MEAN procedure.

Intake measures and production traits were analysed using the MIXED procedure, with a mixed model for repeated measures. The model included the fixed effect of treatment and the random effect of cow to account for repeated measures on the same cow. Least square means and standard errors were obtained using the MIXED procedure.

Regression equations for the dependent variables; total urine N, estimated urine N, UUN, and MUN and ruminal NH_3 concentrations, were estimated for each of the treatments and all cows using the GLM procedure. The following linear regression model was used to examine the relationships between N intake and total urine N, estimated urine N, total UUN, and MUN, as well as the relationships between MUN and ruminal NH_3 and total urine N:

$y = a + bx$, where

y = dependent variable

a = intercept of the equation

b = regression coefficient

x = independent variable (N intake)

3.3 Results

3.3.1 Intake, digestibility of feed, liveweights and faecal weight

Intake, digestibility of feed, liveweights and faecal weights for the two treatments are presented in Table 13. The DMI and N intake from pasture were not different between treatments. This was intentional to provide a basal level of N in the diet from pasture. The total N intake was increased in the Urea supplement treatment by adding 0.35 kg urea to the diet daily (0.16 kg urea N) (Table 13). Addition of feed grade urea to the pasture-based diet resulted in a greater mean N intake (0.11 kg/d increase; P <0.001) in the Urea supplement treatment. Consequently, the total N content and CP content of the diet was greater in the Urea supplement treatment compared to the Control (P <0.001).

Table 13: Intake, liveweight, digestibility of feed and faecal weight in the Control and Urea supplement treatments. Dry matter intake (DMI), nitrogen (N), crude protein (CP) dry matter (DM), and dry matter digestibility (DMD).

Item	Control	Urea supplement	SEM	P-value
Intake, kg/d				
DMI	19.1	18.7	0.580	0.60
Pasture N	0.56	0.55	0.017	0.60
Urea N	-	0.12	0.003	<0.001
Total N	0.56	0.67	0.017	<0.001
N content, g/kg DM	29.4	36.1	0.129	<0.001
CP content, %	18.4	22.6	0.080	<0.001
Liveweight, kg	522	527	10.9	0.78
Fresh faecal weight, kg/d	44.5	41.1	3.00	0.43
Faecal DM, %	9.67	10.4	0.62	0.40
DMD, %	77.5	77.5	0.53	0.99
Apparent N digestibility, %	75.4	75.3	1.45	0.94

Liveweight, fresh faecal weight, faecal DM %, DMD % and apparent N digestibility were not affected by N intake. This was expected due to the same basal pasture diet fed to both treatments during the collection period and similar faecal weights. The calculated DMD % presented in Table 13 is similar to the DMD % obtained from the NIRS pasture analysis as displayed in Table 11.

3.3.2 Water balance

The water imbibed, water intake from feed, water output in milk, urine and faeces, and water balance defined as the water intake minus water output are presented in Table 14. The water intakes, water outputs and water balance were not different between treatments.

Table 14: Water balance in the Control and Urea supplement treatments.

Item	Control	Urea supplement	SEM	P-value
Water intake				
Feed, kg/d	102	101	2.18	0.68
Imbibed, L/d	33.0	30.0	2.17	0.35
Water output				
Milk, kg/d	25.0	22.9	1.48	0.33
Urine, kg/d	34.0	35.7	2.24	0.60
Faeces, kg/d	40.2	36.9	2.90	0.43
Water balance, kg/d	34.7	35.3	3.33	0.90

3.3.3 Milk production

Mean milk yields and composition values for the two treatments are presented in Table 15. Milk yields, compositions and somatic cell counts were not different between the two treatments. Milk urea concentration (P <0.001) and total urea secreted in milk (P <0.001) were greater in the Urea supplemented treatment.

Table 15: Yield of milk and milk components of the Control and Urea supplement treatments during the 7 day nitrogen balance period.

Item	Control	Urea supplement	SEM	P-value
Milk yield, kg/d				
Total yield	25.0	22.9	1.48	0.33
Milk a.m.	16.6	15.8	0.89	0.53
Milk p.m.	8.39	7.11	0.65	0.17
Milk fat	1.05	0.97	0.05	0.21
Milk crude protein	0.83	0.80	0.03	0.63
Milk composition, %				
Milk fat	4.23	4.32	0.28	0.81
Milk crude protein	3.33	3.55	0.16	0.34
Milk true protein	3.11	3.37	0.19	0.36
Milk casein	2.65	2.79	0.13	0.42
Milk lactose	5.04	4.93	0.06	0.21
Milk urea, mmol/L	3.93	5.60	0.21	<0.001
Total milk urea, mmol/d	98.4	130	12.2	0.07
Somatic cell count (000)	22.3	67.7	20.7	0.13

3.3.4 Urine excretion

The yield and composition of urine components excreted are displayed in Table 16. The mean total urine excreted was not affected by treatment. The yields and concentrations of N^{15}, creatinine, NH_3, calcium and magnesium were not different between the two treatments. There was a 59% increase

in urine urea concentration in the Urea supplement treatment compared with the Control (P <0.001). Consequently, the total urine urea excreted per day was greater (P <0.001) in the Urea supplement treatment compared with the Control (5347.9 and 3073.0 mmol/day, respectively).

Table 16: Yield of urine (mmol/d) and urine components of the Control and Urea supplement treatments during the 7 day nitrogen balance period.

Item	Control	Urea supplement	SEM	P-value
Yield				
Total creatinine, mmol/d	66.1	67.7	3.53	0.74
Total ammonia, mmol/d	453	588	99.3	0.34
Total calcium, mmol/d	88.2	84.7	11.3	0.83
Total magnesium, mmol/d	131	127	16.2	0.89
Total urea, mmol/d	3073	5347	205	<0.001
Urine composition				
N^{15}, %	0.37	0.37	0.001	0.26
Creatinine, µmol/L	1999	1917	114	0.61
Ammonia, µmol/L	13918	16720	2842	0.49
Calcium, mmol/L	2.67	2.38	0.32	0.53
Magnesium, mmol/L	3.92	3.60	0.41	0.58
Urea, mmol/L	95.1	151	8.60	<0.001

3.3.5 Nitrogen balance

The N partitioning into urine, faeces, milk and rumen fluid are displayed in Table 17. The total N intake was different between the Control and Urea supplement treatment (P <0.001). Faecal N concentration was marginally greater (P = 0.05; +0.08 g/kg DM) in the Urea supplemented treatment, but faecal N output was not affected by treatment. The urea N concentration and urea N yields were different in urine. The Urea supplement treatment had a greater concentration of urea N and consequently, higher urea N yield compared with the Control (P <0.001). The effect of total N intake on total UUN concentration was analysed using linear regression and is presented in Figure 25. The linear response of total UUN in relation to total N intake was significant (P <0.001).

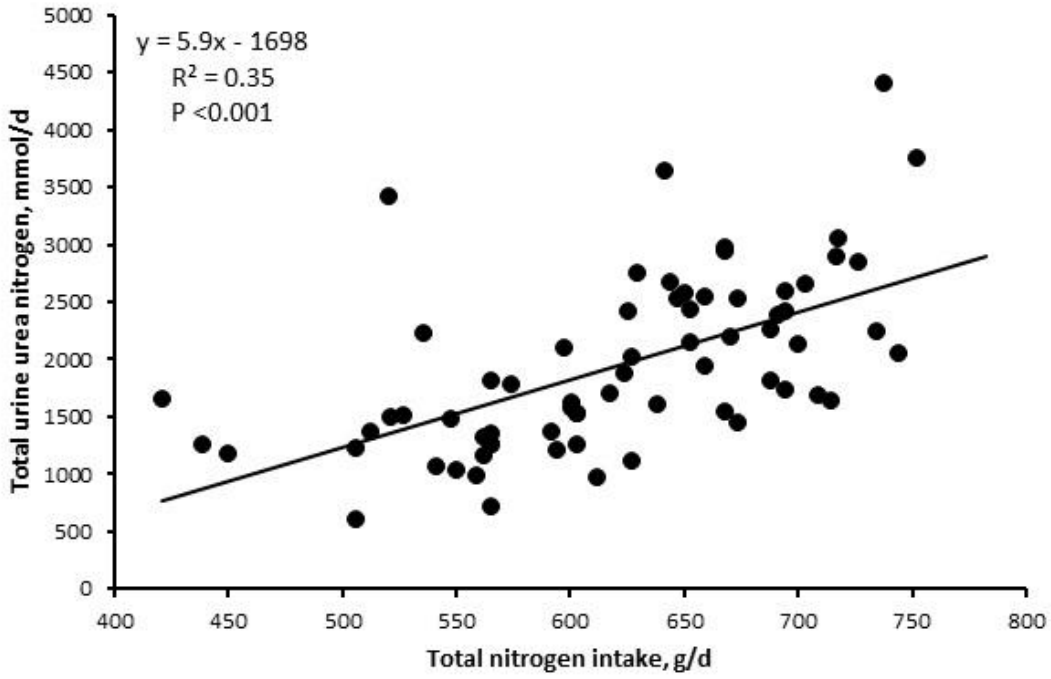


Figure 25: Relationship between total urine urea nitrogen (mmol/d) and total nitrogen intake (g/d) for all cows.

The concentrations of N in urine and the total urinary N excreted per day were different between the two treatments. The concentration of N in urine was 6.1 and 5.1 g/L for the Urea supplement treatment and Control, respectively ($P < 0.05$). In addition, the total urine N excreted was greater in the Urea supplement treatment compared with the Control (215.3 and 168.4 g/day, respectively; $P < 0.05$).

The N-balance was not affected by treatment (130 and 126 g/day for the Control and Urea supplement treatments, respectively). The N-balance was higher than would be expected for lean tissue gain, suggesting that the total urine N measured may be inaccurate. As a result, total urine N was estimated using the total milk N and faecal N values. Estimated total urine N was different between the Urea supplement treatment and Control (422 and 309 g/day, respectively; $P < 0.001$). There was a substantial difference between measured and calculated urinary N excreted (96 and 84% increase in the calculated urinary N compared to measured urinary N for the Control and Urea supplement treatment, respectively). The linear response of total N intake on total urinary N and estimated urine N are presented in Figure 26. The regression coefficient for the relationship between total N intake and estimated urine N ($R^2 = 0.82$) is stronger compared with the regression coefficient for the relationship between total N intake and total urinary N ($R^2 = 0.17$).

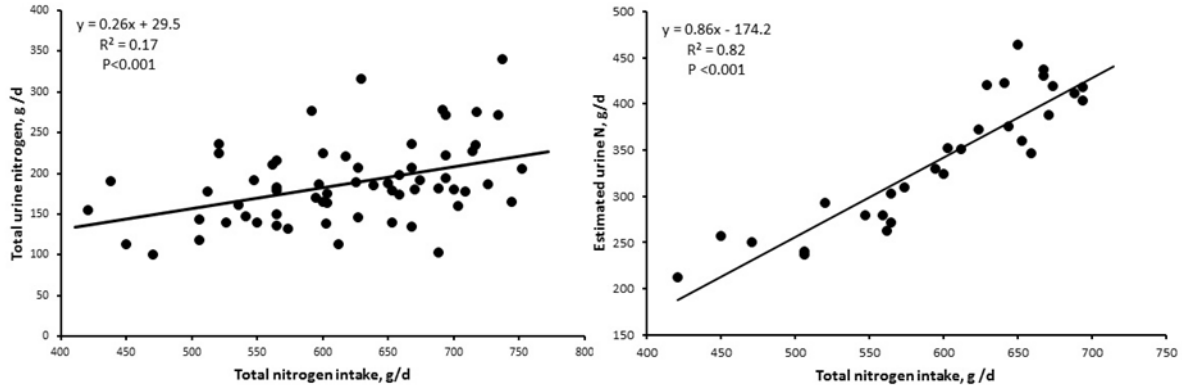


Figure 26: Relationships between total urine nitrogen (g/day), estimated urine nitrogen (g/day) and total nitrogen intake (g/day) for all cows.

The milk urea concentration was greater in the Urea supplement treatment compared with the Control (5.6 and 3.9 mmol/L, respectively). Milk urea N concentration was greater (P <0.001) in the Urea treatment however, total MUN was not. The percentage of N in milk and the total milk N was not affected by treatment.

Table 17: Nitrogen parameters pertaining to partitioning in urine, faeces, milk and rumen fluid of cows grazing pasture in the Control and Urea supplement treatments.

Item	Control	Urea supplement	SEM	P-value
Intake				
N intake, g/d	563	674	17.2	<0.001
Urine				
Urea N, mmol/L	43.7	69.5	3.94	<0.001
Total urea N, mmol/d	1414	2460	94.2	<0.001
Urine N, g/L	5.1	6.1	0.30	0.02
Total urine N, g/d	**168**	**215**	**10.8**	**0.003**
Estimated N urine, g/d	309	422	14.2	<0.001
Faeces				
Faecal N, g/kg	3.04	3.12	0.04	0.05
Total faecal N, g/d	**133**	**132**	**9.91**	**0.91**
Milk				
Urea N, mmol/L	1.81	2.58	0.10	<0.001
Total urea N, mmol/d	45.4	59.9	5.62	0.07
Milk N, %	0.52	0.56	0.03	0.34
Total milk N, g/d	**123**	**126**	**5.31**	**0.62**
N balance, g/d	141	207	18.1	0.02
Rumen				
Urea, mmol/L	11.3	20.8	2.95	0.03
NH_3, mg/dL	28.8	44.2	4.34	0.02
NH_3-N, mg/dL	23.7	36.4	3.57	0.02
Nitrogen-use efficiency, %	20.4	16.8	0.64	<0.001

Nitrogen = N; ammonia = NH_3

The effects of total N intake and urinary N yield on MUN concentration were analysed using linear (Figure 27) and quadratic regressions. The relationship between total N intake and MUN was significant (P <0.001). A small improvement in the fit of the regression equation (linear R^2 = 0.47 vs. quadratic R^2 = 0.55) was observed when a quadratic relationship between total N intake and MUN was analysed. As the total N intake increased, MUN concentration increased linearly as presented in Figure 27. The linear response of total urinary N in relation to the MUN concentration was significant (P <0.001) based on a single regression analysis for all cows. The urinary N excreted increased 1.02 kg/day per unit (mmol/L) increase in MUN as presented in Figure 27.

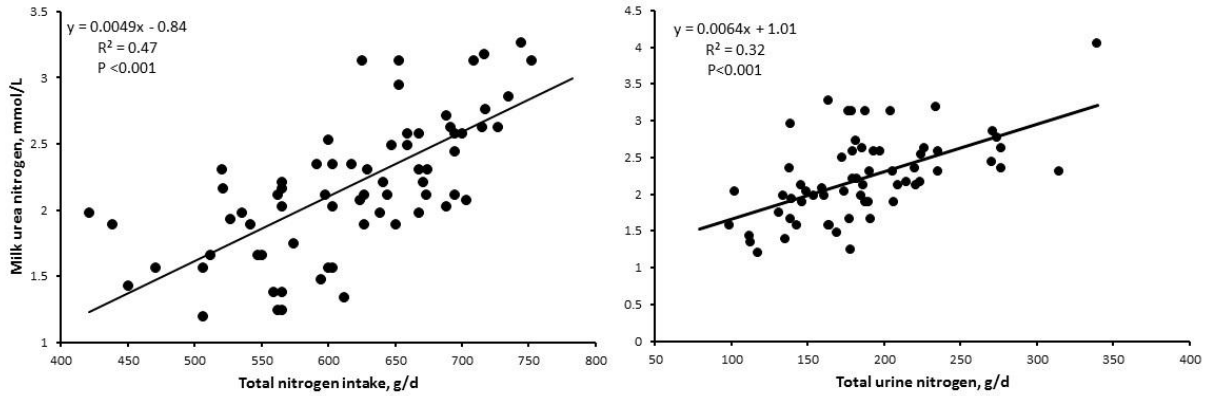


Figure 27: Relationships between milk urea nitrogen (mmol/L) and total nitrogen intake (g/day) and total urine nitrogen (g/day) for all cows.

The rumen urea, NH_3 and NH_3-N concentrations were affected by treatment (P <0.05). The effect of ruminal NH_3-N concentration on MUN was analysed using linear regression (Figure 28). The relationship between ruminal NH_3-N concentration and MUN was significant (P <0.001).

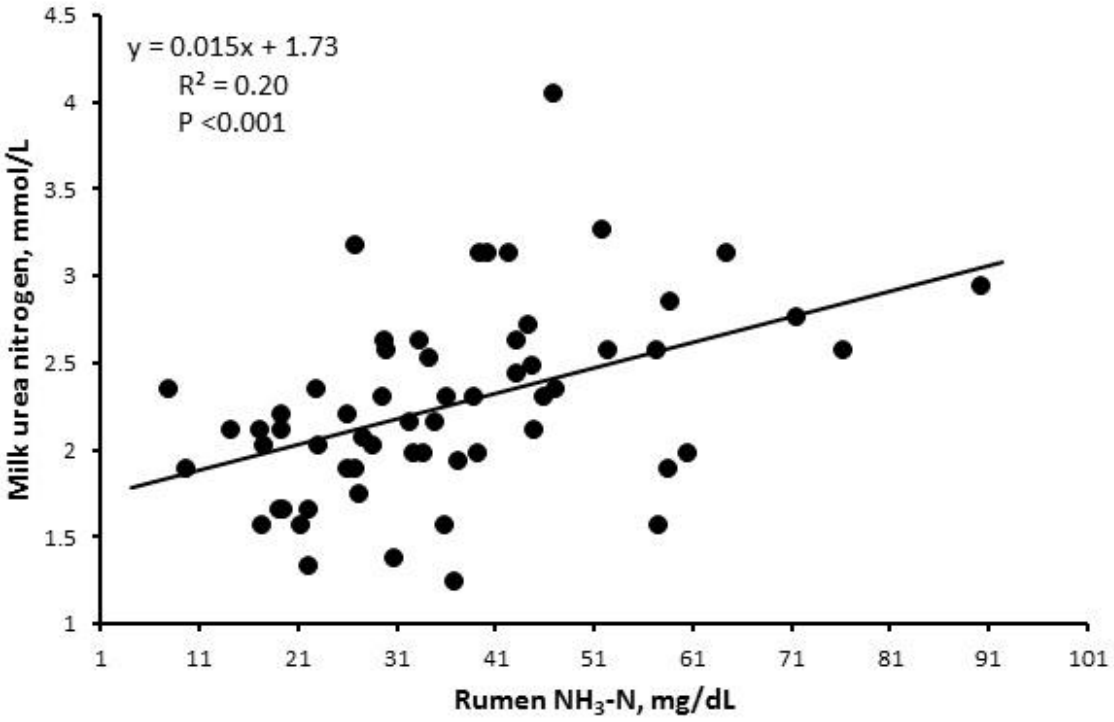


Figure 28: Relationship between milk urea nitrogen (mmol/L) and rumen ammonia (NH_3-N) concentrations (mg/dL) for all cows.

The N-use efficiency was 3.6% units higher in the Control compared with the Urea supplement treatment ($P < 0.05$).

3.4 Discussion

New Zealand's pasture-based system is predominantly based around cows grazing outdoors. Grazing dairy cows consume diets high in protein (≥18.5% CP) (Roche *et al.,* 2009), as a result of grazing management, improved cultivars and increased rates of N fertiliser application in conjunction with dairy farm intensification (Whitehead, 1995). As the CP content of pasture and, in particular, proportions of NPN in the sward increase, the N-use efficiency of dairy cows decreases (Lantinga and Groot, 1996; Hoekstra *et al.,* 2007). This results in increased deposition of urinary N on pasture (Monaghan *et al.,* 2005). Urination on pasture and races by grazing animals is a major cause of N losses through NO_3^- leaching (Pakrou and Dillon, 2000; Di and Cameron, 2005). The growth and intensification of dairying from 1994 to 2015 has resulted in an increase in both stocking rate (18% increase) and the use of N fertiliser (an almost 7-fold increase) (Statistics NZ, 2012; LIC and DairyNZ, 2015), which further excerbates NO_3^- leaching. Nitrate causes accelerated eutrophication of surface water which is of major concern to freshwater ecosystems (Gregg *et al.,* 1993). As a result understanding N partitioning in dairy cows is of major importance to the dairy industry. The experiment described in Chapter 3.2 was designed to ensure that all other nutritional characteristics of the forage remained the same, while urea supplementation as a NPN model allowed the concentrations of NPN in the fresh pasture to increase. This allowed the effect of dietary CP concentration on the partitioning of N in dairy grazing systems to be evaluated.

3.4.1 Nitrogen intake

In the present study, a basal pasture diet with moderate CP levels was supplemented with urea to allow the levels of N in the diet to be increased, while maintaining similar concentrations of other nutrients. This allowed the effects of increasing dietary CP content to be studied without confounding effects of pasture composition and energy content. This is similar to the method used by Burgos *et al.* (2007); however, pasture instead of TMR was used as the basal diet. Supplementation of a low basal CP pasture diet with 0.12 kg urea, where dietary CP level was increased from 18.4% to 22.6% of DM, had no effect on DMI. This is in agreement with Burgos *et al.* (2007), where dietary CP level was increased from 15 to 21% of DM, and also with Olmos Colmenero and Broderick (2006), who increased dietary CP level from 13.5 to 19.4%. However, contradicted findings by Broderick (2003), who reported increasing DMI with increasing dietary CP concentration. However, the dietary CP level in the study by Broderick (2003) was in the range 15.1-18.4% which is below the dietary CP level investigated in the current study.

3.4.2 Faecal nitrogen

Increasing dietary CP concentration had no effect on faecal N. This is in agreement with Marini and Van Amburgh (2003) and Broderick (2003), who increased dietary CP level from 15.1 to 18.4% of DM. Broderick (2003) reported that faecal N concentration increased significantly up to 16.7% CP, with no further increase at 18.4% CP. Kebreab *et al.* (2001) found a bi-linear relationship between N intake and faecal N, with a point of inflection up to about 420 g N/day, after which point another linear but less steep relationship was observed. Kebreab *et al.* (2001) reported that up until 400 g N/day, the faecal N output was proportional to the amount of N in the rumen, mainly due to an increase in microbial N synthesis. As the intake exceeded 400 g N/day, microbial N flow from the rumen became constant, which could help explain the insignificant difference in faecal N between the two treatments in the current study where N intake was in the range 560-670 g/day (Kebreab *et al.,* 2001). Dietary indigestible protein N and undigested microbial N are two major contributors of total faecal N (NRC*,* 2001). Due to the addition of highly degradable urea to the Urea supplementation treatment, the indigestible protein N content contributing to total faecal N was likely to have remained unchanged between the Control and Urea supplementation treatments.

The MP synthesis was likely to have reached an optimum in the Control treatment, due to an excessive dietary CP content (>14% CP) of 18.4% (Satter & Slyter, 1974) and, therefore, increasing CP content and, more importantly, RDP in the Urea supplementation treatment (22.6% CP), would have resulted in no additional microbial N flow from the rumen in the Urea supplement treatment compared with the Control. As a result, the contribution of undigested microbial N to total faecal N likely remained constant (Kebreab *et al.,* 2001; Burke *et al.,* 2002). No changes in the amounts of indigestible protein N and undigested microbial N leaving the rumen would explain the similar faecal N concentrations between treatments.

3.4.3 Milk production

No effect of urea supplementation on milk yield, fat, and CP contents was evident when dietary CP was increased from 18.4 to 22.6% of DM, in agreement with findings from Burgos *et al.* (2007). Broderick (2003) reported a linear increase in DMI with increasing dietary CP concentration, when dietary CP was increased from 15.1 and 16.7 to 18.4% of DM, however milk yield, milk fat and milk CP contents only increased up to 16.7% CP, with no further increase at 18.4% CP. Burgos *et al.* (2007) reported that milk fat and protein were unaffected by increasing dietary CP concentration. However, Broderick (2003) and Burgos *et al.* (2007) reported a pattern, where milk yield increased at a substantially lower rate, or even decreased at higher dietary CP concentrations than at lower dietary CP concentrations, is in agreement with the current study, the much larger databases of NRC (2001),

and the reviews of Ipharraguerre and Clark (2005) and Castillo *et al.* (2000). The 2001 NRC Dairy Committee predicted that maximum milk yield is achieved at 23% CP in the diet and Ipharraguerre and Clark (2005) predicted a maximum milk yield at 22.8% CP, which is similar to the CP concentration of the Urea supplementation (22.6% CP) treatment; however, milk yield decreased compared with the Control (18.4% CP) (NRC, 2001; Ipharraguerre and Clark, 2005). Ipharraguerre and Clark (2005) reported that considerable variation in the relationship between the dietary CP concentration and milk yield can be accounted for by the quality, amount and source of the CP. Unlike the experiments undertaken by Broderick (2003), Burgos *et al.* (2007) and the review by Ipharraguerre and Clark (2005), where mostly TMR and silage diets were fed, the current study fed a pasture-based diet and increased CP concentration using urea supplementation. This indicates that feeding urea with pasture to increase dietary CP above 18.4% does not improve milk yield. Macdonald *et al.* (1998) added urea as a source of protein to maize silage diets to provide dietary CP concentrations in the range 13.3-16.9% of DM. Macdonald *et al.* (1998) reported that urea as a source of protein had no effect on milk, milk fat and milk protein production. The lack of response may be due to the amount and source of the protein (Ipharraguerre and Clark, 2005). The high proportions of NPN, fed as urea, in the current study and the study by Macdonald *et al.* (1998), would result in higher fractions of protein being rapidly degraded in the rumen and subsequently high levels of ruminal NH_3 production (Van Vuuren *et al,.* 1992). Ammonia in excess of microbial requirements results absorption of excess NH_3 across the rumen wall (Pisulewski *et al.,* 1981). Therefore, when RDP is not limiting, the addition of RDP to the diet, would not increase MP outflow from the rumen which may explain the lack of milk production response (Macdonald *et al.,* 1998; NRC, 2001).

There was no effect of dietary CP concentration on milk N in the current study, which is in agreement with a study undertaken by Olmos Colmenero and Broderick (2006). The lack of increase in milk N secretion, coupled with a lack of increase in milk yield and milk CP concentrations, indicates that RDP was not limiting milk yield (Kalscheur *et al.,* 2006; Olmos Colmenero and Broderick, 2006). Bi-linear responses in milk N as a result of dietary CP concentrations were obtained using larger datasets by Castillo *et al.* (2000) and Kebreab *et al.* (2001). Up to a consumption of about 420 g N/day, the increase in the milk N output was in proportion to the amount received from microbial and dietary N. As RDP concentration increases and the microbial synthesis reaches optimum, the MP contribution to the milk N concentration remains constant, resulting in a less steep rate of increase in milk N output (Kebreab *et al.,* 2001). Subsequently, N-use efficiency declines because excess N consumed is largely excreted in urine (Castillo *et al.,* 2000; Kebreab *et al.,* 2001). This was also evident in the current study, where N-use efficiency declined 17.6% in the Urea supplement

treatment compared with the Control. This can be explained by the increase in RDP concentration in the Urea supplement treatment due to the supplementation of urea in the diet.

3.4.4 Nitrogen balance

The measured total urinary N excreted (N balance) and calculated urinary N excreted (estimated urinary N) is presented in Table 17. The measured total urinary N excreted was 168 and 215 g/day for the Control and Urea supplementation treatments, respectively. There was a substantial difference between measured and calculated urinary N excreted (96 and 84% increase in the calculated urinary N for the Control and Urea supplement treatment, respectively). This substantial difference between the measured and calculated values for total urinary N indicates an underestimation of urinary N. Spanghero and Kowalski (1997) reported an average positive N-balance of 38.8 g of N/day from a meta-analysis on N-balance trials (35 studies). They deemed that this estimate of N in lean tissue gain was too high, indicating a source of error with urinary N predictions.

In the current experiment, based on the calculated N in lean tissue gain (the difference between N intake and N excreted in milk and faeces), and the average N-balance observed in the analysis by Spanghero and Kowalski (1997), it is apparent that the measurement of urinary N is a source of error. In the current experiment, the calculated N-balance was estimated at 141 and 207 g N/day for the Control and Urea supplementation treatments, respectively (Table 17). When converted to protein (coefficient = 6.25), a protein gain of approximately 900 and 1300 g/day is obtained for the Control and Urea supplementation treatments, respectively (Blaxter, 1989). Based on a protein to water ratio of 1:3 (Blaxter, 1989), the protein gain of ~900 and 1300 g/day allow a lean tissue gain of approximately 3600 and 5000 g/day for the Control and Urea supplementation treatments, respectively.

For the period 2nd November to 11th November the average lean tissue gain were 422 and 822 g/day for the Control and Urea supplementation treatments, respectively. Based on the assumptions above and using a back calculation method the average N in lean tissue gain was 16.9 and 32.9 g N/day for the Control and Urea supplementation treatments, respectively (Blaxter, 1989). As indicated by the average N in lean tissue gain calculated above and the small change in liveweight during the current experiment, the estimated N in lean tissue gain calculated using the N-balance values was overestimated (Table 17). This is in agreement with the results of Spanghero and Kowalski (1997), where the average N in lean tissue gain of 38.8 g N/day was calculated across 35 N-balance studies with a range of N-balance values from -57 to 205.1 g/day, and it was concluded that

this estimate was too high. Underestimation of urinary N was the likely source of error in the current study, resulting in overestimation of N in lean tissue gain.

3.4.5 Urinary nitrogen

The relationships between dietary CP and urinary N and UUN could be used as models to predict UUN excretion. Total UUN excretion was affected by increasing CP content. The total UUN excretion increased linearly as N intake increased, as presented in Figure 25. This is in agreement with the study by Marini and Van Amburgh (2003), where it was reported that increasing N content in the diet increased UUN excretion linearly ($P < 0.001$). It has been reported in literature that increasing N intake has little or no effect on the non-urea component of urine, whereas UUN accounts for 92-99% of the additional N in urine (Marini and Van Amburgh, 2003). This is expected, as urea is the main N constituent in which excess N is removed in ruminants (Broderick, 2003; Marini *et al.,* 2006). Therefore, this would explain the similarities between the relationships between total urinary N and N intake, and total UUN and N intake.

Total urinary N and estimated urinary N were affected by increasing CP content. In agreement with the findings of Marini and Van Amburgh (2003); increasing N content in the diet increased urinary N excretion ($P < 0.001$). The relationships between total urinary N and estimated urinary N and total N intake were linear as presented in Figure 26. The regression coefficient indicated a larger improvement for the relationship between total N intake and estimated urinary N, compared with the relationship between total N intake and total urinary N (R^2 = 0.82 vs. 0.17, respectively). Due to the underestimation of total urinary N as described earlier, the estimated urinary N was used when comparing literature. In disagreement with the relationship presented between total N intake and estimated urinary N, Castillo *et al.* (2000) and Kebreab *et al.* (2001) both reported a quadratic relationship, where urinary N increased linearly, with a point of inflection around 400 g N/day, after which urinary N increased exponentially. A decreasing rate of N partitioned into faeces and milk was also reported in the studies by Castillo *et al.* (2000) and Kebreab *et al.* (2001). The lack of effect of dietary CP concentration on milk and faecal N in the current study fits this relationship. However, the relationship between dietary N and urinary N was linear, and not quadratic, in agreement with the studies by Olmos Colmenero and Broderick (2006) and Marini and Van Amburgh (2003).

The studies by Castillo *et al.* (2000) and Kebreab *et al.* (2001) involved analysing the relationship between N intake and urinary N through meta-analysis. A large dataset, with a wide range of N intake values, between 200-750 g N/day, was studied. In the current study, the N intake data ranged from 563 to 674 g N/day and, therefore, the dataset in the current study would fall into the area above the point of inflection described by Kebreab *et al.* (2001). This is a possible explanation for the

linear and not quadratic relationship evident in the current study. In the current study, N intake was greater than 400 g N/day for all treatments, so that a 20% increase in N intake resulted in a 40% increase in estimated urinary N excretion. The relationship presented and the exponential relationships described by Castillo *et al.* (2000) and Kebreab *et al.* (2001) for urinary N output, emphasise that urinary N is the principal route for excess N output (Susmel *et al.,* 1995; Castillo *et al.,* 2000; Kebreab *et al.,* 2001).

Rumen degradable protein entering the rumen is absorbed across the rumen wall as NH_3 if provided in excess of microbial requirements. Subsequently, this is converted to urea in the liver and excreted in urine (Higgs *et al.,* 2013). Rumen undegraded protein and MP entering the small intestine, may be absorbed as amino acids and partitioned in milk, deposited in tissue, or excess N excreted (McDonald *et al.,* 2011). If in excess of the requirements of the animal, amino acids from RUP and MP will be converted to urea and excreted in urine (Hall and Huntington, 2008). This emphasises the importance of reducing N intake to avoid providing N in surplus to requirements. A small reduction in urinary N excretion and subsequent N leaching from dairy cows grazing pasture could be achieved by using moderate amounts (0-200 kg/ha/year) of N fertiliser on pasture (Kebreab *et al.,* 2001; Buckthought *et al.,* 2015). However, these must both be achieved while having minimal or limited effects on pasture and milk production (Castillo *et al.,* 2000). Alternatively, if N intake in the cow cannot be reduced, N utilisation must be improved by increasing productive output (N partitioned to milk) (Kebreab *et al.,* 2001; Marini and Van Amburgh, 2003). The relationship between dietary CP content and urinary N indicate a strong correlation, which is to be expected, as N intake is the primary factor affecting urinary N output and could provide a useful on-farm tool to predict urinary N excretion (Higgs *et al.,* 2013). However, dietary CP could be a difficult variable to measure on pasture-based dairy farms and therefore, is not practical. This has generated interest in using the relationship between MUN and urinary N as a tool to predict the urinary N excretion in dairy cows.

3.4.6 Milk urea nitrogen

Milk urea N is an easy on-farm variable to measure, due to the ability to analyse bulk milk samples for urea through enzymatic or physical methods (Broderick and Clayton, 1997; Kauffman and St-Pierre, 2001; Nousiainen *et al.,* 2004). Therefore, it has been suggested that MUN could be used as a diagnostic tool to predict dietary CP content of pasture on farm and urinary N excretion (Kauffman and St-Pierre, 2001). In the current study, MUN concentration increased 42.5% as the dietary CP concentration increased from 18.4 to 22.6%. As presented in Figure 27, the relationship between MUN concentration and N intake was linear and ranged from 1.2 to 4.0 mmol/L (3.4 to 11.3 mg/dL), when the N intake increased from 562.6 to 673.9 g N/day.

This is supported by the findings of Burgos *et al.* (2007) and Broderick (2003), who reported a linear relationship for cows fed 15 to 21% and 15.1 to 18.4% dietary CP, respectively. A small improvement in the fit of the regression equation was evident in the current study (linear R^2 = 0.47 vs. quadratic R^2 = 0.55), which is similar to the findings of Broderick (2003), who reported a small improvement in the fit of the regression equations (linear R^2 = 0.79 vs. quadratic R^2 = 0.80) when analysing MUN concentrations ranging from 9.2 to 15.9 mg/dl. However, Broderick (2003) and the current study analysed MUN concentrations <25 mg/dL, which, based on previous literature, supports a linear relationship.

Burgos *et al.* (2007) analysed MUN concentrations from 5.3 to 31.8 mg/dL and, therefore, had a wider range of values than either Broderick (2003) or the current study to evaluate the relationship between dietary CP concentration and MUN. Burgos *et al.* (2007) reported that the differences between the relationships may relate to the range of dietary CP concentrations used to develop the equation, because when MUN concentrations >25 mg/dL were not included in the study by Burgos *et al.* (2007), the relationship was linear. Nousiainen *et al.* (2004) stated that even though their findings presented a linear relationship, there is a tendency for curvilinear association of MUN at high CP concentrations in the diet and, therefore, provided that the prediction equation is applied to similar nutritional circumstances under which it was developed, measurements of MUN could be used to assess the dietary CP content of pasture on farm (Kauffman and St-Pierre, 2001; Burgos *et al.,* 2007).

The current study evaluated MUN in the range 3.35 to 11.3 mg/dL; therefore, based on the findings of Burgos *et al.* (2007), it would be expected that the relationship would be linear. However, the size of the dataset may have been a limitation in the current study and, as a result, the range of MUN would have been insufficient to adequately challenge the relationship between MUN and total N intake at higher MUN concentrations. The relationship presented would not be suitable as a standardised MUN model to predict dietary CP content in grazing dairy cows, unless applied to a similar range of MUN concentrations (Broderick, 2003).

Several studies support that dietary CP content is the best single predictor of MUN (Broderick, 2003; Nousiainen *et al.,* 2004). As a result, it has been suggested that MUN could be used to predict urinary N excretion in relation to a particular dietary CP content (Nousiainen *et al.,* 2004). Understanding the relationship between MUN concentration and total urinary N would provide the link between dietary CP content, MUN concentration and urinary N (Kauffman and St-Pierre, 2001). If urinary N excretion is proportional to blood urea N concentration and MUN is proportional to blood urea N, this should allow MUN to linearly predict urinary N (Jonker *et al.,* 1998). This assumes

that the kidney has a constant urea filtration rate (millilitres of blood filtered per minute), regardless of urine volume, and that blood urea diffuses rapidly into milk via the mammary gland (Jonker *et al.,* 1998).

The studies undertaken by Jonker *et al.* (1998), Kauffman and St-Pierre (2001) and Nousiainen *et al.* (2004), all reported a strong linear relationship between MUN and urinary N. In the current study there was a linear relationship between MUN and urinary N over the range of MUN values observed (R^2 = 0.32). Based on MUN, the relationship indicated that urinary N excretion increased 17.8 g per unit (mg/dL) increase in MUN. Corresponding equations reported by Jonker *et al.* (1998), Kauffman and St-Pierre (2001) and Nousiainen *et al.* (2004), provide similar slopes (13.4, 12.5 and 17.6, respectively), although different intercepts as presented in Figure 29.

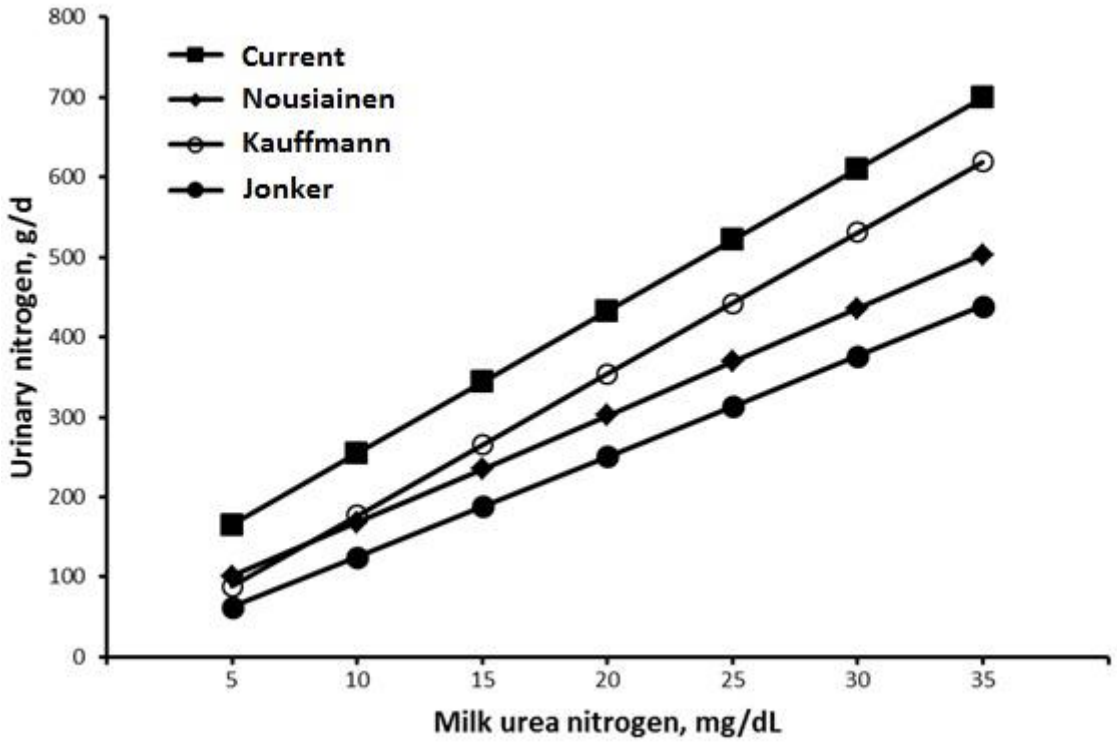


Figure 29: Comparison of the predictions of urinary nitrogen excretion (g/day) based on milk urea nitrogen (mg/dL) according to the current study (y = 75.6 + 17.8x; R^2 = 0.32), along with studies by Jonker *et al.* (1998), Kauffman and St-Pierre (2001) and Nousiainen *et al.* (2004).

These differing numeric relationships demonstrate the model errors that need to be considered when using these models to predict a response (Nousiainen *et al.,* 2004). Due to differences between studies, where models have been developed under a specific set of conditions (i.e. dietary treatments and physiological factors), the models are not robust enough to predict urinary N

excretion for regulatory purposes (Burgos *et al.,* 2007). In addition, the models relate specifically to the dataset analysed and there are potential problems of over-extrapolation of the model using data that are outside of the range measured, resulting in over prediction of urinary N (Jonker *et al.,* 1998). This is clear in Figure 29 where the model for the current study, which is representative of a pasture-based system, would likely overestimate urinary N excretion if applied to cows fed TMR. A major difference between pasture diets and TMR would be in protein degradability, which influences N utilisation (Kebreab *et al.,* 2001). Kebreab *et al.* (2001) showed a 24% decline in urinary N excretion as a result of feeding low compared to high, degradable protein, with no compromise in milk output. The model in the current study is related to MUN concentrations in the range 3.35 – 11.3 mg/dL; however this range of MUN is insufficient to adequately challenge the TMR relationships between MUN and urinary N presented in literature.

In agreement with the studies of Nousiainen *et al.* (2004), in the current study, total N intake was a better predictor of estimated urinary N than was MUN (R^2 = 0.82 vs. 0.32). This raises the question of whether MUN or dietary CP content would be a better predictor of urinary N. Regional councils have begun to produce strict regulations with regards to the quantities of N that can leach on dairy farms, where models such as the MUN model would be useful. The accuracy of the relationships presented in the current study and in literature is unknown and prompts the need for further research before they could be used to monitor farmers. It is also important to consider the timing of N excretion and urine N induced NO_3^- leaching losses, due to leaching losses being more likely in late autumn, winter and early spring (Buckthought *et al.,* 2015). Understanding the relationships between N intake, MUN and urinary N in the animal and the contribution of urinary N in soil to NO_3^- leaching, is crucial if N leaching is to be successfully monitored and mitigated (Buckthought *et al.,* 2015).

3.4.7 Ruminal nitrogen

In the current study, MUN concentration increased linearly as ruminal NH_3-N concentration increased as presented in Figure 28. To my knowledge, there is no published literature illustrating the same relationship. However, linear relationships between MUN concentrations and blood urea N concentrations have been reported (Kauffman and St-Pierre, 2001; Burgos *et al.,* 2007). When the amount of NH_3 released in the rumen exceeds the microbial requirements, a greater proportion of NH_3 is absorbed through the rumen wall and converted to urea in the liver. Subsequently, urea concentrations in circulation increase (Burgos *et al.,* 2007). Due to urea readily diffusing across cellular membranes, milk urea equilibrates with urea in the blood and, as a result, MUN concentration is proportional to plasma urea N (Jonker *et al.,* 1998). Because of this process and the similar relationship between MUN and plasma urea N, the close association between MUN

concentration and ruminal NH_3-N concentration implies that ruminal NH_3-N concentration is proportional to the urea entering the blood and subsequently equilibrating into milk (Gustafsson and Palmquist, 1993).

Rumen urea, NH_3 and NH_3-N were all affected by increasing CP content. This is not unexpected, because urea added to the diet to increase the CP content is high in RDP (NRC, 2001). As CP and RDP increased, there would be greater instantaneous degradation of readily soluble N to NH_3, explaining the increased NH_3 and NH_3-N concentrations between the Control and Urea supplement treatments. When Boucher *et al.* (2007) added incremental amounts of urea to a basal diet of corn silage fed to four lactating Holstein cows, it was reported that increasing the amounts of urea fed resulted in quadratic increases in rumen NH_3-N concentrations. The CP contents of the diets ranged from 14.9 to 17.3% CP. The wide data range of ruminal NH_3-N concentrations in the current study (6.58 – 74.23 mg/dL) did not allow the detection of a non-linear effect; however, the NH_3-N concentration did increase as N intake increased, which is in agreement with the study by Boucher *et al.* (2007) and Kang-Meznarich and Broderick (1980). The discrepancy with previous studies may be due to the methodology used to collect ruminal fluid. In the study by Boucher *et al.* (2007) ruminal fluid was collected every 6 hours over a 4 day collection period; whereas, in the current study, samples were collected daily prior to feeding. Due to the fluctuations in rumen NH_3 during the day, particularly after feeding, the ruminal samples collected were not representative of daily NH_3-N concentrations (Kang-Meznarich and Broderick, 1980).

Studies conducted *in vivo* and *in vitro* often use the point of NH_3 accumulation in the rumen as a point of reference corresponding to the optimal ruminal NH_3-N concentration to support maximum MP synthesis (Satter and Slyter, 1974). Conflicting results have been reported with regards to the ruminal NH_3-N concentrations required to optimise microbial growth, with a range from 5-13 mg/dL (Kang-Meznarich and Broderick, 1980; Reynal *et al.,* 2003; Boucher *et al.,* 2007). *In vitro*, neither urea recycling nor absorption occurs; therefore, the dietary protein level at which NH_3 starts to accumulate *in vitro* does not necessarily reflect the same dietary protein level at which NH_3 would start to accumulate *in vivo* (Satter and Slyter, 1974). There are also differences in literature due to differences in dietary composition, the experimental methods, and the manner in which NH_3-N concentrations were altered (Satter and Slyter, 1974; Boucher *et al,* 2007). Therefore, in a practical sense, the optimal NH_3-N concentrations required to maximise MP synthesis are influenced by the chemical and structural characteristics of the feed (Reynal and Broderick, 2005). In the current study, the NH_3-N concentrations were 23.7 and 36.4 mg/dL for the Control and Urea supplement treatments, respectively. Although the optimal NH_3-N concentration required to maximise MP

synthesis varies in literature, the levels recorded in the current experiment far exceed the optimal concentrations reported in literature. Although microbial synthesis was not measured, it is likely that ruminal NH_3-N concentrations were in excess of microbial requirements and this is further supported by the high levels of N excreted in urine.

3.4.8 Conclusion

The current study investigated N partitioning in grazing dairy cows and the effect of N intake on urinary N, faecal N and milk N. Results confirmed that increasing N intake resulted in linear increases in MUN, urinary N and UUN. Results also confirmed that urinary N increases linearly as a result of MUN. Further research is needed to determine a robust prediction equation for the relationship between N intake and MUN in grazing dairy cows, if this relationship were to assess the dietary CP content of pasture on farm. There is a need for additional research to develop robust prediction equations for the relationships between MUN and urinary N, and urinary N and N intake. These relationships could provide useful tools to predict urinary N excretion as a result of a particular dietary CP content due to the strong relationships between these variables, depending on the information available. The data available in literature based on grazing dairy cows is limited and the relationships predicted in the current study have limited use due to the small range of data. There is a need for further research providing prediction equations in grazing dairy cows and validating which of the relationships available in literature would be a better predictor of urinary N, before they could be used as regulatory tools.

Chapter 4: General discussion and conclusions

Excessive feeding of protein and in particular NPN, results in inefficient N-use by the dairy cow. New Zealand's pasture-based system results in the feeding of pasture that often contain levels of CP ≥18.5 % of DM (Corson *et al.,* 1999), a large proportion of which can be present as NPN (≥30 %) (Van Vuuren *et al.,* 1991; NRC, 2001). These high concentrations of CP are a result of several feed management practices undertaken on farm to increase pasture production (Lambert *et al.,* 2004). In conjunction with dairy farm intensification, increasing rates of N fertiliser application, grazing management and the selection of genetically improved cultivars have contributed to cows grazing pasture high in CP concentrations and, in particular, NPN (Whitehead, 1995; Lambert *et al.,* 2004). Non-protein N increases the RDP content of the diet and is degraded rapidly in the rumen (Sniffen *et al.,* 1992). Higher proportions of NPN in the sward result in rapid release of NH_3 (NRC, 2001) and, consequently, accumulation of NH_3 in the rumen results in NH_3 in excess of rumen microbial requirements (Van Vuuren *et al.,* 1991). As a result, NH_3 is absorbed across the rumen wall (Satter and Slyter, 1974), converted to urea in the liver and excreted in urine (Tamminga, 1996). Consequently, this has implications for the productive performance of dairy cattle and has become a serious environmental issue in New Zealand, contributing to N losses to waterways and N_2O emissions to the atmosphere (Huhtanen and Hristov, 2009; Agle *et al.,* 2010). In addition, high levels of NPN could also lead to a reduction in DMI, due to the negative physiological feedback mechanisms reported to prevent reoccurrence of NH_3 toxicity (i.e. negative conditioned aversion) (Kertz *et al.,* 1982). Chapter 2 investigated the effect of increasing concentrations of soluble NPN in fresh pasture on DMI and milk production of grazing dairy cows. Chapter 3 investigated the potential of a diet to contribute to NO_3^- leaching (through loss of N from urine), by developing a better understanding of N partitioning and the relationships between N intake and N outputs in grazing dairy cows.

The methodological aspects of the current studies were considered prior to commencement, to minimise sources of error. However, both studies presented in Chapter 2 and 3, were subject to inaccuracies due to the experimental approach.

In Chapter 2, various methodological aspects limited the accuracy of the data analysed and the major limiting factor was the inability to accurately predict DMI in grazing cows. The DMI was estimated by back calculation of the energy requirements of the cows due to the inability to accurately measure individual intakes in grazing cows (Oudshoorn *et al.,* 2013). This limited the results in the study and, in particular, the model predicting milk yield based on DMI and urea intake, as milk yield and DMI are directly correlated. This experiment would need to be repeated and DMI

measured for this model to be scientifically and statistically supported. The short-term nature of the study limited the ability to fully investigate the relationships between urea intake and milk yield. In the current study, cows were gradually acclimated to urea treatments over a 25 day experimental period and fed their total urea allocation for 3-7 days. In the study undertaken by Polan *et al.* (1976), cows were fed assigned rations for 20 weeks and, as a result, the relationships between urea intake, milk yield and DMI were more robust. The changes in liveweight used to predict DMI were limited by the short-term nature of the current study, due to the large variations in liveweight during the experimental period. It is likely that daily differences in intake and gut fill affected the liveweight measurements taken during this short-term experiment, resulting in inaccuracies in the energy balance equation (Thomson and Barnes, 1993). For future studies focused on understanding the effect of high NPN contents in cows grazing pasture, the cows may need to be assigned to their treatment diets for a longer period of time and individually housed and fed pasture, rather than estimating DMI, as demonstrated by Polan *et al.* (1976).

In Chapter 3, several issues discussed in literature that arose from previous N-balance studies were considered before conducting the study to avoid sources of error in the N input and output measurements. The study was conducted using animals that were at the same stage of lactation and not more than 6 months pregnant (Castillo, 1999). An animal in late gestation will experience N retention by the foetus and associated structures (~2 g N/day at the 20^{th} week of pregnancy) (NRC, 1985). The N retention becomes quantitatively more important during late gestation. Urea supplementation was used to increase the N component of the diet ensuring that the level of N in the diet was increased while maintaining similar concentrations of all other nutrients. This ensured that differences in pasture composition between treatments were minimised and prevented the research from being confounded by other ingredients of the diet as demonstrated by Kebreab *et al.* (2001) and Higgs *et al.* (2013). Kebreab *et al.* (2001) demonstrated that when N fertiliser was used to increase dietary N content in pasture, a change in composition of the sward resulted in the energy type and degradability being altered, which subsequently affected N utilisation.

Cows were housed in metabolism stalls allowing precise control of N intake and made it possible to quantitatively separate and recover all faeces and urine improving the accuracy of the experiment (Spek *et al.,* 2013). A 10 day acclimation period followed by a 7 day collection period allowed a large dataset to be generated and it was anticipated that this would allow relationships to be predicted more accurately. Faeces were collected through the use of faecal separators and a sample was collected every 24 hours to minimise NH_3 losses and prevent underestimation of faecal N (Spanghero and Kowalski, 1999). However, inaccuracies due to the urine collection and preservation

method resulted in underestimation of urinary N. Urine was collected in containers through the use of urine and faecal separators. Samples were taken from the bulk urine collected. It is recommended that a strong acid such as HCl or H_2SO_4 is added to urine to reduce pH to below 2, to minimise N losses (Knowlton *et al.,* 2010); however, using collection containers containing large volumes of urine resulted in the addition of large volumes of acid being impractical (Spanghero and Kowalski, 1997) and it presented a health and safety hazard for research staff. Based on the results in the current study, it was assumed that volatile losses of NH_3 from the collection containers as a result of enzyme-catalysed urea hydrolysis were the cause of the underestimated urinary N values. The resulting urinary N measurements emphasise the importance of minimising N losses from urine. In future experiments, an alternative method for measuring urine volume and urine N concentrations may be required. In the experiment conducted by Knowlton *et al.* (2010), where 3 urine collection methods (chilled, acidified before collection or acidified after 6 hours of collection) were compared, it was reported that all three methods did not affect urinary N or UUN concentration (P = 0.97 and 0.36, respectively) and they were equally effective in preserving N during urine collection. For future studies focused on understanding N partitioning, the method where urine is chilled by placing the collection containers in ice-filled foam coolers would be appropriate and avoids the hazard of acid use (Knowlton *et al.,* 2010). Alternatively, more frequent sampling should be undertaken (~every 6 hours) (Boucher *et al.,* 2007).

The study in Chapter 2 investigated the effect of urea intake on milk yield and analysed the relationship between the two variables, as well as the relationship between urea intake, DMI and milk yield. Milk yield decreased when urea supplementation increased beyond 350 g/day, and the interaction evident in milk yield was mirrored in yields of fat, CP and lactose. This study confirms that excessive urea intake has the potential to reduce milk yield in grazing dairy cows. Further research is needed to determine if high soluble NPN concentrations in fresh pasture would affect DMI and milk yield in the same way.

The study in Chapter 3 investigated N partitioning in grazing dairy cows and the effect of N intake on urinary N, faecal N and milk N. This study confirmed that increasing N intake resulted in linear increases in MUN, urinary N and UUN. Urinary N was strongly correlated with N intake, resulting in a 28% increase in urinary N excretion as dietary CP content increased from 18.4 to 22.6% of DM. A reduction in urinary N excretion and subsequent N leaching from dairy cows grazing pasture could be achieved by reducing N intake. This could be achieved by using moderate amounts of N fertiliser on pasture or extending the time interval between N fertiliser application and grazing however, this must have minimal or limited effects on milk production. Alternatively, if N intake cannot be

reduced, N utilisation must be improved by increasing productive output (N partitioned to milk). This will require further improvement of N-use efficiency through improved animal nutrition, breeding improved grasses and cow genetic selection (Cheng *et al.,* 2014).

Further research is needed to develop more robust prediction equations for the relationships between MUN and urinary N, and urinary N and N intake. These relationships could provide useful tools to predict urinary N excretion as a result of a particular dietary CP content due to the strong relationships between these variables, depending on the information available. Further research is needed to determine a robust prediction equation for the relationship between N intake and MUN in grazing dairy cows, if this relationship were to assess the dietary CP content of pasture on farm. In addition, further research must be undertaken to determine a robust prediction equation for the relationship between MUN and urinary N in grazing dairy cows, if this relationship were to assess urinary N output on farm. The data available for grazing dairy cows are limited and the relationships predicted in the current study have limited use due to the small range of data. There is a need for further research providing models to predict urinary N in grazing dairy cows and validating which of the relationships available in literature would be a better predictor of urinary N before they could be used as regulatory tools. Would MUN or N intake be a better predictor of urinary N?

APPENDICES

Appendix 1: Analytical procedures adapted from (Dairy One, 2014)

Dry matter

A. Oven - 60ºC for 4 hours (forced air).

1. Goering, H.K. and P.J. Van Soest. 1970. Forage Fiber Analyses (apparatus, reagents, procedures, and some applications). ARS/USDA Handbook No. 379, Superintendent of Documents, US Government Printing Office, Washington, D.C. 20402. p15.

2. NFTA Method 2.2.1.1 – Partial DM using Forced-air Drying Ovens.

B. Oven - 135ºC for 2 hours - AOAC 930.15 – Loss on Drying (Moisture) for Feeds.

C. Oven - 105ºC for 3 hours – NFTA Method 2.2.2.5 – Dry Matter by Oven Drying for 3hr at 105C.

D. Near Infrared Reflectance Spectroscopy (NIRS) - AOAC 991.01 – Moisture in Forage.

Protein

Crude protein

1. **Leco FP-528 Nitrogen/Protein Analyzer**

- AOAC 990.03 – Protein (Crude) in Animal Feed
- Leco Application Note "Nitrogen/Protein in Animal Feeds" Form 203-821-146, 07/03 Rev1.

Leco Corporation, 300 Lakeview Avenue, St. Joseph, MI 49085 **www.leco.com**

2. **Leco TruMac N Macro Determinator**

- AOAC 990.03 – Protein (Crude) in Animal Feed
- Leco Application Note "Nitrogen/Protein in Feeds, Grains, and Oil Seeds" Form 203-821-392, 09/10-Rev0.

Leco Corporation, 300 Lakeview Avenue, St. Joseph, MI 49085 **www.leco.com**

3. **NIRS - Foss NIRSystems Model 6500 with Win ISI II v1.5** – AOAC 989.03

Soluble protein

1. **Cornell Sodium Borate-Sodium Phosphate Buffer Procedure.**

- Soy products incubated at 39°C. All other samples incubated at ambient temperature.
- Residues analysed for CP using Leco TruMac N Macro Determinator.
- Cornell Nutrition Conference Proceedings, 1990, pp. 85-86.

2. **NIRS - Foss NIRSystems Model 6500 with Win ISI II v1.**5 - AOAC 989.03.

Fibre

Acid detergent fibre (ADF)

1. **ANKOM Technology Method 5 –** Acid Detergent Fiber in Feeds - Filter Bag Technique for A200 (4-13-11).
- Solutions as in AOAC 973.18 – Fiber (Acid Detergent) and Lignin (H2SO4) in Animal Feed.
- Samples individually weighed at 0.5g into filter bags and digested for 75 minutes as a group of 24 in 2L of ADF solution in ANKOM A200 Digestion Unit.
- Samples are rinsed three times with boiling water for 5 minutes in filter bags followed by a 3 minute acetone soak and drying at 100ºC for 2 hours.

ANKOM Technology, 2052 O'Neil Road, Macedon, NY 14502 **www.ankom.com**

2. **AOAC 973.18. Liquid samples.** Whatman 541 filter paper and buchner funnels.
3. NIRS - Foss NIRSystems Model 6500 with Win ISI II v1.5 - AOAC 989.03.

Neutral detergent fibre (NDF)

1. **ANKOM Technology Method 6 –** Neutral Detergent Fiber in Feeds - Filter Bag Technique for A200 (4-13-11).
- Solutions as in Van Soest, P.J., J.B. Robertson, and B.A. Lewis. 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. J.Dairy Science 74:3583-3597.
- Samples individually weighed at 0.5g into filter bags and digested for 75 minutes as a group of 24 in 2L of NDF solution in ANKOM A200 Digestion Unit.
- Four ml of Alpha Amylase and 20g sodium sulfite are added at the start of digestion.
- Samples are rinsed three times with boiling water for 5 minutes.
- Alpha Amylase is added to the first 2 rinses.
- Water rinses are followed by a 3 minute acetone soak and drying at 100ºC for 2 hours.

2. Journal of Dairy Science 74:3583 - 3597, 10/91. Liquid samples.

3. NIRS - Foss NIRSystems Model 6500 with Win ISI II v1.5 - AOAC 989.03.

Lignin

1. **ANKOM Technology Method 9** – Method for Determining Acid Detergent Lignin in the DaisyII Incubator – 04/11.

- Solution as in AOAC 973.18 – Fiber (Acid Detergent) and Lignin (H2SO4) in Animal Feed.
- ADF performed as in IV.A.1. ADF residue digested as a group of 24 in 72% w/w sulphuric acid for 3 hours in ANKOM DaisyII Incubator at ambient temperature.

2. **AOAC 973.18.** No asbestos.
3. **NIRS - Foss NIRSystems Model 6500 with Win ISI II v1.5** - AOAC 989.03.

Minerals

A. Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn, Mo, Co, S, Al, B, Cr, Sr

1. **Analyzed using a Thermo ICAP 6300 Inductively Coupled Plasma (ICP) Radial Spectrometer** after microwave digestion.

- CEM Microwave Accelerated Reaction System (MARS) with MarsXpress Temperature Control using 50ml calibrated Xpress Teflon PFA vessels with Kevlar/fiberglass insulating sleeves.
- Method utilized based upon CEM Application Notes for Acid Digestion on the following matrices - Feed Grain, Alfalfa, Corn Flour, Milk Powder, Soybean Meal, Flour, Hair, Potato Chips, Wheat Crackers, Peanut Butter, Urine, Dog Feces, Wine.
- Forage Lab Procedures Page 5 of 14 Sample weights – 0.5g for forages, ingredients, byproducts (1.0g for Co or Cr); 0.5g for grain mixes; 0.2g for mineral mixes.
- Samples first pre-digested at ambient temperature 10 minutes with 8ml nitric acid (HNO3) and 2ml hydrochloric acid (HCl) and then an additional 10 minutes with 1ml 30% hydrogen peroxide (H2O2).
- After pre-digestion complete, samples ramped to 190ºC in 15 minutes and finally held at digestion temperature of 190ºC for 15 minutes at 1600W.
- Vessels brought to 50-ml volume, aliquot used for analysis.

CEM, 3100 Smith Farm Road, Matthews, NC 28106 **www.cem.com** Thermo Fisher Scientific Inc., 81 Wyman Street, Waltham, MA 02454 **www.thermoscientific.com**

2. **NIRS - (Ca, P, Mg, K, S) - Foss NIRSystems Model 6500 with Win ISI II v1.5** - AOAC 989.03.

B. Chloride Ion (Cl^-)

1. **Brinkmann Metrohm 716 Titrino Titration Unit**

- 0.5g dried, ground sample extracted in 50ml 0.1N HNO3 followed by potentiometric titration with AgNO3 using Brinkmann Metrohm 716 Titrino Titration Unit with silver electrode.

- Metrohm Application Bulletin No. 130 by Metrohm Ltd., C-H-9101 Herisau, Switzerland.
- Metrohm USA, 6555 Pelican Creek Circle, Riverview Fl, 33578 **www.metrohmusa.com**
- The method by Metrohm is similar to the concepts found in: Cantliffe, D.J., Macdonald, G.E. and Peck, N.H. 1970. The potentiometric determination of nitrate and chloride in plant tissue. New York's Food and Life Sciences Bulletin. No.3, September 1970. Plant Sciences. Vegetable Crops Geneva. No. 1: 5-7.

2. **NIRS - Foss NIRSystems Model 6500 with Win ISI II v1.5** - AOAC 989.03.

Supplemental services

Ash

1. **AOAC Method 942.05** – Ash of Animal Feed.
2. **NIRS - Foss NIRSystems Model 6500 with Win ISI II v1.5** - AOAC 989.03.

Starch

1. **YSI 2700 SELECT Biochemistry Analyzer**

- YSI Incorporated Life Sciences, 1725 Brannum Lane, Yellow Springs, Ohio 45387 Application Note Number 319. **www.ysilifescience.com**
- Samples are pre-extracted for sugar by incubation in 40ºC water bath and filtration on Whatman 41 filter paper.
- Residues are thermally solubilized using an autoclave, then incubated with glucoamylase enzyme to hydrolyze starch to produce dextrose (glucose).
- Prepared samples injected into sample chamber of YSI Analyzer where dextrose diffuses into a membrane containing glucose oxidase.
- The dextrose is immediately oxidized to hydrogen peroxide and D-glucono-4-lactone.
- The hydrogen peroxide is detected amperometrically at the platinum electrode surface.
- The current flow at the electrode is directly proportional to the hydrogen peroxide concentration, and hence to the dextrose concentration. Starch is determined by multiplying dextrose by 0.9.

2. **NIRS - Foss NIRSystems Model 6500 with Win ISI II v1.5** - AOAC 989.03.

Appendix 2: Urea dosing schedule for Chapter 2

Table 18: Urea dosing schedule for the Medium and High treatments during the experimental period.

		High treatment			**High treatment**			**Medium treatment**		
No. cows		2			3			5		
	Dose time	0930	1300	1630	0930	1300	1630	0930	1300	1630
Date	**Day**	**Urea dosage, g**								
8/10/2009	1	44		44						
9/10/2009	2	44		44						
10/10/2009	3	44		44						
11/10/2009	4	44		44						
12/10/2009	5	88		88	44		44			
13/10/2009	6	88		88	44		44			
14/10/2009	7	88		88	44		44			
15/10/2009	8	117	117	117	44		44			
16/10/2009	9	117	117	117	88		88			
17/10/2009	10	117	117	117	88		88			
18/10/2009	11	117	117	117	88		88			
19/10/2009	12	150	150	150	117	117	117			
20/10/2009	13	150	150	150	117	117	117			
21/10/2009	14	150	150	150	117	117	117			
22/10/2009	15	200	200	200	117	117	117	44		44
23/10/2009	16	200	200	200	150	150	150	44		44
24/10/2009	17	200	200	200	150	150	150	44		44
25/10/2009	18	200	200	200	150	150	150	44		44
26/10/2009	19	230	230	230	150	150	150	88		88
27/10/2009	20	230	230	230	200	200	200	88		88
28/10/2009	21	230	230	230	200	200	200	88		88
29/10/2009	22	230	230	230	200	200	200	117	117	117
30/10/2009	23	230	230	230	230	230	230	117	117	117
31/10/2009	24	230	230	230	230	230	230	117	117	117
01/11/2009	25	230	230	230	230	230	230	117	117	117

High treatment (2 cows) = cow no. 2628 ad 3517; High treatment (3 cows) = cow no. 3950, 5643 and 8643

Appendix 3: Urea dosing schedules for Chapter 3

Table 19: Urea dosing schedule for the Urea supplement treatment during the acclimation period.

		Urea supplement		
	Dose time	0900	1300	1630
Date	**Day**		**Urea, g**	
22/10/2009	1	44		44
23/10/2009	2	44		44
24/10/2009	3	44		44
25/10/2009	4	44		44
26/10/2009	5	88		88
27/10/2009	6	88		88
28/10/2009	7	88		88
29/10/2009	8	117	117	117
30/10/2009	9	117	117	117
31/10/2009	10	117	117	117
01/11/2009	11	117	117	117

Table 20: Urea dosing schedule for the Urea supplement treatment during the metabolism stall and collection period.

		Urea supplement				
	Cow no.	17	4109	4110	4519	5206
Date	**Day**			**Total urea dose, g**		
2/11/2009	12	350	350	350	350	350
3/11/2009	13	350	350	350	350	350
4/11/2009	14	350	350	350	350	350
5/11/2009	15	350	350	350	350	350
6/11/2009	16	250	250	250	250	250
7/11/2009	17	250	250	250	250	250
8/11/2009	18	250	250	250	250	250
9/11/2009	19	250	250	250	250	250
10/11/2009	20	250	250	250	250	250
11/11/2009	21	250	250	250	250	250

Appendix 4: Additional data for Chapter 2

Table 21: Yield of milk (kg/day), milk components, pasture intake and pasture N content from dairy cows offered increasing amount of urea as a supplement to pasture during early lactation.

Item	Treatment	Period						
		1	2	3	4	5	SE	P - value
Milk a.m., kg/d	Control	14.8	14.8	16.2	16.0	15.9	0.396	<0.001
	Medium	15.7	15.7	17.4	16.4	17.7	0.438	<0.001
	High	14.2	13.5	14.2	12.3	9.6	0.391	<0.001
	SE	0.406	0.406	0.406	0.406	0.406		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
Milk p.m., kg/d	Control	7.38	6.26	7.82	7.44	8.39	0.361	<0.001
	Medium	6.26	6.06	7.53	7.10	7.09	0.398	<0.001
	High	6.63	5.50	6.07	5.64	4.12	0.355	<0.001
	SE	0.370	0.370	0.370	0.370	0.370		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
Milk N, kg/d	Control	0.12	0.11	0.13	0.13	0.12	0.038	<0.001
	Medium	0.12	0.11	0.14	0.13	0.13	0.041	<0.001
	High	0.11	0.10	0.10	0.09	0.06	0.037	<0.001
	SE	0.035	0.035	0.035	0.035	0.035		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
Milk composition, %								
Casein	Control	2.70	2.61	2.64	2.61	2.55	0.099	<0.001
	Medium	2.84	2.69	2.80	2.76	2.65	0.110	<0.001
	High	2.78	2.62	2.54	2.46	2.37	0.099	<0.001
	SE	0.102	0.102	0.102	0.102	0.102		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
Milk N	Control	0.54	0.52	0.53	0.52	0.50	0.021	<0.001
	Medium	0.57	0.54	0.57	0.56	0.53	0.021	<0.001
	High	0.54	0.52	0.51	0.50	0.48	0.023	<0.001
	SE	0.021	0.021	0.021	0.021	0.021		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
Intake								
Pasture N, g/d	Control	603	573	541	520	532	14.9	<0.001
	Medium	569	539	528	490	500	16.7	<0.001
	High	539	495	465	418	360	14.9	<0.001
	SE	15.4	15.4	15.4	15.4	15.4		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		
N content, g/kg DM	Control	34.7	36.0	31.4	30.9	32.0	0.543	<0.001
	Medium	34.7	36.0	31.9	34.6	41.4	0.607	<0.001
	High	36.4	42.2	43.1	49.9	59.9	0.543	<0.001
	SE	0.561	0.561	0.561	0.561	0.561		
	P - value	<0.001	<0.001	<0.001	<0.001	<0.001		

BIBLIOGRAPHY

Abdoun, K., Stumpff, F., and Martens, H. (2007). Ammonia and urea transport across the rumen epithelium: a review. *Animal Health Research Reviews* 7, 43-59.

Agle, M., Hristov, A. N., Zaman, S., Schneider, C., Ndegwa, P., and Vaddella, V. K. (2010). The effects of ruminally degraded protein on rumen fermentation and ammonia losses from manure in dairy cows. *Journal of Dairy Science* 93, 1625-1637.

Bach, A., Calsamiglia, S., and Stern, M. D. (2005). Nitrogen metabolism in the rumen. *Journal of Dairy Science* 88, E9-E21.

Ballantine, D., and Davies-Colley, R. J. (2014). Water quality trends in New Zealand Rivers: 1989-2009. *Environmental Monitoring and Assessment* 186, 1939-1950.

Bartley, E. E., Davidovich, A. D., Barr, G. W., Griffel, G. W., Dayton, A. D., Deyoe, C. W., and Bechtle, R. M. (1976). Ammonia toxicity in cattle. I. Rumen and blood changes associated with toxicity and treatment methods. *Journal of Animal Science* 43, 835-841.

Blaxter, K. L. (1989). *Energy metabolism in animals and man*. Cambridge, New York; Cambridge University Press.

Blethen, D. B., Wohlt, J. E., Jasaitis, D. K., and Evans, J. L. (1990). Feed protein fractions: relationship to nitrogen solubility and degradability. *Journal of Dairy Science* 73, 1544-1551.

Bonhomme, A. (1990). Rumen ciliates: their metabolism and relationships with bacteria and their hosts. *Animal Feed Science and Technology* 30, 203-266.

Boucher, S.E., Ordway, R.S., Whitehouse, N.L., Lundy, F.P., Kononoff, P.J., and Schwab, C.G. (2007). Effect of incremental urea supplementation of a conventional corn silage-based diet on ruminal ammonia concentration and synthesis of microbial protein. *Journal of Dairy Science* 90, 5619-5633.

Breves, G., and Wolffram, S. (2006). *Transport systems in the epithelia of the small and large intestines.* Wageningen, The Netherlands: Wageningen Academic Publishers.

Broderick, G. A. (2003). Effects of varying dietary protein and energy levels on the production of lactating dairy cows. *Journal of Dairy Science* 86, 1370-1381.

Bryant, R. H., Walpot, V., Dalley, D. E., Gibbs, S. J., and Edwards, G. R. (2010). Manipulating dietary nitrogen in perennial ryegrass pastures to reduce nitrogen losses in dairy cows in spring. *Proceedings of the 4th Australasian Dairy Science Symposium*, 97-100.

Buckthought, L.E., Clough, T.J., Cameron, K.C., Di, H.J., Shephard, M.A., 2015. Urine patch and fertiliser N interaction: Effects of fertiliser rate and season of urine application on nitrate leaching and pasture N uptake. *Agriculture, Ecosystems and Environment* 203, 19-28.

Burgos, S. A., Fadel, J. G., and Depeters, E. J. (2007). Prediction of ammonia emissions from dairy cattle manure based on milk urea nitrogen: relation of milk urea nitrogen to urine urea nitrogen excretion. *Journal of Dairy Science* 90, 5499-5508.

Burke, J. L., Waghorn, G. C., and Chaves, A. V. (2002). Improving animal performance using forage-based diets. *Proceedings of the New Zealand Society of Animal Production* 62, 267-272.

Burns, N. M., Rutherford, J. C., and Clayton, J. S. (1999). A monitoring and classification system for New Zealand Lakes and Reservoirs. *Journal of Lake and Reservoir Management* 15, 255-271.

Cabrita, A. R. J., Dewhurst, R., Abreu, J. M. F., and Fonsexa, A. J. M. (2006). Evaluation of the effects of synchronising the availability of N and energy on rumen function and production responses of dairy cows - a review. *Animal Research* 55, 1-24.

Calsamiglia, S., Ferret, A., Reynolds, C. K., Kristensen, N. B., and Van Vuuren, A. M. (2010). Strategies for optimizing nitrogen use by ruminants. *Animal* 4, 1184-1196.

Castillo, A. R. (1999). *Improving nitrogen utilisation in dairy cows.* (Unpublished doctoral dissertation). The University of Reading, Berkshire, United Kingdom.

Castillo, A. R., Kebreab, E., Beever, D. E., and France, J. (2000). A review of efficiency of nitrogen utilisation in lactating dairy cows and its relationship with environmental pollution. *Journal of Animal and Feed Science* 9, 1-32.

Chalupa, W., Baile, C. A., McLaughlin, C. L., and Brand, J. G. (1979). Effect of introduction of urea on feeding behaviour of Holstein heifers. *Journal of Dairy Science* 62, 1278-1284.

Chapman, D., McCarthy, S., and Kay, J. (2014). Hidden dollars in grazing management: Getting the most profit from your pastures. *Proceedings of the SIDE conference*, 21-36.

Chaves, A. V., Waghorn, G. C., Brookes, I. M., and Woodfield, D. R. (2006). Effect of maturation and initial harvest dates on the nutritive characteristics of ryegrass (*Lolium perenne* L.). *Animal Feed Science and Technology* 127, 293-318.

Cheng, L., Sheahan, A. J., Gibbs, S. J., Rius, A. G., Kay, J. K., Meier, S., Edwards, G. R., Dewhurst, R., and Roche, J. R. (2013). Technical note: Nitrogen isotope fractionation can be used to predict

nitrogen-use efficiency in dairy cows fed temperate pasture. *Journal of Animal Science* 91, 5785-5788.

Cheng, L., Woodward, S.J.R., Dewhurst, R., Zhou, H., Edwards, G.R., 2014. Nitrogen partitioning, energy use efficiency and isotopic fractionation measurements from cows differing in genetic merit fed low-quality pasture in late lactation. *Animal Production Science* 54, 1651-1656.

Choung, J. J. C., D. G., Thomas, P. C., and Bradbury, I. (1990). The effects of intraruminal infusions of urea on the voluntary intake and milk production of cows receiving grass silage diets. *Journal of Dairy Research* 57, 455-464.

Christensen, C. L., Hanly, J., Hedley, M. J., and Horne, D. (2011). *Nitrate leaching and pasture production from two years of duration-controlled grazing.* Palmerston North, New Zealand; Fertiliser and Lime Research Centre.

Clark, D. A. (1997). How much nitrogen is enough? *Proceedings of the Ruakura Farmers Conference* 49, 41-46.

Cocimano, M. R., and Leng, R. A. (1967). Metabolism of urea in sheep. British Journal of Nutrition 21, 353-371.

Corson, D. C., Waghorn, G. C., Ulyatt, M. J., and Lee, J. (1999). NIRS: Forage analysis and livestock feeding. Proceedings of the New Zealand Grassland Association 61, 127-132.

Cronje, P. (2000). *Ruminant physiology; digestion, metabolism, growth and reproduction.* New York, United States of America: Cabi Publishing.

Crosse, S., O'Farrell, K., and Dillion, P. (1994). Why calving date and compact calving are so important to profitable dairying. *Irish Grassland and Animal Production Association* 28, 3-8.

DairyNZ (2014). *Strategy for sustainable dairy farming 2013-2020*. Hamilton, New Zealand: Dairy New Zealand.

Dairy One (2014). *Analytical Procedures - Forage, Manure, Water*. Ithaca, United States of America: Dairy One.

Darwinkel, A., (1975). *Aspects of assimilation and accumulation of nitrate in some cultivated plants.* Wageningen, The Netherlands: Centre for Agricultural Publishing and Documentation.

Di, H. J., and Cameron, K. (2005). Reducing environmental impacts of agriculture by using a fine particle suspension nitrification inhibitor to decrease nitrate leaching from grazed pastures. *Agriculture, Ecosystems and Environment* 109, 202-212.

Dijkstra, J., Boer, H., van Bruchem, J., Bruining, M., and Tamminga, S. (1993). Absorption of volatile fatty acids from the rumen of lactating dairy cows as influenced by volatile fatty acid concentration, pH and rumen liquid volume. *British Journal of Nutrition* 69, 385-396.

Dijkstra, J., Oenema, O., van Groenigen, J. W., Spek, J. W., Van Vuuren, A. M., and Bannink, A. (2013). Diet effects on urine composition of cattle and N_2O emissions. *Animal* 7, 292-302.

Dillon, P., Crosse, S., and Stakelum, G. (1995). The effect of calving date and stocking rate on the performance of spring-calving dairy cows. *Grass and Forage Science* 50, 286-299.

Environment Canterbury. (2014). *Canterbury Land & Water Regional Plan*. Christchurch, New Zealand: Environment Canterbury Regional Council.

Fanchone, A., Noziere, P., Portelli, J., Duriot, B., Largeau, V., and Doreau, M. (2013). Effects of nitrogen underfeeding and energy source on nitrogen ruminal metabolism, digestion, and nitrogen partitioning in dairy cows. *Journal of Animal Science* 91, 895-906.

Fulkerson, W. J., and Donaghy, D. J. (2001). Plant-soluble carbohydrate reserves and senescence - key criteria for developing an effective grazing management system for ryegrass-based pastures: a review. Australian Journal of Agricultural Research 41, 261-275.

Gehman, A. M., Bertrand, J. A., Jenkins, T. C., and Pinkerton, B. W. (2004). The effect of carbohydrate source on nitrogen capture in dairy cows on pasture. *Journal of Dairy Science* 89, 2659-2667.

Gregg, P. E. H., Hedley, M. J., Cooper, A. B., and Cooke, J. (1993). Transfer of nutrients from grazed pastures to aquatic surface waters and their impact on water quality. *Proceedings of the New Zealand Fertiliser Manufacturer's Conference* 22, 64-75.

Gustafsson, A.H., and Palmquist, D.L., (1993). Diurnal variation of rumen ammonia, serum urea, and milk urea in dairy cows at high and low yields. *Journal of Dairy Science* 76, 475-484.

Hall, M. B., and Huntington, G. B. (2008). Nutrient synchrony: sound in theory, elusive in practice. *Journal of Animal Science* 86, E287-E292.

Haussinger, D. (1990). Nitrogen metabolism in liver: structural and functional organisation and physiological relevance. *Biochemistry Journal* 267, 281-290.

Haynes, R. J., and Williams, P. H. (1993). Nutrient cycling and soil fertility in the grazed pasture ecosystem. *Advances in Agronomy* 49, 119-199.

Heeren, J. A. H., Podesta, S. C., Hatew, B., Klop, G., van Laar, H., Bannink, A., Warner, D., and Dijkstra, J. (2014). Rumen degradation characteristics of ryegrass herbage and ryegrass silage are affected by interactions between stage of maturity and nitrogen fertilisation rate. *Animal Production Science* 54, 1263-1267.

Helmer, L. G., and Bartley, E. E. (1971). Progress in the utilisation of urea as a protein replacer for ruminants: a review. *Journal of Dairy Science* 54, 25-51.

Higgs, R. J., Sheahan, A. J., Mandok, K., Van Amburgh, M. E., and Roche, J. R. (2013). The effect of starch-, fiber-, or sugar-based supplements on nitrogen utilisation in grazing dairy cows. *Journal of Dairy Science* 96, 3857-3866.

Hoekstra, N. J., Schulte, R. P. O., Struik, P. C., and Lantinga, E. A. (2007). Pathways to improving the nitrogen efficiency of grazing bovines. *European Journal of Agronomy* 26, 363-374.

Hogan, J. P. (1961). The absorption of ammonia through the rumen of the sheep. *Australian Journal of Biological Science* 14, 448-460.

Holmes, C. W., Davey, A. W. F., and Grainger, C. (1981). The efficiency with which feed is utilised by the dairy cow. *Proceedings of the New Zealand Society of Animal Production* 41, 16-27.

Huhtanen, P., and Hristov, A. N. (2009). A meta-analysis of the effects of dietary protein concentration and degradability on milk protein yield and milk nitrogen efficiency in dairy cows. *Journal of Dairy Science* 92, 3222-32.

Ipharraguerre, I. R., and Clark, J. H. (2005). Impacts of the source and amount of crude protein on the intestinal supply of nitrogen fractions and performance of dairy cows. *Journal of Dairy Science* 88, E22-E37.

Jonker, J. S., Kohn, R. A., and Erdman, R. A. (1998). Using milk urea nitrogen to predict nitrogen excretion and utilization efficiency in lactating dairy cows. *Journal of Dairy Science* 81, 2681-2692.

Kalscheur, K. F., Baldwin, R. L., Glenn, B. P., and Kohn, R. A. (2006). Milk production of dairy cows fed differing concentrations of rumen-degraded protein. *Journal of Dairy Science* 89, 249-259.

Kang-Meznarich, J. H., and Broderick, G. A. (1980). Effect of incremental urea supplementation on ruminal ammonia concentration and bacterial protein formation. *Journal of Animal Science* **51,** 422-431.

Kauffman, A. J., and St-Pierre, N. R. (2001). The relationship of milk urea nitrogen to urine nitrogen excretion in Holstein and Jersey cows. *Journal of Dairy Science* 84, 2284-2294.

Kebreab, E., Castillo, A. R., Beever, D. E., Humphries, D. J., and France, J. (2000). Effects of management practices prior to and during ensiling and concentrate type on nitrogen utilisation in dairy cows. *Journal of Dairy Science* 83, 1274-1285.

Kebreab, E., France, J., Beever, D. E., and Castillo, A. R. (2001). Nitrogen pollution by dairy cows and its mitigation by dietary manipulation. *Nutrient Cycling in Agroecosystems* 60, 275-285.

Keim, J. P., and Anrique, R. (2011). Nutritional strategies to improve nitrogen use efficiency by grazing dairy cows. *Chilean Journal of Agricultural Research* 71, 623-633.

Kennedy, P. M., and Milligan, L. P. (1980). The degradation and utilisation of endogenous urea in the gastrointestinal tract of ruminants: a review. *Canadian Journal of Animal Science* 60, 205-221.

Kertz, A. F. (2010). Review: urea feeding to dairy cattle: A historical perspective and review. *The Professional Animal Scientist* 26, 257-272.

Kertz, A. F., Davidson, L. E., Cords, B. R., and Puch, H. C. (1983). Ruminal infusion of ammonium chloride in lactating cows to determine effect of pH on ammonia trapping. *Journal of Dairy Science* 66, 2597-2601.

Kertz, A. F., Koepke, M. K., Davidson, L. E., Betz, N. L., Norris, J. R., Skoch, L. V., Cords, B. R., and Hopkins, D. T. (1982). Factors influencing intake of high urea-containing rations by lactating dairy cows. *Journal of Dairy Science* 65, 587-604.

Knowlton, K. F., McGilliard, A. D., Zhao, Z., Hall, K. G., Mims, W., and Hanigan, M. D. (2010). Effect of nitrogen preservation during urine collection from Holstein heifers fed diets with high or low protein content. *Journal of Dairy Science* 93, 323-329.

Kolver, E. S., Muller, L. D., Varga, G. A., and Cassidy, T. J. (1998). Synchronisation of ruminal degradation of supplemental carbohydrate with pasture nitrogen in lactating dairy cows. *Journal of Dairy Science* 81, 2017-2028.

Lambert, M. G., Clark, D. A., and Litherland, A. J. (2004). Advances in pasture management for animal productivity and health. *New Zealand Veterinary Journal* 52, 311-319.

Lantinga, E. A., and Groot, J. C. J. (1996). Optimization of grassland production and herbage feed quality in an ecological context. Symposium p*roceeding of the Wageningen Institute of Animal Sciences* 84, 58-66.

Ledgard, S., Shils, R., Eriksen, J., and Luo, J. (2009). Environmental impacts of grazed clover/grass pastures. *Irish Journal of Agricultural and Food Research* 48, 209-226.

Leng, R. A., and Nolan, J. V. (1984). Symposium: protein nutrition of the lactating dairy cow. *Journal of Dairy Science* 67, 1072-1089.

LIC (1994). *New Zealand dairy statistics 1993-1994.* Hamilton, New Zealand: Livestock Improvement Corporation Limited.

LIC and DairyNZ (2013). *New Zealand dairy statistics 2012-13.* Hamilton, New Zealand: Livestock Improvement Corporation and Dairy New Zealand.

LIC and DairyNZ (2015). *New Zealand dairy statistics 2014-15.* Hamilton, New Zealand: Livestock Improvement Corporation and Dairy New Zealand.

MacDonald, K. A., Penno, J. W., Kolver, E. S., Carter, W. A., and Lancaster, J. A. S. (1998). Balancing pasture and maize silage diets for dairy cows using urea, soybean meal or fishmeal. *Proceedings of the New Zealand Society of Animal Production* 58, 102.

Macdonald, K. A., Penno, J. W., Lancaster, J. A., and Roche, J. R. (2008). Effect of stocking rate on pasture production, milk production, and reproduction of dairy cows in pasture-based systems. *Journal of Dairy Science* 91, 2151-2163.

Marini, J. C., Sands, J. M., and Van Amburgh, M. E. (2006). *Urea transporters and urea recycling in ruminants.* Wageningen, The Netherlands: Wageningen Academic Publishers.

Marini, J. C., and Van Amburgh, M. E. (2003). Nitrogen metabolism and recycling in Holstein heifers. *Journal of Animal Science* 81, 545-552.

Matthews, P. N. P., Harrington, K. C., and Hampton, J. G. (1999). *Management of grazing systems*. Auckland, New Zealand: Oxford University Press.

Mayne, C. S., McCoy, M. A., Lennox, S. D., Mackey, D. R., Verner, M., Catney, D. C., McCaughey, W. J., Wylie, A. R. G., Kennedy, B. W., and Gordon, F. J. (2002). Fertility of dairy cows in Northern Ireland. *Veterinary Record* 150, 707-713.

McDonald, P., Edwards, R. A., Greenhalgh, J. F. D., Morgan, C. A., Sinclair, L. A., and Wilkinson, R. G. (2011). *Animal Nutrition* (7th ed.). Harlow, New York: Prentice Hall/Pearson.

MFE (2014). *Climate Change Information.* Wellington, New Zealand: Ministry for the Environment (MFE).

MFE (2013). *Freshwater reform 2013 and beyond*. Wellington, New Zealand: Ministry for the Environment (MFE).

Moller, S., Edwards, N. J., Parker, W. J., Hodgson, J., and Wilson, G. F. (1996). Nitrogen application to dairy pasture - the effect of rate and timing of spring nitrogen applications on the concentration of pasture nutrients. *Proceedings of the New Zealand Society of Animal Production* 56, 276-279.

Monaghan, R. M., Paton, R. J., Smith, L. C., Drewry, J. J., and Littlejohn, R. P. (2005). The impacts of nitrogen fertilisation and increased stocking rate on pasture yield, soil physical condition and nutrient losses in drainage from a cattle-grazed pasture. *New Zealand Journal of Agricultural Research* 48, 227-240.

Nicol, A. M., and Brookes, I. M. (2007). *The metabolisable energy requirements of grazing livestock.* Christchurch, New Zealand: New Zealand Society of Animal Production (Inc.).

Nocek, J. E., and Russell, J. B. (1988). Protein and energy as an integrated system. Relationship of ruminal protein and carbohydrate availability to microbial protein synthesis and milk production. *Journal of Dairy Science* 71, 2070-2107.

Nousiainen, J., Shingfield, K. J., and Huhtanen, P. (2004). Evaluation of milk urea nitrogen as a diagnostic of protein feeding. *Journal of Dairy Science* 87, 386-398.

NPS-FM (2014). *National Policy Statement for Freshwater Management 2014*. Wellington, New Zealand: Ministry for the Environment (MFE).

NRC (2001). *Nutrient requirements of dairy cattle* (7th rev. ed.). Washington, United State of America: National Academy Press.

NRC (1985). *Ruminant nitrogen usage*. Washington, United State of America: National Academy Press.

Olmos Colmenero, J. J., and Broderick, G. A. (2006). Effect of dietary crude protein concentration on milk production and nitrogen utilisation in lactating dairy cows. Journal of Dairy Science 89, 1704-1712.

Oudshoorn, F. W., Cornou, C., Hellwing, A. L. F., Hansen, H. H., Munksgaard, L., Lund, P., and Kristensen, T. (2013). Estimation of grass intake on pasture for dairy cows using tightly and loosely mounted di- and tri-axial accelerometers combined with bite count. *Computers and Electronics in Agriculture* 99, 227-235.

Pacheco, D., Burke, J. L., Death, A. F., and Cosgrove, G. P. (2007). Comparison of models for estimation of urinary nitrogen excretion from dairy cows fed fresh forages. *Proceedings of the Australasian Dairy Science Symposium,* 417-422.

Pakrou, N., and Dillon, P. (2000). Key processes on the nitrogen cycle in an irrigated and non-irrigated grazed pasture. *Plant and Soil* 224, 231-250.

PCE (2004). *Growing for good: Intensive farming, sustainability and New Zealand's environment*. Wellington, New Zealand: Parliamentary Commissioner for the Environment (PCE).

PCE (2013). *Water quality in New Zealand: land use and nutrient pollution*. Wellington, New Zealand: Parliamentary Commissioner for the Environment (PCE).

Penno, J. W., and Kolver, E. S. (2000). Future farm systems. *Proceedings of the Ruakura Farmers Conference* 50, 1-14.

Petersen, S. O., Sommer, S. G., Aaes, O., and Soegaard, K. (1998). Ammonia losses from urine and dung of grazing cattle: effect of N intake. *Atmospheric Environment* 32, 295-300.

Peyraud, J. L., and Astigarraga, L. (1998). Review of the effect of nitrogen fertilisation on the chemical composition, intake, digestion and nutritive value of fresh herbage: consequences on animal nutrition and nitrogen balance. *Animal Feed Science and Technology* 72, 235-259.

Pisulewski, P. M., Okorie, A. U., Buttery, P. J., Haresign, W., and Lewis, D. (1981). Ammonia concentration and protein synthesis in the rumen. *Journal of the Science of Food and Agriculture* 32, 759-766.

Polan, C. E., Miller, C. N., and McGilliard, M. L. (1976). Variable dietary protein and urea for intake and production in Holstein cows. *Journal of Dairy Science* 59, 1910-1914.

Qiu, W., Di, H. J., Cameron, K., and Hu, C. (2010). Nitrous oxide emissions from animal urine as affected by season and a nitrification inhibitor dicyandiamide. *Journal of Soils and Sediments* 10, 1229-1235.

Ramsbottom, G., Horan, B., Berry, D. P., and Roche, J. R. (2015). Factors associated with the financial performance of spring-calving, pasture-based dairy farms. *Journal of Dairy Science* 98, 3526-3540.

Recktenwald, E. B., Ross, D. A., Fessenden, S. W., Wall, C. J., and Van Amburgh, M. E. (2014). Urea-N recycling in lactating dairy cows fed diets with 2 different levels of dietary crude protein and starch with or without monensin. *Journal of Dairy Science* 97, 1611-1622.

Reid, J. T. (1953). Urea as a protein replacement for ruminants: a review. Journal of Dairy Science 36, 955-996.

Reynal, S. M., Broderick, G. A., Ahvenjarvi, S., and Huhtanen, P. (2003). Effect of feeding protein supplements of differing degradability on omasal flow of microbial and undegraded protein. *Journal of Dairy Science* 86, 1292-1305.

Reynolds, C. K., and Kristensen, N. B. (2008). Nitrogen recycling through the gut and the nitrogen economy of ruminants: an asynchronous symbiosis. *Journal of Animal Science* 86, E293-305.

Roche, J. R., Berry, D. P., and Kolver, E. S. (2006). Holstein-Friesian strain and feed effects on milk production, body weight, and body condition score profiles in grazing dairy cows. *Journal of Dairy Science* 89, 3532-3543.

Roche, J. R., Dillon, P., Stockdale, C. R., Baumgard, L. H., and VanBaale, M. J. (2004). Relationships among international body condition scoring systems. *Journal of Dairy Science* 87, 3076-3079.

Roche, J. R., Turner, L. R., Lee, J. M., Edmeades, D. C., Donaghy, D. J., Macdonald, K. A., Penno, J. W., and Berry, D. P. (2009). Weather, herbage quality and milk production in pastoral systems. 2. Temporal patterns and intra-relationships in herbage quality and mineral concentration parameters. *Animal Production Science* 49, 200-210.

Romagnolo, D., Polan, C. E., and Barbeau, W. E. (1994). Electrophoretic analysis of ruminal degradability of corn proteins. *Journal of Dairy Science* 77, 1093-1099.

Ruiz, R., Tedeschi, L. O., Marini, J. C., Fox, D. G., Pell, A. A., Jarvis, G., and Russell, J. B. (2002). The effect of a ruminal nitrogen deficiency in dairy cows: evaluation of the Cornell Net Carbohydrate and Protein System ruminal nitrogen deficiency adjustment. *Journal of Dairy Science* 85, 2986-2999.

Russell, J. B., O'Connor, J. D., Fox, D. G., Van Soest, P. J., and Sniffen, C. J. (1992). A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. *Journal of Animal Science* 70, 3551-3561.

Satter, L. D., and Slyter, L. L. (1974). Effect of ammonia concentration on rumen microbial protein production in vitro. *British Journal of Nutrition* 32, 199-208.

Selbie, D. R., Buckthought, L. E., and Shepherd, M. A. (2015). The challenge of the urine patch for managing nitrogen in grazed pasture systems. *Advances in Agronomy* 129, 229-292.

Scarsbrook, M., and Melland, A. (2015). Dairying and water quality issues in Australia and New Zealand. *Animal Production Science* 55, 856-868.

Sheahan, A. J., Kolver, E. S., and Roche, J. R. (2011). Genetic strain and diet effect on grazing behaviour, pasture intake and milk production. *Journal of Dairy Science* 94, 3583-3591.

Sniffen, C. J., O'Connor, J. D., Van Soest, P. J., Fox, D. G., and Russel, J. B. (1992). A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. *Journal of Animal Science* 70, 3562-3577.

Spanghero, M., and Kowalski, Z. M. (1997). Critical analysis of N balance experiments with lactating cows. *Livestock Production Science* 52, 113-122.

Spek, J. W., Bannink, A., Gort, G., Hendriks, W. H., and Dijkstra, J. (2013). Interaction between dietary content of protein and sodium chloride on milk urea concentration, urinary urea excretion, renal recycling of urea, and urea transfer to the gastrointestinal tract in dairy cows. *Journal of Dairy Science* 96, 5734-45.

Statistics NZ (2012). *Infoshare.* Wellington, New Zealand: Statistics New Zealand.

Susmel, P., Spanghero, M., Stefanon, B., and Mills, C. R. (1995). Nitrogen balance and partitioning of some nitrogen catabolites in milk and urine of lactating cows. *Livestock Production Science* 44, 207-219.

Tamminga, S. (1979). Protein degradation in the forestomachs of ruminants. *Journal of Animal Science* 49, 1615-1630.

Tamminga, S. (1992). Nutrition management of dairy cows as a contribution to pollution control. *Journal of Dairy Science* 75, 345-357.

Tamminga, S. (1996). A review on environmental impacts of nutritional strategies in ruminants. *Journal of Animal Science* 74, 3112-3124.

Tamminga, S. (1986). Utilization of naturally occurring non-protein nitrogen compounds by ruminants. *Archives of Animal Nutrition* 35, 169-175.

Thomson, N. A., and Barnes, M. L. (1993). Effect of distance walked on dairy production and milk quality. *Proceedings of the New Zealand Society of Animal Production* 53, 69-72.

Ulyatt, M. J. (1997). Can protein utilisation from pasture be improved? *Proceedings of the New Zealand Society of Animal Production* 57, 4-8.

United Nations (2014). *United Nations Comtrade Database.* New York, United States of America: United Nations.

Van Horn, H. H., Foreman, C. F., and Rodriguez, J. E. (1967). Effect of high-urea supplementation on feed intake and milk production of dairy cows. *Journal of Dairy Science* 50, 709-714.

Van Vuuren, A. M., Krol-Kamer, F., Van Der Lee, R. A., and Corbijn, H. (1992). Protein digestion and intestinal amino acids in dairy cows fed fresh *Lolium perenne* with different nitrogen contents. *Journal of Dairy Science* 75, 2215-2225.

Van Vuuren, A. M., Tamminga, S., and Ketelaar, R. S. (1991). In sacco degradation of organic matter and crude protein of fresh grass (*Lolium perenne*) in the rumen of grazing dairy cows. *Journal of Agricultural Science* 116, 429-436.

Verburg, P., Hamill, K., Unwin, M., and Abell, J. (2010). *Lake water quality in New Zealand 2010: Status and trends*. (NIWA Client Report no. HAM2010-107). Wellington, New Zealand: Ministry for the Environment.

Vibart, R. E., Koolaard, J., Barrett, B. A., and Pacheco, D. (2009). Exploring the relationships between plant chemical composition and nitrogen partitioning in lactating dairy cows fed ryegrass-based diets. *Proceedings of the New Zealand Society of Animal Production* 69, 188-195.

Visek, W. J. (1968). Some aspects of ammonia toxicity in animal cells. *Journal of Dairy Science* 51, 286-294.

Visek, W. J. (1984). Ammonia: Its effects on biological systems, metabolic hormones, and reproduction. *Journal of Dairy Science* 67, 481-498.

Waldo, D. R. (1968). Symposium: nitrogen utilization by the ruminant nitrogen metabolism in the ruminant. *Journal of Dairy Science* 68, 265-275.

Wheadon, N. W., Cheng, L., Dewhurst, R., and Edwards, G. R. (2013). Brief communication: estimation of protein utilisation efficiency and metabolisable protein efficiency in lactating cows grouped by breeding worth. *Proceedings of the New Zealand Society of Animal Production* 73, 100-102.

Whitehead, D. C. (1995). *Grassland Nitrogen*. Wallingford, United Kingdom: CAB International.

Whitehead, D. C., and Raistrick, N. (1993). Nitrogen in the excreta of dairy cattle: changes during short-term storage. *Journal of Agricultural Science* 121, 73-81.

Wilman, D. (1965). The effect of nitrogenous fertiliser on the rate of growth of Italian ryegrass. *Journal of the British Grassland Society* 20, 248-254.

Wilcock, R. J., Monaghan, R. M., Quinn, J. M., Srinivasan, M. S., Houlbrooke, D. J., Duncan, M. J., Wright-Stow, A. E., and Scarsbrook, M. R. (2013). Trends in water quality of fiver dairy farming streams in response to adoption of best practice and benefits of long-term monitoring at the catchment scale. *Marine and Freshwater Research* 64, 401-412.

Wilson, G. F., Martz, F. A., Campell, J. R., and Becker, B. A. (1975). Evaluation of factors responsible for reduced voluntary intake of urea diets for ruminants. *Journal of Animal Science* 41, 1431-1437.

Word, J. D., Martin, L. C., Williams, D. L., Williams, E. I., Panciera, R. J., Nelson, T. E., and Tillman, A. D. (1969). Urea toxicity studies in the bovine. *Journal of Animal Science* 29, 786-791.

Zhu, L. H., Armentano, L., Bremmer, D. R., Grummer, R. R., and Bertics, S. J. (2000). Plasma concentration of urea, ammonia, glutamine around calving, and the relation of hepatic triglyceride, to plasma ammonia removal and blood acid-base balance. *Journal of Dairy Science* 83, 734-740.