Can Naturally Occurring Glucosinolate Related Compounds from Brassica Crops Act As Biological Nitrification Inhibitors and Reduce Nitrous Oxide Emissions?

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SHEREE BALVERT

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Abstract

Nitrous oxide (N_2O) is problematic as it is a potent greenhouse gas with a global warming potential about 298 times that of carbon dioxide, and it also contributes to the depletion of stratospheric ozone. The use of nitrification inhibitors to reduce nitrification, and consequently N_2O emissions, has been extensively researched. Much of this work was carried out using synthetic inhibitors and these inhibitors have proved to be very effective. Some plants also have the ability to inhibit nitrification through release of secondary metabolites produced in plant tissues. This is termed biological nitrification inhibition (BNI). Brassica crops are considered plants with potential biological nitrification inhibitors, as they contain glucosinolates (GLS) whose hydrolysis products (e.g. isothiocyanates and nitriles) have been shown to reduce soil nitrification. Brassicas may, therefore, provide a practical forage based tool for mitigating nitrification and N_2O emissions. The aim of this study was to determine whether brassicas and GLS hydrolysis products inhibited nitrification, and whether N_2O emissions were reduced as a result of that inhibition. This aim was achieved through a series of laboratory incubations and a field trial that sequentially tested whether GLS hydrolysis products, brassicas, or urine derived from cows fed brassicas, reduced nitrification and N_2O emissions.

The first study was a laboratory incubation where urea (600 μg N g^{-1} soil) along with a selection of GLS hydrolysis products (at 2 rates: 30 and 60 μg N g^{-1} soil) were applied to soil. Ammonia oxidising bacteria populations, soil mineral N concentrations, N_2O emissions and soil respiration were monitored throughout the 40 day incubation. The results showed that some GLS hydrolysis products inhibited soil nitrification and reduced N_2O emissions by up to 51%. The effective products identified in the laboratory study were then tested in a field using a small plot trial. Artificial urine (600 kg N ha^{-1}; 10 L m^2) and GLS hydrolysis products (60 kg ha^{-1}) were applied to the plots, and N mineralisation and N_2O emissions were measured. No inhibition of nitrification or reduction in N_2O emissions were observed in the field study. In the laboratory study, there was evidence that the reduction in N_2O emissions was a result of inhibition of nitrification, however, the results suggested that the inhibition by GLS hydrolysis products was not strong and was short-lived.

Multiple applications may therefore be required to achieve a meaningful reduction in N_2O emissions from urine affected soil.

The second study examined whether brassica tissues incorporated into soil inhibited nitrification and reduced N_2O emissions. A laboratory incubation was conducted where three types of brassica tissue and ryegrass (as a control) were incorporated into soil with urea added (600 μg N g^{-1} soil). Ammonia oxidising bacteria populations, soil mineral N concentrations, N_2O emissions and soil respiration were monitored throughout the 52 day incubation. The results showed that incorporation of brassica tissues reduced urea-derived N_2O emissions, relative to ryegrass tissues, by up to 62%. However, there was no evidence that this reduction was a result of inhibition of nitrification, rather it was likely due to the difference in labile C provided by the different plants. While there was a reduction in urea-derived N_2O emissions, total N_2O emissions increased as a result of tissue incorporation into soil and this trade-off must be investigated if brassica tissues are considered as an option for N_2O reduction.

The final study examined whether the BNI capacity of GLS hydrolysis products was transferred into the urine of cows grazing brassica crops and subsequently reduced N_2O emissions from the deposited urine patch. A secondary objective was to determine if soil growing brassica crops contained BNI compounds that decreased N_2O emissions following addition of urine. These were addressed in a laboratory incubation where urine (600 kg N ha^{-1}) derived from animals fed on pasture or a kale crop, was applied to soils growing either pasture or a kale crop. Ammonia oxidising bacteria populations, soil mineral N concentrations, N_2O emissions and soil respiration were monitored throughout the 60 day incubation. Urine from cows fed kale did not show decreased N_2O emissions compared to urine from cows fed on pasture when applied to soil. N_2O emissions were higher from the kale-cropped soil than the pasture soil, which was attributed to the higher fertility status of the cropped soil. Overall, these results provided no evidence that feeding kale to grazing animals reduced nitrification rates or N_2O production following urine inputs to soil.

In summary, this study showed that GLS hydrolysis products exhibit BNI capacity when applied directly to soil which is in agreement with other published studies. Glucosinolate hydrolysis products also reduced N_2O emissions when applied to soil

with urea. There was no evidence that BNI capacity remained following decomposition of brassica tissues incorporated into soil or when brassica derived cow urine was applied to soil. Other literature investigating the incorporation of brassica tissues into soil generally supports BNI activity, however, those studies have all been under low N conditions. Although urea-derived N_2O emissions were reduced following incorporation of brassica tissues into soil, this reduction in emissions could not be attributed to inhibition of nitrification. The impact of brassica fed urine on soil nitrification and N_2O emissions remains inconclusive. Further work examining the mechanism by which GLS hydrolysis products inhibit nitrification, and how that may be exploited, is required to determine whether GLS hydrolysis products or brassicas may be a useful N_2O mitigation tool.

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Chapter 1

Introduction

1.1 Introduction

Nitrogen (N) is a naturally occurring element that is an essential nutrient for the growth and reproduction of living organisms. Most N resides in the atmosphere as the unreactive dinitrogen (N_2) and is unavailable to most organisms. Naturally, the majority of N added to the plant-soil system, is provided through biological fixation of atmospheric N (e.g. Haynes, 1986; Robertson & Vitousek, 2009). The invention and utilisation of nitrogenous fertilisers have allowed for the increased food production needed to feed the world's growing population, however, this has also resulted in an increase of reactive N in the biosphere. Excess reactive N has many environmental consequences, which threatens air and water quality, biological diversity, and human health (Galloway *et al.*, 2004; Bouwman *et al.*, 2009). Agricultural systems are particularly 'leaky' with regard to N, and losses of N from these systems represent a loss of potential production as well as a significant threat to the environment. In a New Zealand context, losses of N from agriculture particularly impacts water quality via the loss of nitrate to waterways, and contribute to greenhouse gas emissions (Selbie *et al.*, 2015) as agricultural N is the predominant source of nitrous oxide (N_2O) production (Ministry for the Environment, 2017).

Nitrous oxide is a naturally occurring trace gas found in the atmosphere. It is a potent greenhouse gas with a global warming potential about 298 times that of CO_2 (l00 year time span) (Myhre *et al.*, 2013), and significantly contributes to the depletion of stratospheric ozone (Ravishankara *et al.*, 2009). Nitrous oxide is biologically produced during the cycling of N in soil, particularly from the microbial processes of nitrification and denitrification (Firestone & Davidson, 1989). There has been considerable research on the contribution of N_2O emissions from agricultural systems both in New Zealand and internationally to greenhouse gas emissions (e.g. Cardenas *et al.*, 2013; Schils *et al.*, 2013; Krol *et al.*, 2016; Luo *et al.*, 2017). Ruminants, that dominate New Zealand agricultural land, are inefficient users of dietary N and consume more N than they need for growth and

production. As a result, up to 95% of ingested N is excreted in urine and dung (Oenema *et al.*, 2005) and once deposited on soil, contribute to N_2O production and emissions (de Klein & Ledgard, 2005). The amount of N excreted in urine is dependent on plant and dietary N content. Generally, as dietary N increases, the amount of N excreted in urine also increases (Luo & Kelliher, 2010). In New Zealand, about 10% of the national greenhouse gas production can be attributed to N_2O emissions derived from deposited excreta (Ministry for the Environment, 2017). Identifying approaches that decrease this production is a key strategy for reducing New Zealand's national greenhouse gas emissions.

During the last decade there has been a significant focus on the use of the nitrification inhibitors to reduce N_2O emissions from urine patches (e.g. de Klein *et al.*, 2011; Di & Cameron, 2011; Misselbrook *et al.*, 2014; Luo *et al.*, 2015a). Dicyandiamide (DCD), for example, has been shown to reduce emissions from urine patches by an average of 57% (de Klein *et al.*, 2011; Di & Cameron, 2011; Luo *et al.*, 2013). Work on DCD and other synthetically produced inhibitors was halted in 2013 when residues were found in milk powder that could be of concern to consumers. Consequently, alternative naturally occurring inhibitors (also known as biological nitrification inhibitors; BNIs) are now being sought. Preliminary research has suggested grazing of diverse pastures and forages reduced N_2O emissions (Di *et al.*, 2016), which may be caused by inhibition of N cycling processes when secondary metabolites of these plants are excreted in urine patches and deposited onto soil (e.g. Prasad & Power, 1995; Ryan *et al.*, 2006). Brassica crops are one such potential BNI option as these plants contain high concentrations of glucosinolates (GLS) in plant tissues whose hydrolysis products (isothiocyanates, thiocyanates and nitriles) have been shown to inhibit soil nitrification processes (e.g. Bending & Lincoln, 2000; Reardon *et al.*, 2013) and potentially N_2O emissions. Glucosinolate hydrolysis products are excreted in the urine of animals that consume brassicas (Duncan & Milne, 2007) and so may provide a practical forage based tool for mitigating N_2O emissions targeted to urine patches and could be applied to New Zealand pastoral grazing systems.

The overarching hypothesis of this thesis was:

Naturally occurring glucosinolate derivatives from brassica crops act as biological nitrification inhibitors and reduce nitrous oxide emissions from urine patches.

1.1.1 Objective 1

Several studies have shown that some GLS hydrolysis products inhibit nitrifying bacteria and nitrification when applied to soil (Bending & Lincoln, 2000; Brown & Morra, 2009; O'Sullivan *et al.*, 2016). However, there have been no studies carried out on soils receiving high N inputs (e.g. urine deposition), or that assessed whether inhibition of nitrification would also reduce N_2O emissions. Therefore, this objective firstly addresses whether GLS hydrolysis products inhibit nitrification and N_2O production under high N loadings, whether that inhibition is standard across all hydrolysis products, and whether any synergistic effects accrue with simultaneous addition of several hydrolysis products.

Therefore, the first key objective of this thesis was:

1. *To determine whether secondary metabolites from brassica forage crops (specifically, glucosinolate and its hydrolysis products) have an inhibitory effect on soil nitrification and N_2O emissions when applied to soil with high N inputs.*

This objective was addressed firstly in a laboratory incubation study, and then with a follow-up plot field study. A laboratory incubation allowed for comparison of products under controlled conditions, and is relatively inexpensive allowing for more treatments and replication. N_2O and soil mineral N dynamics were measured following addition of urea solution at a concentration of 600 μg g^{-1} soil, and GLS hydrolysis products to soil. Incubated soils were also subsampled for determination of variation in ammonia oxidising bacteria populations under the different treatments via quantitative polymerase chain reaction (PCR). The most successful products were then assessed in a field study. The field study was carried out on two soil types, one free draining and one poorly drained, and artificial cattle urine was used as the N source. A static chamber method was employed for measurement of N_2O emissions, and soil mineral N dynamics were monitored.

1.1.2 Objective 2

Secondly, there are several reports of inhibition of soil N cycling following incorporation of brassica tissues into soil, which was attributed to GLS hydrolysis products released upon the break-down of the tissues (e.g. Brown & Morra, 2009; Snyder *et al.*, 2010). Again, these previous studies were all carried out on soils with relatively low N inputs. Velthof *et al.* (2002) was the only identified study that had examined the effect of tissue incorporation on N_2O production. However, this was also under low N conditions and N was applied as NO_3^- which did not allow for assessment of nitrification inhibition properties.

Therefore, the second key objective of this thesis was:

2. *To determine the effects of incorporating brassica crop tissues into soil on subsequent nitrification and nitrous oxide emissions under high N loadings.*

This objective was addressed in a laboratory incubation study which allowed for comparison of products under controlled conditions, and is relatively inexpensive allowing for more treatments and replication. Tissues from brassica crops and ryegrass were collected, finely chopped, and incorporated into soil with or without the addition of urea solution at a concentration of 600 μg g^{-1} soil. N_2O emissions and soil mineral N dynamics were subsequently measured. Comparison of N_2O production from soils both with and without urea allowed for distinguishing between urea N derived emissions and those derived from added plant N. Incubated soils were also subsampled for determination of variation in ammonia oxidising bacteria populations under the different treatments via quantitative PCR.

1.1.3 Objective 3

Only two studies have been published comparing the N_2O emissions from urine of animals fed brassicas with those of urine from animals fed on a traditional pasture based diet (Luo *et al.*, 2015b; Hoogendoorn *et al.*, 2016). One of these studies (Luo *et al.*, 2015b) showed a significant reduction in N_2O emissions from urine derived from animals fed on a brassica diet. The second study (Hoogendoorn *et al.*, 2016), however, showed opposing results with no reduction in N_2O emissions. The

objective of the final section of this thesis was, therefore, to assess, under controlled laboratory conditions, whether urine from animals fed on a predominantly brassica diet applied to soil, resulted in lower N_2O emissions than that from animals fed on a predominantly ryegrass/clover pasture diet.

The third key objective of this thesis was therefore:

3. *To determine whether urine from animals fed on a predominantly brassica diet applied to soil resulted in lower N_2O emissions than urine from animals fed on a predominantly ryegrass/clover pasture diet.*

This objective was also addressed in an incubation study. Urine was obtained from animals fed on the different diets and was applied to soil at consistent rates of N. Comparing using a consistent rate of N eliminated the factor of differing urinary N concentrations and the uncertainty around the effect of N concentration on emission factors. A smaller component of this study investigated whether there was a compounding effect of applying brassica fed urine to soil collected from beneath growing brassica crops. N_2O and soil mineral N dynamics were measured following urine application to the different soils. Incubated soils were also subsampled for determination of variation in ammonia oxidising bacteria populations under the different treatments via quantitative PCR.

1.2 Thesis structure

This thesis consists of a series of novel experiments that were designed to test the above hypothesis and is structured into six chapters, with this being the introductory chapter. The second chapter is a literature review summarising the current knowledge of N dynamics and N_2O emissions, and evidence for the potential of plant secondary metabolites to be used as an inhibitor of nitrification and N_2O mitigation tool. Parts of this literature review focussed on dietary manipulation as a tool for reducing N_2O emissions and contributed to a book chapter in "*Greenhouse gas emissions and nitrogen losses from grazed dairy and animal housing systems*" edited by J. Luo and Y. Li. The full document has been included in the Appendices for reference (Appendix A).

The main research findings are presented as stand-alone chapters in manuscript form and address Objectives 1, 2 and 3 above, respectively. As a consequence of this format there is some repetition in the introductions, methods and reference lists. Titles of the research chapters are as follows:

Chapter 3: Balvert, S. F., Luo, J., & Schipper, L. A. (2017). Do glucosinolate hydrolysis products reduce nitrous oxide emissions from urine affected soil? *Science of the Total Environment, 603*, 370-380.

Chapter 4: Can incorporating brassica tissues into soil reduce nitrification rates and nitrous oxide emissions?
Journal of Environmental Quality, doi:10.2134/jeq2018.04.0143

Chapter 5: Are nitrous oxide emissions from soil lower when amended with urine from brassica fed cows than pasture fed cows?

Chapter 6 concludes by summarising the results of the three main research chapters, as well as providing some discussion and concluding remarks on whether naturally occurring GLS related compounds from brassica crops can act as biological nitrification inhibitors and reduce N_2O emissions.

Further information is included in the appendices, such as, author contribution information for the research chapters (Appendix B).

1.3 References

Bending, G. D., & Lincoln, S. D. (2000). Inhibition of soil nitrifying bacteria communities and their activities by glucosinolate hydrolysis products. *Soil Biology and Biochemistry, 32*, 1261-1269.

Bouwman, A. F., Beusen, A. H. W., & Billen, G. (2009). Human alteration of the global nitrogen and phosphorus soil balances for the period 1970–2050. *Global Biogeochemical Cycles, 23*, GB0A04.

Brown, P. D., & Morra, M. J. (2009). Brassicaceae tissues as inhibbitors of nitrification in soil. *Journal of Agricultural and Food Chemistry, 57*, 7706-7711.

Cardenas, L. M., Gooday, R., Brown, L., Scholefield, D., Cuttle, S., Gilhespy, S., Matthews, R., Misselbrook, T., Wang, J., Li, C., Hughes, G., & Lord, E. (2013). Towards an improved inventory of N2O from agriculture: Model evaluation of N_2O emission factors and N fraction leached from different sources in UK agriculture. *Atmospheric Environment, 79*, 340-348.

de Klein, C. A. M., Cameron, K. C., Di, H. J., Rys, G., Monaghan, R. M., & Sherlock, R. R. (2011). Repeated annual use of the nitrification inhibitor dicyandiamide (DCD) does not alter its effectiveness in reducing N_2O emissions from cow urine. *Animal Feed Science and Technology, 166–167*, 480-491.

de Klein, C. M., & Ledgard, S. (2005). Nitrous oxide emissions from New Zealand agriculture – key sources and mitigation strategies. *Nutrient Cycling in Agroecosystems, 72*, 77-85.

Di, H. J., & Cameron, K. C. (2011). How does the application of different nitrification inhibitors affect nitrous oxide emissions and nitrate leaching from cow urine in grazed pastures? *Soil Use and Management, 28*, 54-61.

Di, H. J., Cameron, K. C., Podolyan, A., Edwards, G. R., de Klein, C. A. M., Dynes, R., & Woods, R. (2016). The potential of using alternative pastures, forage crops and gibberellic acid to mitigate nitrous oxide emissions. *Journal of Soils and Sediments, 16*, 2252-2262.

Duncan, A. J., & Milne, J. A. (2007). Effects of oral administration of brassica secondary metabolites, allyl cyanide, allyl isothiocyanate and dimethyl disulphide, on the voluntary food intake and metabolism of sheep. *British Journal of Nutrition, 70*(2), 631-645.

Firestone, M. K., & Davidson, E. A. (1989). Microbial basis of NO and N_2O production and consumption. In M. O. Andreae & D. S. Schimel (Eds.), *Exchange of trace gases between terrestrial ecosystems and the atmosphere* (pp. 7-21). New York, United States of America: John Wiley and Sons Ltd.

Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., Asner, G. P., Cleveland, C. C., Green, P. A., Holland, E. A., Karl, D. M., Michaels, A. F., Porter, J. H., Townsend, A. R., & Vöosmarty, C. J. (2004). Nitrogen cycles: past, present, and future. *Biogeochemistry, 70*, 153-226.

Haynes, R. J. (1986). Origin, Distribution and Cycling of Nitrogen in Terrestrial Ecosystems. In R. J. Haynes & F. L. Orlando (Eds.), *Mineral Nitrogen in the Plant-Soil System.* (pp. 1). London,: Academic Press Inc.

Hoogendoorn, C. J., Luo, J., Lloyd-West, C. M., Devantier, B. P., Lindsey, S. B., Sun, S., Pacheco, D., Li, Y., Theobald, P. W., & Judge, A. (2016). Nitrous oxide emission factors for urine from sheep and cattle fed forage rape (Brassica napus L.) or perennial ryegrass/white clover pasture (Lolium perenne L./Trifolium repens). *Agriculture, Ecosystems & Environment, 227*, 11-23.

Krol, D. J., Carolan, R., Minet, E., McGeough, K. L., Watson, C. J., Forrestal, P. J., Lanigan, G. J., & Richards, K. G. (2016). Improving and disaggregating N_2O emission factors for ruminant excreta on temperate pasture soils. *Science of the Total Environment, 568*, 327-338.

Luo, J., & Kelliher, F. M. (2010). *Partitioning of animal excreta N into urine and dung and developing the N_2O inventory*. MPI Technical Paper No: 2014/05. Wellington: Ministry for Primary Industries (MPI).

Luo, J., Ledgard, S. F., & Lindsey, S. B. (2013). Nitrous oxide and greenhouse gas emissions from grazed pastures as affected by use of nitrification inhibitor and restricted grazing regime. *Science of the Total Environment, 465*, 107-114.

Luo, J., Ledgard, S., Wise, B., Welten, B. G., Lindsey, S., Judge, A., & Sprosen, M. S. (2015a). Effect of dicyandiamide (DCD) delivery method, application rate, and season on pasture urine patch nitrous oxide emissions. *Biology and Fertility of Soils, 51*, 453-464.

Luo, J., Sun, X. Z., Pacheco, D., Ledgard, S., Lindsey, S., Hoogendoorn, C. J., Wise, B., & Watkins, N. (2015b). Nitrous oxide emission factors for urine and dung from sheep fed either fresh forage rape (*Brassica napus* L.) or fresh perennial rygrass (*Lolium perenne* L.). *Animal, 9*, 534-543.

Luo, J., Wyatt, J., van der Weerden, T. J., Thomas, S. M., de Klein, C. A. M., Li, Y., Rollo, M., Lindsey, S., Ledgard, S. F., Li, J., Ding, W., Qin, S., Zhang, N., Bolan, N., Kirkham, M. B., Bai, Z., Ma, L., Zhang, X., Wang, H., Liu, H., & Rys, G. (2017). Chapter Five - Potential Hotspot Areas of Nitrous Oxide Emissions From Grazed Pastoral Dairy Farm Systems. In D. L. Sparks (Ed.), *Advances in Agronomy* (pp. 205-268): Academic Press.

Ministry for the Environment. (2017). *New Zealand's Greenhouse Gas Inventory: 1990-2015*. Wellington, New Zealand. 542p., from http://www.mfe.govt.nz/sites/default/files/media/Climate%20Change/FINAL%20GHG%20inventory%20-%2025%20May.pdf.

Misselbrook, T. H., Cardenas, L. M., Camp, V., Thorman, R. E., Williams, J. R., Rollett, A. J., & Chambers, B. J. (2014). An assessment of nitrification inhibitors to reduce nitrous oxide emissions from UK agriculture. *Environmental Research Letters, 9*, 115006.

Myhre, G., Shindell, D., Breon, F.-M., Collins, W., Fuglestvedt, J., Huang, J., Koch, D., Lamarque, J.-F., Lee, D., Mendoza, B., Nakajima, T., Robock, A., Stephens, G., Tekemura, T., & Zhang, H. (2013). Anthropogenic and natural radiative forcing. In T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex & P. M. Midgley (Eds.), *Climate Change 2013: The physical science basis. Contribution of Working Group I to the fifth assessment report of the Intergovernmental Panel on Climate Change.* (pp. 659-740). Cambridge: Cambridge University Press.

O'Sullivan, C. A., Duncan, E. G., Whisson, K., Treble, K., Roper, M. M., & Peoples, M. B. (Compiler) (2016). *Changes in N cycling in the rhizosphere of canola lead to decreased N reaquirements in a following wheat crop*. 7th International Nitrogen Initiative Conference: Solutions to improve nitrogen use efficiency for the world. Melbourne, Australia.

Oenema, O., Wrage, N., Velthof, G. L., Van Groenigen, J. W., Dolfing, J., & Kuikman, P. J. (2005). Trends in global nitrous oxide emissions from animal production systems. *Nutrient Cycling in Agroecosystems, 46*, 119-199.

Prasad, R., & Power, J. F. (1995). Nitrification inhibitors for agriculture, health, and the environment. *Advances in Agronomy, Volume 54*, 233-281.

Ravishankara, A. R., Daniel, J. S., & Portmann, R. W. (2009). Nitrous oxide (N_2O): The dominant ozone-depleting substance emitted in the 21st century. *Science, 326*, 123-125.

Reardon, C. L., Strauss, S. L., & Mazzola, M. (2013). Changes in available nitrogen and nematode abundance in response to *Brassica* seed meal amendment of orchard soil. *Soil Biology and Biochemistry, 57*, 22-29.

Robertson, G. P., & Vitousek, P. M. (2009). Nitrogen in agriculture: Balancing the cost of an essential resource. *Annual Review of Environment and Resources, 34*, 97-125.

Ryan, M. H., Kirkegaard, J. A., & Angus, J. F. (2006). *Brassica* crops stimulate soil mineral N accumulation. *Australian Journal of Soil Research, 44*, 367-377.

Schils, R. L. M., Eriksen, J., Ledgard, S. F., Vellinga, T. V., Kuikman, P. J., Luo, J., Petersen, S. O., & Velthof, G. L. (2013). Strategies to mitigate nitrous oxide emissions from herbivore production systems. *Animal, 7*(Supplements1), 29-40.

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

Snyder, A. J., Johnson-Maynard, J. L., & Morra, M. J. (2010). Nitrogen mineralization in soil incubated with 15N-labeled Brassicaceae seed meals. *Applied Soil Ecology, 46*, 73-80.

Velthof, G., Kuikman, P., & Oenema, O. (2002). Nitrous oxide emission from soils amended with crop residues. *Nutrient Cycling in Agroecosystems, 62*, 249-261.

Chapter 2

Literature Review

2.1 Introduction

Nitrogen (N) is an essential element needed for the growth and reproduction of living organisms (e.g. Gruber & Galloway, 2008; Robertson *et al.*, 2013; Ussiri & Lal, 2013; Selbie *et al.*, 2015a). Most of the N on the planet, however, occurs in the atmosphere as unreactive di-nitrogen gas (N_2) and is generally in relatively short supply in forms that are accessible by most organisms. Thus productivity in many of the world's ecosystems are limited by N (Galloway *et al.*, 2004; Bouwman *et al.*, 2009). Atmospheric N_2 must be fixed by soil micro-organisms or by chemical processes before it can be utilised (Figure 2.1). The global N cycle was greatly changed with the invention of the Haber-Bosch process in the 20th century, which allows for the industrial conversion of atmospheric N_2 to a biologically available or 'reactive' N form (ammonia; NH_3). Nitrogen in this form is extremely mobile (Ussiri & Lal, 2013) and cascades through many chemical forms (Gruber & Galloway, 2008; Sutton *et al.*, 2011; Robertson *et al.*, 2013). Once N is fixed from atmospheric N_2, a single molecule may be transformed and utilised many times before being returned to atmosphere as N_2 (Compton *et al.*, 2011). Consequently, excess reactive N is created, which has many complex environmental effects and threatens air and water quality, biological diversity, and human health (Bouwman *et al.*, 2009; Robertson & Vitousek, 2009; Sutton *et al.*, 2011). It has been estimated that the anthropogenic creation of reactive N has doubled the natural rate of N entering the land-based N cycle (Galloway *et al.*, 2004; Steinfeld *et al.*, 2006; Qiao *et al.*, 2015).

Nitrogen is extremely important in agricultural systems to enhance production and increased N fixation has had undeniable benefits. The invention and implementation of artificial N fertilisers (through the Haber-Bosch process) have led to a massive intensification of agricultural production (Compton *et al.*, 2011; Sutton *et al.*, 2011; Robertson *et al.*, 2013; Qiao *et al.*, 2015) with approximately 75% of the fertiliser N created being used in agricultural systems. The remainder is utilised for energy production and other industrial processes (Galloway *et al.*, 2004). Although this acceleration of the N cycle has allowed for the production of

the quantity of food required for the rapidly growing human population, it raises concern as to the impact to terrestrial and aquatic environments (Gruber & Galloway, 2008; Bouwman *et al.*, 2009). The main reason being that much of the reactive N entering agricultural systems is not fully used, but due to its mobility, can instead be rapidly lost to other ecosystems (Galloway *et al.*, 2004), affecting the system's functioning and biodiversity (Robertson & Vitousek, 2009).

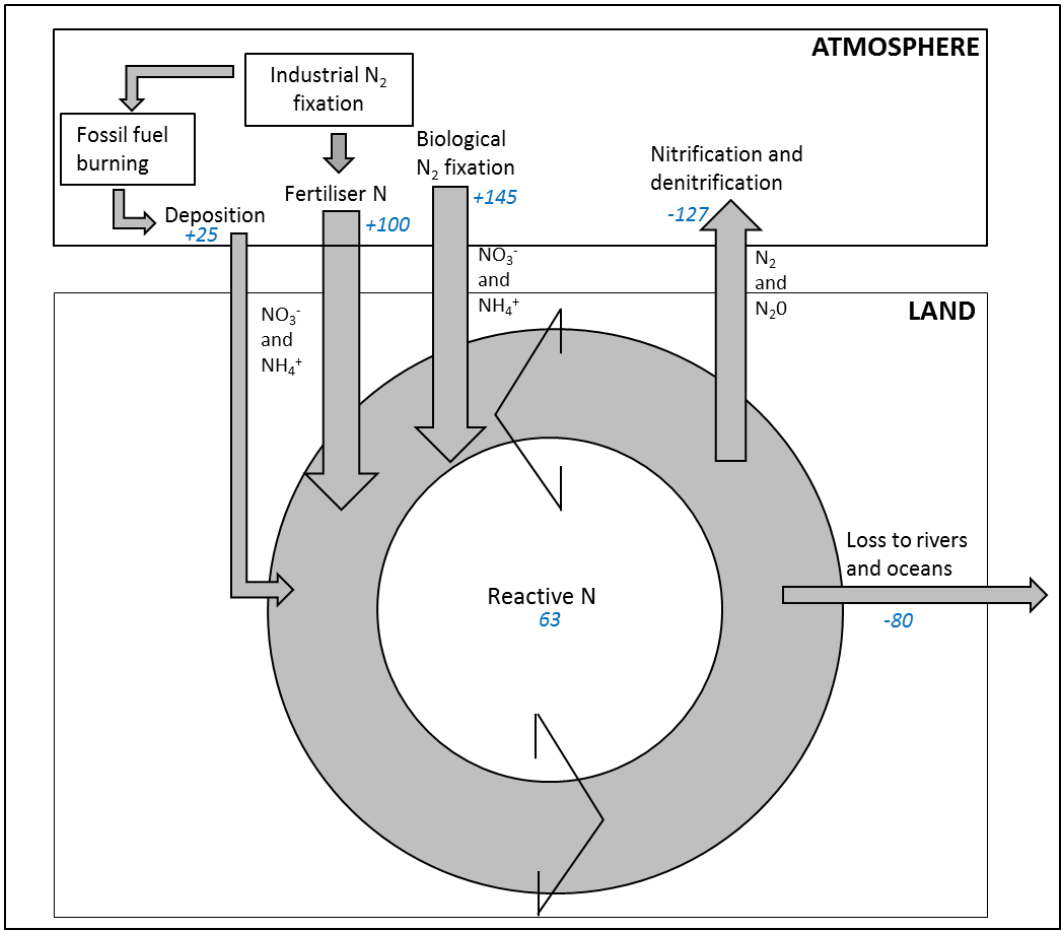

Figure 2.1: Depiction of the global N cycle on land and in the atmosphere. Major processes that transform molecular N into reactive N, and back, are shown. The numbers are in Tg N per year (diagram adapted from: Gruber & Galloway, 2008).

Recently, the concept of planetary boundaries has been introduced. These are planet wide environmental boundaries or 'tipping points', beyond which human society may be at risk (de Vries *et al.*, 2013; Steffen *et al.*, 2015). Based on our growing understanding of the earth system, a planetary boundary has been established for reactive N, which has already been exceeded. Evidence suggests that this breach is

likely a result of a few agricultural regions, particularly those with very high N application rates (Steffen *et al.*, 2015).

One important loss pathway is the production and emission of nitrous oxide (N_2O) from agricultural soils to the atmosphere. Nitrous oxide is a naturally occurring trace gas found in the earth's atmosphere. However, it is also a potent greenhouse gas with a global warming potential approximately 298 times that of carbon dioxide (CO_2) (100 year time span) (Myhre *et al.*, 2013). As such, it is the third most important human induced greenhouse gas, following CO_2 and methane (CH_4) (Singh & Verma, 2007; de Klein *et al.*, 2008; Schreiber *et al.*, 2012; Schils *et al.*, 2013). It is estimated that N_2O is responsible for about 6% of the warming due to greenhouse gases (Robertson & Vitousek, 2009). Nitrous oxide is also the dominant stratospheric ozone depleting substance (Ravishankara *et al.*, 2009).

Nitrous oxide is produced biologically during the cycling of N in soil ecosystems, arising primarily from the soil microbial processes of nitrification and denitrification (See following section - Reay *et al.*, 2012; Misselbrook *et al.*, 2014). In most soils, the availability of mineral N substrate is an important controller of N_2O formation (Mosier *et al.*, 1998) so soils with high N inputs from agricultural activities are the major source of N_2O to the atmosphere, contributing ~50-70% of the total global N_2O budget (Robertson & Vitousek, 2009; Schreiber *et al.*, 2012; Ussiri & Lal, 2013).

Emissions of N_2O are categorised as being either 'direct' or 'indirect'. Direct emissions arise from N directly applied to land, whereas indirect emissions come from N that is leached or volatilised (IPCC, 2008). In agricultural systems, direct sources of N_2O emissions from applied N include: deposition of animal excreta during grazing, application of inorganic N fertiliser and high N effluents, and decomposition of crop residues. Nitrous oxide emissions can also be derived from N applied indirectly, including: atmospheric N deposition and nitrate leaching (de Klein *et al.*, 2008; Matthews *et al.*, 2010). Generally, N_2O emissions are dominated by large emissions from a small area or "hotspot" and over a short time period (Velthof *et al.*, 1996; Groffman *et al.*, 2009; Matthews *et al.*, 2010; Luo *et al.*, 2017). The concept of N_2O hotspots is similar to that of critical source areas of nutrient loss, where small areas contribute disproportionately to nutrient loss relative to their size. With phosphorus for example, it is considered that approximately 80% of

losses to waterways come from approximately 20% of the total farm area (McDowell & Srinivasan, 2009). Animal excreta deposited onto grazed pastures are considered a hotspot for N_2O production as excreta provides high localised concentrations of available N and C in soils. In New Zealand about 64% of the total N_2O emissions comes from urine and dung deposits (Ministry for the Environment, 2017). This is because excreta from grazing ruminants is very high in N due to relatively low utilisation of N in feed. The other main contributor to agricultural N_2O emissions is N fertiliser inputs (de Klein & Ledgard, 2005; de Klein *et al.*, 2008).

Atmospheric N_2O concentrations have been steadily increasing since pre-industrial times (270 ppb to 320 ppb today) (Robertson *et al.*, 2013) in part due to the ongoing intensification of agricultural systems and also from burning of fossil fuels. Due to the environmental impacts of the elevated atmospheric N_2O concentration, this represents a threat to the sustainability of these systems. It is therefore clear that there is a need to address the mitigation of agriculturally derived N_2O, however, global demand for food means any reduction cannot be at the expense of productivity.

Compared to other developed countries, New Zealand's greenhouse gas inventory is unusual as it has a high proportion of the (largely) agriculturally derived gases methane (CH_4; 43%) and N_2O (11%) (Ministry for the Environment, 2017). For most developed nations, CO_2 is the major greenhouse gas contributor representing about 65% of total emissions (Saggar *et al.*, 2004). Thus, any reduction in N_2O production will have an important impact on New Zealand's greenhouse gas emissions.

The following literature review will include:

1. An overview of N cycling in agricultural systems.
2. A summary of N_2O producing processes and drivers of N_2O production.
3. A discussion of approaches for mitigating N_2O production with a focus on nitrification inhibitors.
4. The potential role of secondary metabolites found in brassica plants in mitigating N_2O production.

2.2 Nitrogen cycling in agricultural systems

New Zealand soils contain between 0.1 and 0.6% N in the top 150 mm, over 95% of which is in the organic N pool, including: stabilised organic matter, plant and animal residues, and microbial biomass (Haynes, 1986a; McLaren & Cameron, 1990). All except the stabilised organic matter (which makes up about two thirds of the organic N pool) are readily mineralisable (Haynes, 1986a). Mineral N forms include ammonium (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-) which represents a small and transient pool, as well as dinitrogen (N_2), nitric oxide (NO) and N_2O which exist in gaseous forms in soil pore spaces. NH_4^+ and NO_3^- are the forms that are directly available to plants. The transfer of N from one form to another within an ecological system is commonly referred to as the N cycle. Nearly all transformations of N are driven by micro-organisms as part of their metabolism. Micro-organisms require N for synthesis of structural components, or as an energy source for growth (Ussiri & Lal, 2013). Soil microbial activity is largely driven by abiotic factors, the most important being soil temperature and soil moisture content. The rate of N cycling therefore fluctuates with changes in conditions such as soil wetting and drying, and soil disturbance.

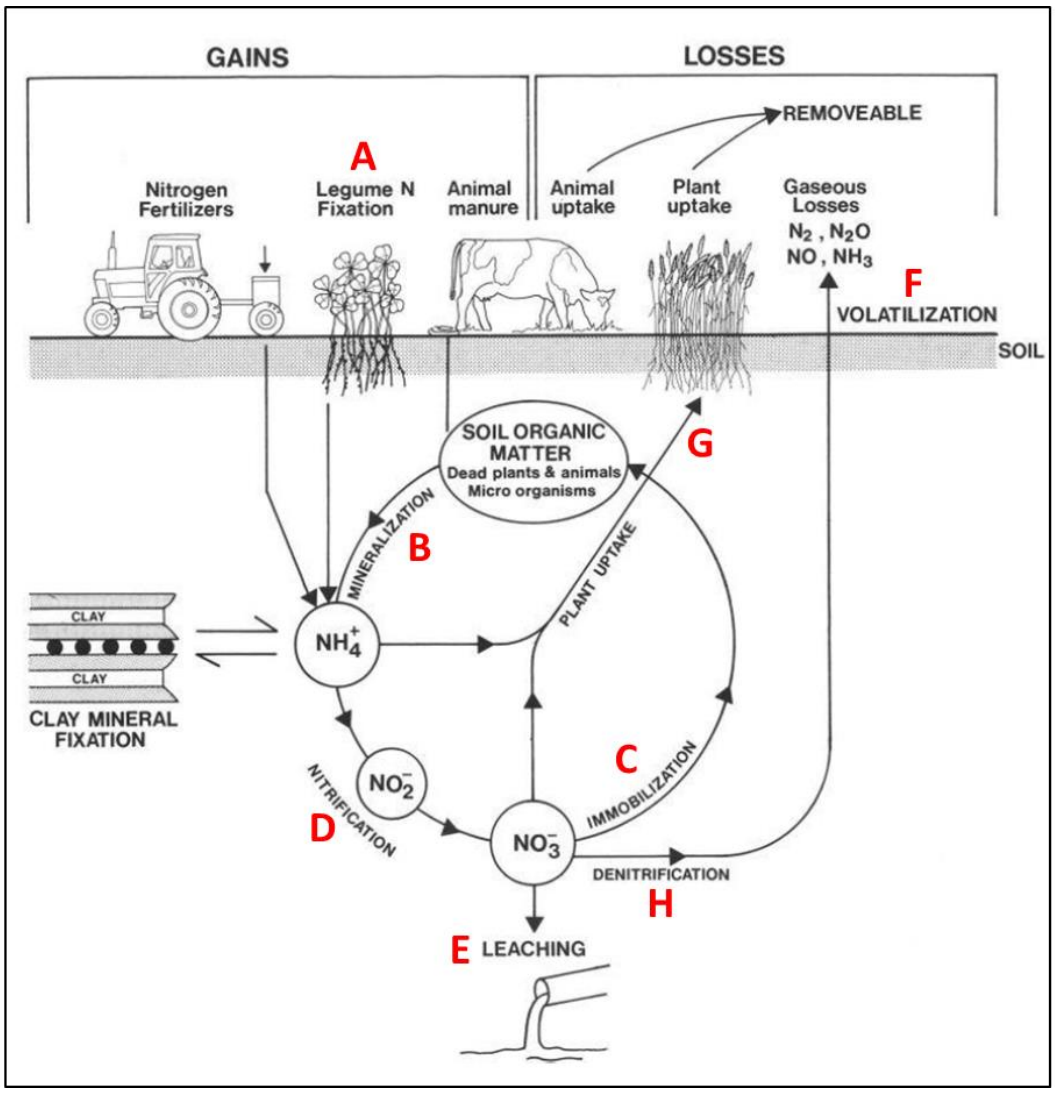

Figure 2.2: Generalised N cycle in grazed pastoral systems (Adapted from McLaren & Cameron, 1990). Letters refer to the different processes discussed in Section 2.2.

2.2.1 Sources of nitrogen

There is recent research that suggests that up to 17% of N inputs may come from the weathering and denudation of parent material (Houlton *et al.*, 2018), however, this is a relatively new area of research and requires further confirmation. Regardless, in soil ecosystems, the majority of new N comes from outside the plant-soil system, largely through the fixation of atmospheric N_2 (Figure 2.2 "A") (Robertson & Vitousek, 2009). There are only a few micro-organisms that have the ability to biologically convert atmospheric N_2 to reactive forms of N. These micro-organisms may be free living (e.g. *Clostiridium* spp), or living in symbiosis with plants (e.g. *Rhizobium* spp, which live in association with legumes) (Stevenson, 1965; Bouwman *et al.*, 2009; Robertson & Vitousek, 2009). The process of N fixation involves the reduction of gaseous N to ammonia (NH_3) by the nitrogenase

enzyme which occurs in these micro-organisms. Most N fixation, particularly in agricultural systems, is carried out by symbiotic as opposed to free living micro-organisms (Stevenson, 1965). N fixed by symbiotic N fixers is immediately available to plants as these N fixers transfer N to the plants. N fixed by free living N fixers, however, is only available for plant uptake following microbial death and decomposition (Schimel & Bennett, 2004).

In natural systems, the main input of available N is *via* biological N fixation. Wet deposition plays a small role in New Zealand as industrial emissions are relatively low and prevailing winds remove around 75% of the combustion products to the oceans (Parfitt *et al.*, 2006). In many agricultural systems, however, synthetic N fertilisers provide the main N input which are produced by chemical fixation of N (e.g. the Haber-Bosch process). N is also recycled within agricultural systems as it is returned in animal excreta. Grazing ruminants are poor utilisers of dietary N, with only 10–30% of the N consumed being retained in body tissues and in products such as milk. The remainder is excreted in urine or dung (e.g., Selbie et al., 2015).

2.2.2 Transformations of nitrogen

2.2.2.1 Mineralisation and immobilisation

Organic matter is the largest pool of N in soil, the decomposition of which drives the cycling of N (Schimel & Bennett, 2004). The process where 'organic' forms of N such as complex proteins (e.g. R – NH_2) are broken down into mineral or 'inorganic' forms, ultimately ammonia (NH_3), is called mineralisation (Figure 2.2 "B"). The final stage of the process where NH_3 is released is termed ammonification (equation 2.1) and provides energy and a source of N to microorganisms (Cameron, 1992). Ammonification is generally carried out under aerobic conditions by a wide range of heterotrophic micro-organisms that use the energy provided by the reaction and O_2 as an electron donor (Cameron, 1992).

$$R - NH_2 + H_2O \rightarrow NH_3 + R - OH + energy$$

Equation 2.1

The reverse process, where N is taken up by micro-organisms and incorporated into organic constituents of their own body tissues is called immobilisation (Figure 2.2 "C"). These two processes occur simultaneously in soil and local conditions may favour one over the other at any point in time (Haynes, 1986a). When more N is taken up by microbes than is mineralised, 'net immobilisation' occurs, and when more mineral N is released than immobilised, 'net mineralisation' occurs. The ratio of available C to available N present in the soil is a major determinant of which net process will be occurring. While microbes have a biomass C:N of about 6-8, they require only a small amount of N relative to C during catabolism. When decomposing material has a high N content, more N is released than is required by micro-organisms and so net mineralisation occurs. On the other hand, when material has a low N content, the N produced is insufficient and N is taken up from the surrounding soil and immobilised (Whitehead, 1995b).

2.2.2.2 Nitrification

Autotrophic nitrification (Figure 2.2 "D") is the process by which soil micro-organisms convert NH_4^+ to NO_3^- under aerobic conditions. It is a two-step oxidation process which involves the intermediary nitrite (NO_2^-). In the first step, NH_4^+ is oxidised to NO_2^- (with the intermediary hydroxylamine (NH_2OH) which is not stable in soil) by the ammonia monooxygenase enzyme, which is associated with ammonia oxidising bacteria (and some archaea) such as *Nitrosomonas* spp and *Nitrosospira* spp. (Figure 2.3; equation 2.2).

$$NH_4^+ \xrightarrow{ammonium\ mono-oxygenase} NH_2OH \xrightarrow{Hydroxylamine\ oxidoreductase} NO_2^-$$

Figure 2.3: Pathway of the first step of nitrification and the enzymes involved.

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+ + energy$$

Equation 2.2

In the second step, the NO_2^- produced is further oxidised to NO_3^- by nitrifying bacteria including *Nitrobacter* spp. (Equation 2.3), catalysed by the nitrite

oxidoreductase enzyme. This second step occurs very rapidly and so NO_2^- rarely accumulates in soil (Mosier *et al.*, 1998; Singh & Verma, 2007).

$$2NO_2^- + O_2 \rightarrow 2NO_3^- + energy$$

Equation 2.3

As a result of this transformation, the N is converted into a form readily lost from soil by denitrification or leaching (Alexander, 1965; Robertson *et al.*, 2013).

Although both ammonia oxidising bacteria and archaea are known to be present in large numbers in soil, Di *et al.* (2009) observed that in high N environments such as agricultural soils, neither archaea abundance nor activity increased following the addition of an NH_3 substrate. This suggests that nitrification was largely driven by bacteria rather than archaea in N-rich agricultural soils.

Heterotrophic nitrification is carried out by a range of both bacteria and fungi and is considered to play a significant role in acidic soils in particular. Heterotrophic organisms can carry out all steps of nitrification, however, the physiological role of heterotrophic nitrification is unclear as the reactions do not yield energy and so do not contribute to cellular growth (Hayatsu *et al.*, 2008).

2.2.2.3 Dissimilatory nitrate reduction to ammonium

Dissimilatory nitrate reduction to ammonium (DNRA) is a reactive N conserving transformation as N is recycled back through the system as opposed to lost through gaseous forms for example. DNRA involving the reduction of NO_3^- to NO_2^- and NO to NH_4^+ is carried out by fermentative bacteria. DNRA occurs under anaerobic conditions where NO_3^- or NO_2^- are used as an electron acceptor as opposed to oxygen (Cole & Brown, 1980).

2.2.3 Losses of nitrogen

2.2.3.1 Plant uptake

Plants require N for the synthesis of proteins within their tissues and it is generally the nutrient most limiting to plant growth. Plant uptake of N and subsequent harvest

is a pathway by which N is removed from agricultural systems (Figure 2.2 "G"). The N utilised by plants is primarily in inorganic forms, specifically as NH_4^+ and NO_3^-, which are absorbed from the soil solution via the root system. Although plants can take up both NH_4^+ and NO_3^-, NO_3^- must first be converted to NH_4^+, at considerable energy expense, before being converted into amino acids (Haynes, 1986b; Tischner, 2000).

Plants may also take up some organic compounds directly via their roots (Kielland, 1994; Hodge *et al.*, 2000), or in association with some mycorrhizal fungi (Nasholm, 1998). Absorption of gaseous N (primarily NH_3) through plant leaves has also been reported (Sommer & Jensen, 1991), although this equates to only a small proportion of total N uptake. The rate of plant N uptake varies between plant species and between growth stages (Ismande & Touraine, 1994).

Plant tissues generally contain between 1 and 6% N (Goh & Haynes, 1986; McLaren & Cameron, 1990), and depending on factors such as fertiliser (or other N input) regimes, soil type, and climatic conditions, ryegrass-based pastures can take up between 200 and 700 kg N ha^{-1} $year^{-1}$ (Whitehead, 1995a). In environments where available soil N is high, such as under a urine patch, plants may take up N in excess of their requirements and store it as NO_3^- or amides. This is termed 'luxury uptake' (Chapin, 1980; Lipson *et al.*, 1996). However, urine patch N load will generally exceed plant uptake (Selbie *et al.*, 2015a).

2.2.3.2 Leaching

N leaching (Figure 2.2 "E") is the loss of soil N in drainage water and can include both particulate or dissolved forms of organic N as well as mineral forms of N. Nitrate, however, is normally the dominant form of N leached through agricultural soil profiles because it is negatively charged and is thus not retained by soil colloids that are also predominantly negatively charged (Robertson & Vitousek, 2009). The amount of N lost *via* leaching is dependent on the concentration of N (NO_3^-) in drainage water and the amount of drainage. Season and climate are major drivers of N leaching. Drainage is highest, and plant uptake low, during winter and so the largest losses of N by leaching generally occur during this period.

2.2.3.3 Volatilisation

NH_3 volatilisation (Figure 2.2 "F") is the loss of gaseous NH_3 from the soil surface and is another pathway by which N can be lost from agricultural systems. Volatilisation occurs when there is high concentrations of NH_3 present at the soil surface (McInnes *et al.*, 1986). This loss pathway generally occurs when pH is high as NH_3 is in pH dependent equilibrium with NH_4^+ in soils and solutions (equation 2.3), and high pH favours production of aqueous NH_3 that can then be volatilised (Robertson & Vitousek, 2009).

$$NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O$$

Equation 2.4

2.2.3.4 Denitrification

Biological denitrification (Figure 2.2 "H") is the anaerobic microbial reduction of NO_3^- to N_2 gas (Mosier *et al.*, 1998) with other gaseous products being formed during the process. Micro-organisms use NO_3^- as an electron acceptor instead of oxygen and convert NO_3^- to NO_2^-, nitric oxide (NO), N_2O and N_2 gas (Ussiri & Lal, 2013). Each step is catalysed by a specific enzyme (Figure 2.4).

$$NO_3^- \xrightarrow{Nitrate\ reductase} NO_2^- \xrightarrow{Nitrite\ reductase} [NO] \xrightarrow{NO-reductase} N_2O \xrightarrow{N_2O-reductase} N_2$$

Figure 2.4: Pathway of and enzymes involved in biological denitrification in soils (adapted from Saggar *et al.*, 2004).

Chemo-denitrification is a chemical or abiotic process where high levels of NO_2^- in acidic soils forms NO (Ussiri & Lal, 2013). This NO may subsequently react with amino compounds or organic matter to form N_2O and N_2 (Heil *et al.*, 2016). This process is generally minor in comparison to biological denitrification.

Denitrification of NO_3^- to unreactive N_2 removes biologically available/reactive N from soil ecosystems, which is a loss in terms of N use efficiency, but because it returns N to the very large, unreactive, atmospheric N_2 pool, the environmental consequences are benign (Robertson & Vitousek, 2009). However, NO and N_2O

are often released into the atmosphere before they are converted to N_2 so creating another loss pathway.

2.2.3.5 Summary of agricultural nitrogen cycle

The soil N cycle consists of inputs, outputs and internal transformations of N. Inputs to the agricultural N cycle include biological N fixation, N fertilisers, and N deposition. N is then either transformed within the system or removed from the soil system *via* loss pathways. Internal transformation processes are microbially mediated and include mineralisation, immobilisation, nitrification and DNRA. Loss pathways include NO_3^- leaching, plant uptake, volatilisation, and chemical and biological denitrification. Some of these transformations and loss pathways lead to the production of N_2O gas.

2.3 Formation of nitrous oxide

Nitrous oxide is a gaseous intermediate in the reaction sequences of both the microbial processes of nitrification and denitrification, which together contribute about 90% of agricultural N_2O emissions (Figure 2.5). There is also some evidence that small amounts are produced during chemo-denitrification as presented in section 2.2.3.4 (Ussiri & Lal, 2013).

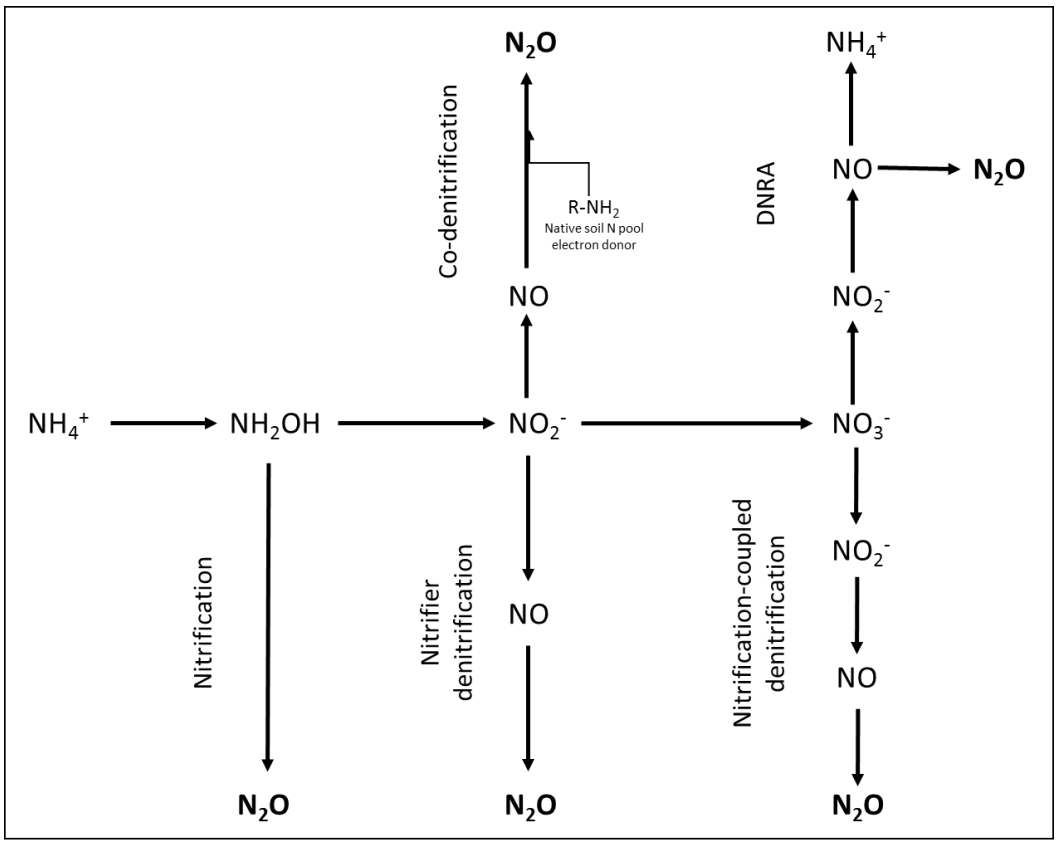

Figure 2.5: Major pathways of N_2O formation (adapted from Ussiri & Lal, 2013 and Selbie et al, 2015b)

The evolution of NO and N_2O during nitrification and denitrification processes is conceptualised using the "hole in the pipe" model (Figure 2.6). In this conceptual model, the rate that N flows through the process 'pipes', or rate of nitrification and denitrification, is determined by the availability of N in the soil. NO and N_2O 'leak' out of holes in the pipe, and the size of the holes is primarily determined by soil water content. Other factors such as soil acidity, and abundance of electron donors and acceptors, also contribute to the relative production and partitioning of the gases (Firestone & Davidson, 1989; Davidson *et al.*, 2000).

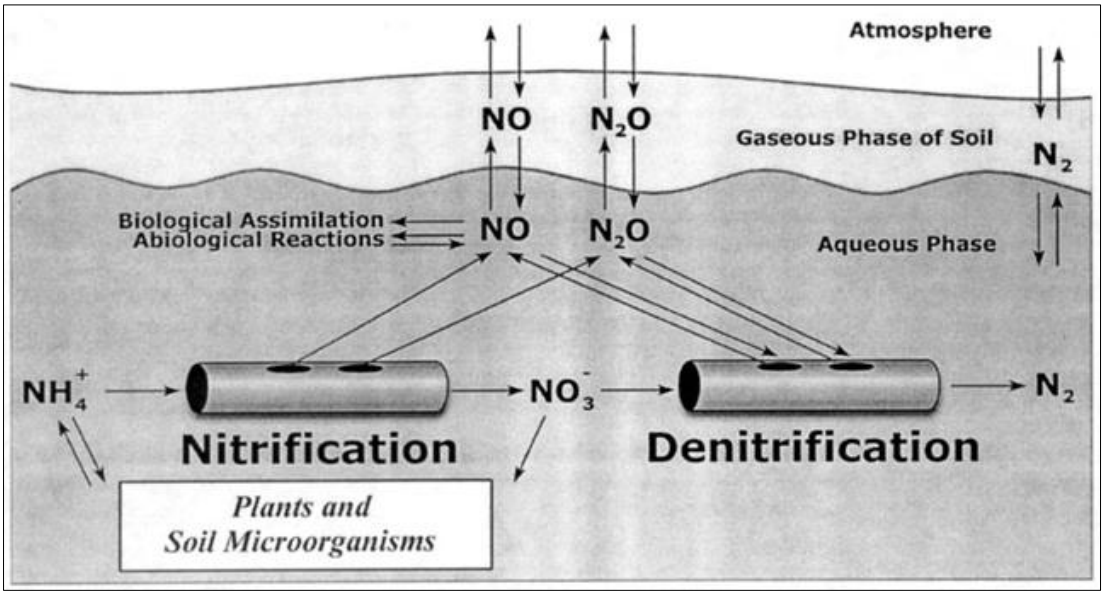

Figure 2.6: Diagram of the "hole in the pipe" conceptual model (From: Davidson *et al.*, 2000).

During the nitrification process, N_2O can be formed during the chemical decomposition of NH_2OH, nitroxyl hydride (HNO), or NO_2^-, intermediates of NH_4^+ oxidation (Wrage *et al.*, 2004; Heil *et al.*, 2016). As nitrification occurs in aerobic conditions, it is the predominant process producing N_2O under drier conditions when soil moisture is low and the diffusion of oxygen is not limited (Mathieu *et al.*, 2006)(discussed further in section 2.3.1). Under anaerobic conditions, nitrification likely only makes a small contribution to direct emissions (Mathieu *et al.*, 2006). However, nitrification has been estimated to contribute up to 80% of soil N_2O emissions (Hu *et al.*, 2015), largely due to the provision of NO_3^- as a substrate for denitrification, the main N_2O forming pathway. Additionally, NO_3^- leaching contributes to indirect N_2O emissions where the NO_3^- is denitrified in receiving wetlands or waterways (Butterbach-Bahl *et al.*, 2013; Fowler *et al.*, 2013).

In contrast, denitrification is largely a process that occurs under wet and anaerobic conditions when a NO_3^- source is present in the soil (Coyne, 2018) Agricultural soils which receive high N loading and have poor drainage, for example soils which are heavily compacted or pugged, are hotspots for denitrification (Luo *et al.*, 2017). Nitrous oxide is produced during denitrification activity as it is an obligate transitional form in the denitrification pathway and so may be released to the atmosphere before it is further reduced to N_2 (Firestone & Davidson, 1989; Hu *et al.*, 2015).

Often, denitrification (section 2.2.3.4) can be coupled with nitrification (section 2.2.2.2) and the NO_3^- produced during nitrification provides the substrate for denitrification. However, an alternative denitrification pathway, called nitrifier denitrification, is the direct reduction of the nitrification intermediary NO_2^- to N_2O and is carried out by the ammonium oxidisers that convert NH_4^+ to NO_2^- during nitrification (Wrage *et al.*, 2004; Ussiri & Lal, 2013).

Co-denitrification is the sequential reduction of NO_3^-, similar to that of conventional denitrification, however, following the formation of NO, a side reaction occurs. One NO atom derived from an inorganic N source (e.g. NO_2^-) binds with an electron donor from the soil native N pool forming N_2O and N_2 (Selbie *et al.*, 2015b).

Dissimilatory nitrate reduction to ammonium (DNRA) has also been reported to occasionally contribute to N_2O production in particular cases (Rutting *et al.*, 2011; Zhu *et al.*, 2013). All of these pathways may be occurring simultaneously in a soil profile as different soil microsites will have different conditions. It is possible that NO_3^- is produced in aerobic zones which then diffuses into anaerobic zones where it is denitrified (Cuttle, 2008).

Production of N_2O by fungi

Denitrification is widespread among prokaryotes. Some soil fungal strains, however, are also capable of denitrification (Shoun *et al.*, 1992; Mothapo *et al.*, 2013; Maeda *et al.*, 2015). No genes coding for N_2O reductase have been identified in fungi so N_2O is the end-product of fungal driven denitrification (e.g. Laughlin & Stevens, 2002; Coyne, 2018). Although fungal N_2O producing activity is 1-5 orders of magnitude lower than prokaryotes (Mothapo *et al.*, 2015; Coyne, 2018), the high biomass of fungi in soil means fungal contributions of N_2O may be significant in some terrestrial systems (Herold *et al.*, 2012).

2.3.1 Conditions influencing nitrous oxide production in soils

Nitrous oxide fluxes from soil are regulated by a range of biological, chemical and physical factors that are constantly changing depending on soil conditions. Specifically, N_2O production is driven by the soil oxygen status/moisture content,

N substrate supply, organic matter, temperature and pH (e.g. proximal regulators). These in turn, are regulated by inputs and management practices (e.g. distal regulators) (Figure 2.7). Nitrification, denitrification and other N_2O forming processes, dominate under differing soil conditions (section 2.3).

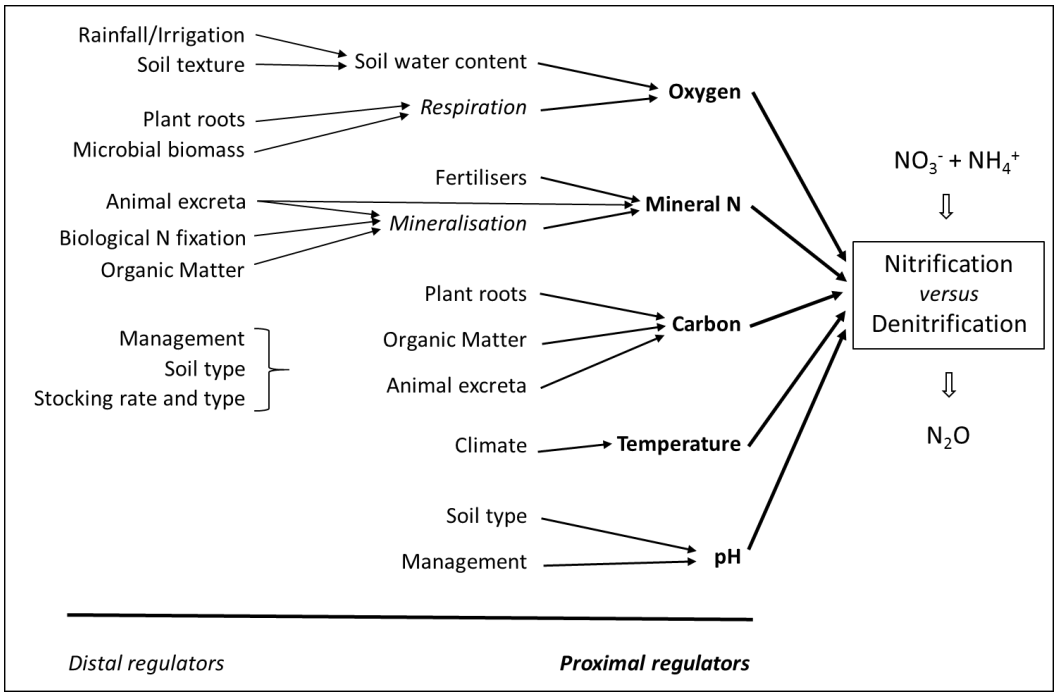

Figure 2.7: Schematic diagram of distal and proximal factors affecting N_2O emissions from agricultural soils (adapted from de Klein *et al.* (2001)).

2.3.1.1 Oxygen status/moisture content

Soil oxygen status is inversely proportional to the soil moisture content primarily because O_2 diffusion through water is much slower than through the gas phase (Weil & Brady, 2016). Soil oxygen status is an important regulator of N_2O emissions as its concentration largely determines which of the main processes is driving N_2O production at that time (Figure 2.8) (Coyne, 2018).

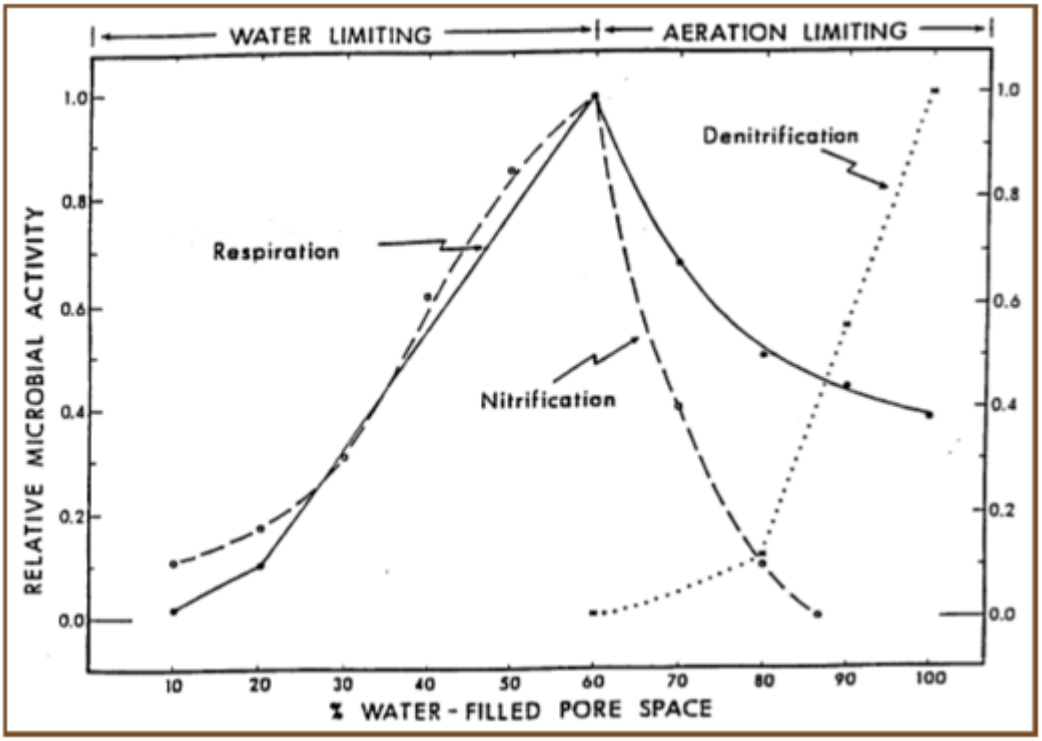

Figure 2.8: Relationship of water filled pore space to soil microbial processes of respiration, nitrification and denitrification (Linn & Doran, 1984).

Highest fluxes of N_2O generally occur under low oxygen or anaerobic conditions because the NO and N_2O reductase enzymes are repressed by the presence of oxygen (Knowles, 1982). Denitrification is favoured, in particular, when soil moisture increases above 60% water filled pore space (Bateman & Baggs, 2005), however, as soil moisture increases, N_2 becomes the dominant gaseous product and so N_2O emissions can reduce (Weier *et al.*, 1993).

Below about 65% water filled pore space, nitrification becomes the main N_2O forming pathway (Linn & Doran, 1984; Bateman & Baggs, 2005). However, microbial activity is stimulated by available water so nitrification will also increase with increasing soil moisture until oxygen becomes limiting.

In aerobic soils, nitrification and denitrification may occur simultaneously in spatially distinct microsites (Parkin, 1987; Carter, 2007). For example, N_2O production by denitrification may occur in anaerobic microsites in otherwise aerobic soil conditions.

2.3.1.2 Nitrogen substrate supply

N substrate availability is a significant driver of nitrification and denitrification processes. Nitrous oxide emissions from nitrification are dependent on the supply of N for nitrifying bacteria in the form of organic residues and NH_4^+ (Alexander, 1965; Zhang *et al.*, 2015a). Nitrous oxide fluxes from denitrification, however, are directly proportional to NO_3^- supply when all other requirements (i.e. water, C source) are not limiting (Malhi *et al.*, 1990; Mosier et al, 1983 in: Granli & Bockman, 1994). Additionally, increased NO_3^- concentration results in an increase in the N_2O:N_2 production ratio due to the inhibition of the N_2O reductase enzyme under high NO_3^- conditions (Knowles, 1982).

Under a urine patch in grazing systems, loadings of N rapidly reach between 200 and 2000 kg N ha^{-1} (average 613 kg N ha^{-1}) (Selbie *et al.*, 2015a), which far exceeds immediate plant requirements and uptake. As a result, high amounts of NH_4^+ and NO_3^- are available for nitrification and denitrification, respectively. Nitrous oxide emissions generally range between 0-4% of N input (de Klein *et al.*, 2001; Cameron *et al.*, 2013).

2.3.1.3 Organic matter

Hetertrophic denitrification is a respiratory process that requires a readily available C source (Tiedje, 1994). Both water soluble C and total organic C are highly, positively correlated with biological denitrification rate (Burford & Bremner, 1975; Senbayram *et al.*, 2012). The following equation demonstrates the amount of available C required for denitrification to occur (Burford & Bremner, 1975):

$$4(CH_2O) + 4NO_3^- + 4H^+ \rightarrow 4CO_2 + 2N_2O + 6H_2O$$

Equation 2.5

Additionally, high concentrations of available C provides an energy source for aerobic respiration. This drives O_2 consumption in soil aggregates to further support denitrification (Parkin, 1987). Therefore, the production of N_2O in soil is partly controlled by the supply of readily decomposable organic matter.

Autotrophic nitrification does not require a C substrate, however, the mineralisation of soil organic N, which supplies NH_4^+ for nitrification, is carried out by a diverse range of micro-organisms which require C for metabolic processes (Brock *et al.*, 1994).

2.3.1.4 Temperature

Nitrification and denitrification (in fact, all biological processes) increase with temperature, therefore, N_2O production also increases with temperature (Granli & Bockman, 1994). In addition, N_2O solubility decreases with increasing temperature resulting in further emissions. Nitrous oxide production therefore closely follows diurnal and seasonal patterns (Goodroad & Keeney, 1984). The optimum temperature (T_{opt}) range for nitrification occurs from 16 to 37ºC, with ammonia oxidising archaea having a significantly higher T_{opt} than ammonia oxidising bacteria (Taylor *et al.*, 2017). Denitrification increases with temperature up to about 60ºC before sharply decreasing (Malhi *et al.*, 1990). Several studies, however, have reported an increase in the N_2O:N_2 production ratio with lower temperature (Keeney *et al.*, 1979; Maag & Vinther, 1996). Therefore, at lower temperatures, total N gas emissions may decrease but N_2O emissions may remain the same.

2.3.1.5 pH

Both nitrification and denitrification rates increase from acidic to slightly alkaline conditions (Goodroad & Keeney, 1984). Nitrifying bacteria metabolism, and so nitrification rate, is at optimum in the neutral to slightly alkaline range (pH 7 to 8) (Goodroad & Keeney, 1984). Denitrification rates also increase with pH and the process has an optimum range of pH 7 to 8 (Weier & Gilliam, 1986). The N_2O/N_2 ratio, however, strongly decreases with increases in pH (Liu *et al.*, 2010), therefore, N_2O emissions decrease with increasing pH.

2.3.1.6 Summary

Nitrous oxide production in soil is largely controlled by soil conditions, such as moisture, temperature and pH. Additionally, N_2O production is dependent on a supply of N and C substrates. These controls can be exploited in order to decrease N_2O emissions from agricultural systems.

2.4 Mitigating nitrous oxide emissions

As agricultural soils account for such a high percentage of anthropogenic N_2O emissions, particularly in New Zealand (Ministry for the Environment, 2017), the agricultural sector therefore offers substantial potential for mitigation of total N_2O emissions. Although animal manures and synthetic fertilisers are important sources of N_2O, they are also a necessity in providing the N needed to support food production for the growing global population, thus any mitigation option needs to be economic, practical, and applicable to a farm system. Reviews by de Klein and Ledgard (2005), Luo *et al*. (2010), Misselbrook *et al.* (2013), Reay *et al.* (2012) and Schils *et al.* (2013) comprehensively discuss strategies for reducing N_2O emissions from agricultural systems, the main points of which are summarised in the following sections (Figure 2.9): -

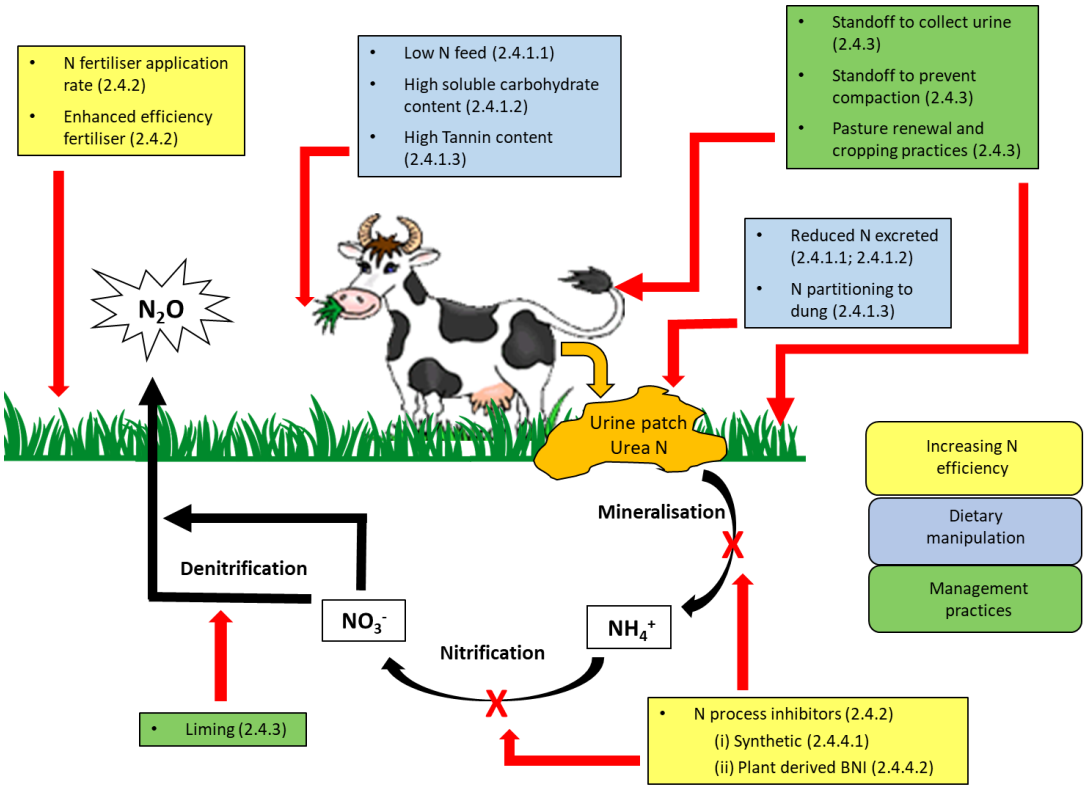

Figure 2.9: Schematic describing overall framework of N_2O mitigation strategies coloured by general strategy (see legend). Numbers in each of the boxes indicates section within this review for further explanation.

2.4.1 Dietary manipulation

Dietary intervention is primarily aimed to either reduce the concentration of N in excreta, or to change the partitioning of N in urine and dung, while preferably having no detrimental effect on productivity. In order to understand the concept of reducing excreta N, and urinary N in particular, it is necessary to understand the N dynamics of the urine patch in grazing systems and how they relate to N_2O emissions. Urine deposited onto grazed pastures is considered a hotspot for N_2O production as urine provides high localised concentrations of available N and C in soils. Additionally, N_2O emissions are correlated with excreta N concentrations (Dai *et al.*, 2013; Di *et al.*, 2016), therefore, reducing N excreted would reduce N_2O emissions. In New Zealand about 64% of the total N_2O emissions come from urine and dung deposition (Ministry for the Environment).

2.4.1.1 Reducing dietary nitrogen and nitrogen in excreta

Due to the strong relationship between N intake and N excretion, one of the ways to reduce N excreted (and hence N available as a substrate for N_2O production) is to reduce the N consumed in the diet through the use of alternative feeds. As N in pasture is generally in excess of animal requirements, reducing N intake should presumably not reduce production. Care, however, must be taken to ensure dietary N/protein is not reduced to a point where it is no longer sufficient to meet the animal's metabolic requirements.

That reducing the N content in the diet through low N supplements reduces total and urinary N excreted in ruminants is well established (e.g. Kebreab *et al.*, 2001; Nielsen *et al.*, 2003; Mulligan *et al.*, 2004; Misselbrook *et al.*, 2005; Steinshamn *et al.*, 2006; Huhtanen *et al.*, 2008; Edwards *et al.*, 2014). Luo *et al.* (2008) showed that using low N feed supplements such as maize in a New Zealand pastoral system, reduced the amount of surplus N excreted and N_2O emissions, while increasing milk production per unit of N and thus increased overall N use efficiency. Additionally, Burke *et al.* (2008) showed a reduction in both urinary and total N excreted (and presumably N_2O emissions), with no effect on production, when supplementing the diet with low N citrus pulp. However, if feed with low N content was considered as a mitigation practice, offsite losses associated with fertiliser used to grow this feed would need to be considered.

2.4.1.2 Nitrogen Partitioning – Protein versus Excreta

The low level of utilisation of dietary N is largely a result of insufficient carbohydrate-derived energy for conversion of the feed N to microbial N in the rumen (Beever *et al.,* 1986). The surplus N is transported through the rumen wall as ammonia and subsequently excreted in urine following hepatic conversion to urea N (Miller *et al.,* 2001). It therefore suggests that increasing dietary soluble carbohydrate content may increase utilisation of feed N in the rumen and thus reduce N excretion.

Miller *et al.* (2001) and Moorby *et al.* (2006) showed that the addition of 'high sugar grasses', or grass varieties with a high content of water soluble carbohydrate, to the diet increased milk production in dairy cows through more efficient utilisation of the feed N in the rumen. They also measured a reduction in total excreted N and urinary N. Additionally, Lee *et al.* (2002) found reduced ammonia-N concentrations in the rumen of steers fed high sugar grasses compared with steers offered the control perennial ryegrass variety, which translated through to reduced urine N excretion. These results are supported by others (e.g. Castillo *et al.*, 2000; Broderick, 2003; Steinshamn *et al.,* 2006) who have reported improved N utilisation and reduced excretal N following supplementation of high energy feeds to cattle. While no direct measurements have been made on the consequences of this for N_2O emissions, it can be hypothesised that a reduction in N excreted would result in reduced N_2O production.

2.4.1.3 Nitrogen partitioning – Dung versus Urine

The relationship between N intake and N excreted is linear for both dung and urine, however, the line is much steeper for urine than for dung (e.g. Dijkstra *et al.*, 2013). This indicates that urine as opposed to dung is the main removal pathway for excess dietary N. Nitrous oxide emissions from dung deposits are considerably lower than from urine patches as the N transformation processes are much slower in dung, and it has been found that the emission factor (percentage of applied N emitted as N_2O-N) for dung is significantly lower than that of urinary N (van der Weerden *et al.*, 2011). It is therefore likely that partitioning more of the dietary N into dung rather than urine could decrease N_2O emissions.

Supplementation of diet with condensed tannins, for example, has been shown to result in a greater partitioning of excreta N in dung rather than urine. Condensed tannins bind with proteins in the rumen, slowing protein degradation and leading to decreased ruminal NH_3 concentration and subsequently decreased urinary N excretion, without affecting production (Carulla *et al.*, 2005; Misselbrook *et al.*, 2005).

2.4.2 Increasing nitrogen use efficiency

Currently, for every 100 units of N used in agriculture, only about 15 are captured in crop, dairy or meat products (Steinfeld *et al.*, 2006; Robertson & Vitousek, 2009). This low ratio points to very low N use efficiency in most agricultural systems. In the dairy industry for example, despite improved genetic potential of cows with high nutrient utilisation, the increased external input of feed concentrates and use of fertiliser N have decreased N use efficiency in these systems (Huhtanen *et al.*, 2008). Increasing N use efficiency is the concept of improving N management by controlling inputs, crops, N-cycling processes, and agricultural practice whilst maintaining high yields. This approach is a key strategy by which the increasing food demand might be met without a corresponding increase in N_2O emissions.

Practices to increase N use efficiency are based around the timing, rate and form of fertiliser being applied. These may include: changing the source of N, e.g., refraining from use of ammonium nitrate fertilisers under wet conditions (Smith *et al.*, 2012), ensuring N addition matches crop/pasture requirements, using slow release fertilisers (Akiyama *et al.*, 2010), or precision placement of fertiliser (Hedley, 2015).

The use of inhibitors of N processes, such as urease and nitrification inhibitors, have the potential to reduce N_2O emissions from both fertiliser and excreta applications (Dell *et al.*, 2014; Li *et al.*, 2014). Urease inhibitors retard the hydrolysis of urea to NH_4^+ whereas nitrification inhibitors retard the conversion of NH_4^+ to NO_3^-. These both prolong the length of time taken for applied N to be converted to NO_3^- thereby allowing for more uptake by plants and improving nutrient use efficiency (Amberger, 1989). This delay results in less available NO_3 for both direct N_2O

emissions and indirect emissions following leaching. Nitrification inhibitors will be discussed in greater detail in section 2.4.4.

2.4.3 Soil management to avoid conditions that favour nitrous oxide emissions

Altering pasture and stock management practices is another method by which N_2O loss might be minimised. Differing soil properties mean soils differ in their risk of emitting N_2O. Poorer-draining clay soils, for example, are more likely to be saturated and support higher denitrification rates and consequently increase direct N_2O emissions. Therefore, management of winter-grazed cattle through restrictive grazing, for example, reduces N_2O emissions as animals are removed from pasture and excreta deposition is reduced during the time when soils are wet and denitrification is high (e.g. Webb *et al.*, 2005; Luo *et al.*, 2008). Effluent captured on feed or stand-off pads can then be utilised during low risk months, which would in turn reduce fertiliser N requirements also (Houlbrooke *et al.*, 2004). More freely draining soils on the other hand, would be at lower risk of high denitrification rates as they are less likely to saturate. However, it should be noted that more freely draining soils have a higher risk of N leaching and therefore a higher risk of indirect N_2O emissions after the leached NO_3^- reaches surface waters.

Soil compaction reduces soil aeration and increases N_2O emissions due to high rates of denitrification (e.g. Menneer *et al.*, 2005; van der Weerden & Styles, 2012). Additionally, soil compaction may damage pastures and reduce pasture N uptake (Nie *et al.*, 2001), resulting in more N available for denitrification. Therefore, altering farm management practices to reduce soil compaction would reduce the likelihood of N_2O emissions. These practices include: rotational instead of set-stock grazing, low stocking density grazing, and removal of stock from pastures during periods when soil moisture is high (Drewry *et al.*, 2008). Land use change, such as intensification *via* irrigation, may also increase soil compaction (Houlbrooke *et al.*, 2011), potentially increasing N_2O emissions.

Conflicting results have been reported as to the contribution of soil tillage and cultivation methods for pasture renewal and crops to N_2O emissions (Luo *et al.*, 2017). High mineralisation of soil and root N following tillage may result in high N_2O emissions (e.g. do Carmo *et al.*, 2005; Mori & Hojito, 2007; Reinsch *et al.*, 2018), whereas tillage practices may increase soil aeration resulting in lower

emissions (e.g. Ball *et al.*, 1999; Thomas *et al.*, 2008; Grave *et al.*, 2018). Nonetheless, the most effective mitigation is to avoid fertiliser applications and excreta deposits from grazing animals immediately prior to renovation in order to reduce the supply of N available for mineralisation and N_2O emissions (Davies *et al.*, 2001).

Liming of acidic soils has been shown to have mixed results in regard to reducing N_2O emissions from agricultural soils (Clough *et al.*, 2003; Coyne, 2018; McMillan *et al.*, 2016). Denitrification rates generally increase when pH is raised, however, the ratio of N_2O to N_2 decreases when pH is raised. Thus, increasing soil pH through the application of lime may reduce N_2O emissions by driving production of N_2 instead of N_2O during denitrification (Kunhikrishnan *et al.*, 2016).

2.4.4 Nitrification inhibitors

A widely researched method for reducing N_2O emissions is to use nitrification inhibitors to reduce nitrification. Nitrification inhibitors are chemicals that slow the first step of nitrification – the oxidation of NH_4^+ to NO_2^- – by either inhibiting or interfering with the metabolism of the micro-organisms involved (Singh & Verma, 2007). Specifically, they inhibit the ammonia mono-oxygenase enzyme activity of *Nitrosomonas* spp. and *Nitrosospira* spp (Qiao *et al.*, 2015). Inhibiting nitrification serves a dual purpose in the mitigation of N_2O as it not only reduces N_2O formed during the second step of nitrification, but also the electron acceptor used in denitrification. Thus, controlling nitrification will presumably also help reduce N_2O production by denitrification (Singh & Verma, 2007; Qiao *et al.*, 2015).

The use of nitrification inhibitors has been shown to be an effective tool for reducing N_2O emissions and to promote better N utilisation in soil (Barneze *et al.*, 2015). As a result, nitrification inhibitors have been recommended by the intergovernmental panel for climate change (IPCC) as a potential mitigation option for reducing agricultural N_2O emissions (IPCC, 2014).

2.4.4.1 Synthetic nitrification inhibitors

Synthetic inhibitors gained significance during the early 1960's in the US, parts of Europe and Japan for improved nutrient use efficiency, and to minimise N leaching

losses (Amberger, 1989; Zerulla *et al.*, 2001). Numerous compounds have been identified and used as nitrification inhibitors, particularly in agricultural soils. Many materials may inhibit nitrification due to being generally biocidal or by causing toxicity to the nitrifying organisms (Huber *et al.*, 1977; Singh & Verma, 2007). Some common nitrification inhibitors include: Dicyandiamide (DCD), dimethylpyrazole phosphate (DMPP), Nitrapyrin (N-serve). Other chemicals that have been shown to inhibit nitrification include: 2-amino-4-chloro-6-methylpyrimidine, terrazole, thiourea, 2-mercaptobenzothiazole, and acetylene (Huber *et al.*, 1977; Prasad & Power, 1995; Wrage *et al.*, 2004). These inhibitors have been shown to suppress ammonia mono-oxygenase enzyme activity in the nitrification pathway. Qiao *et al.* (2015) carried out a meta-analysis of 62 peer reviewed studies, and found that nitrification inhibitors decreased total N loss by 16.5%. Results are highly variable between studies, however, DCD has been shown to reduce N_2O emissions from urine by 60-80% following application to grazed pasture (e.g. de Klein & Ledgard, 2005; Di & Cameron, 2011; Luo *et al.*, 2013; Li *et al.*, 2015). DMPP has been shown to reduce N_2O emissions from cow urine by about 60% (Di & Cameron, 2011). Nitrate leaching, which is an important pathway for indirect N_2O emissions, is also reduced by about 30-70% following use of nitrification inhibitors (e.g. Di & Cameron, 2011; Ledgard *et al.*, 2014).

Some of the common nitrification inhibitors are now commercially available as fertiliser additives and are generally promoted for their agronomic benefits i.e. the maintaining of mineral N in the NH_4^+ form and in the root zone for increased pasture uptake and yield (Moir *et al.*, 2007). The rates required and method of application of nitrification inhibitors vary with each inhibitor, soil type, form of N, organic matter content, soil temperature and soil moisture (Huber *et al.*, 1977).

Synthetic nitrification inhibitors do have some disadvantages. DCD, for example, is not particularly efficient and as targeted application is difficult, high application rates are needed to attain sufficient nitrification inhibition (Zerulla *et al.*, 2001). DCD is also highly soluble in water and during heavy rainfall it may leach from the soil profile, or at least be separated from the NH_4^+ ions being nitrified (Zerulla *et al.*, 2001). Nitripyrin also has disadvantages in that it is highly volatile and so it cannot be combined with solid fertilisers (as it vaporises during processing), making application difficult (Zerulla *et al.*, 2001). Nitrification inhibitors can also have adverse impacts if used inappropriately or incorrectly. This was the case here

in New Zealand in 2012 when DCD residues were found in dairy products which raised concern for food safety. The application of DCD onto pastoral land is now no longer permitted in New Zealand until international agreement is reached on maximum allowable limits (Ministry for Primary Industries, 2014). As a result of this event, researchers are now exploring possible biological or naturally occurring nitrification inhibitors. It is suggested that there are fewer implications for food safety and public perceptions over the use of 'natural' products rather than synthetic products.

2.4.4.2 Biological nitrification inhibitors

The suppression of nitrification has been observed to occur naturally in some ecosystems, with suggestions that the inhibition originated from plants in the ecosystem (Subbarao *et al.*, 2007; Al-Ansari & Abdulkareem, 2014). For example, some species of pine and tropical grasses are believed to be responsible for low nitrification rates (Prasad & Power, 1995; Smits *et al.*, 2010; Subbarao *et al.*, 2013a). This is termed biological nitrification inhibition (BNI). BNI is the ability of certain plant species to release organic molecules from their roots that suppress the function and growth of nitrifying bacteria (Subbarao *et al.*, 2007). This interaction between plants and micro-organisms is broadly characterised as allelopathy (Bremner & McCarty, 1993).

There is extensive evidence to show that plant tissues contain a wide variety of chemical compounds, which are not involved in primary metabolism and these compounds vary according to family and species. These compounds termed 'secondary metabolites', play a variety of roles in protecting plants from a variety of stresses (Bennett & Wallsgrove, 1994; Erickson *et al.*, 2000). It has been hypothesised that BNI has evolved as part of some plants' adaptation mechanisms to conserve and use N efficiently in systems that are naturally limited in mineral N (Subbarao *et al.*, 2007). In these situations, plants that inhibit nitrification of NH_4^+ in the soil of their immediate rhizosphere, would have a competitive advantage over other plants (Bremner & McCarty, 1993), most likely due to the fact that it requires four times more metabolic energy to assimilate NO_3^- into protein than is needed for NH_4^+ (Subbarao *et al.*, 2012).

BNI has the potential to improve agronomic nutrient use efficiency and as a result, reduce both leached and gaseous N loss from agricultural systems. A field study

carried out over two years in China, showed higher vegetable yield and nutrient use efficiency for crops treated with biological nitrification inhibitors in comparison to the synthetic inhibitor DCD (Zhang *et al.*, 2015b). They also showed a reduction in N_2O emissions. Nitrous oxide emissions were also shown to be suppressed by about 90% in field plots of *B. humidicola* (known to release BNI) compared with a pasture species that has not shown to have BNI capacity (Subbarao *et al.*, 2013b). Several BNIs have been isolated from root exudates and plant tissues and BNIs that belong to many different chemical functional groups have been identified (Gopalakrishnan *et al.*, 2007; Subbarao *et al.*, 2013b). BNI synthesis and release appears to be stimulated by high NH_4^+ concentration in the soil (Subbarao *et al.*, 2015).

Residues of plant tissues are also known to release allelo-chemicals which inhibit nitrification in soil (Bremner & McCarty, 1993). As these chemicals are released during destruction or decomposition of the plant, it is thought they are a defence mechanism against herbivores, pests or pathogens but also inhibit nitrification (Bennett & Wallsgrove, 1994; Bending & Lincoln, 2000). Some examples of plants where tissue residues or extracts of tissues inhibit nitrification include neem, karanj and brassicas (Prasad & Power, 1995).

2.5 Glucosinolates and their hydrolysis products

While there are a wide variety of plant extracts that perform as BNIs, here the focus is placed on glucosinolates, because there has been extensive research carried out on these compounds and their breakdown products (Williams *et al.*, 1993; Kirkegaard & Sarwar, 1998; Bending & Lincoln, 2000; Morra & Kirkegaard, 2002; Wittstock & Halkier, 2002; Rumberger & Marschner, 2003; Matthiessen & Shackleton, 2005; Gimsing & Kirkegaard, 2006; Snyder *et al.*, 2010; Sun *et al.*, 2015; Zuluaga *et al.*, 2015). The breakdown products, in particular, have been shown to have some promise as potential nitrification inhibitors (Bending & Lincoln, 2000; Brown & Morra, 2009).

The tissues of *Brassicaceae* family members contain many secondary compounds including glucosinolates. Glucosinolates are sulphur (S) and N containing compounds, which are contained in cell vacuoles of plants. Upon disruption of tissues (i.e. physical damage or herbivory) glucosinolate is released and is

hydrolysed by myrosinase (bound in the cell wall) to form compounds including isothiocyanate (ITC), nitrile, and thiocyanate (Choesin & Boerner, 1991; Bones & Rossiter, 1996; Halkier & Du, 1997; Blažević *et al.*, 2017). These compounds affect the odour, taste and colour of plants, and are generally toxic to browsers and pathogens (Bennett & Wallsgrove, 1994; Bending & Lincoln, 2000). The type and proportions of these hydrolysis products are influenced by the conditions of hydrolysis including pH and temperature (Cole, 1976). The general structure and hydrolysis (catalysed by the myrosinase enzyme) is shown in Figure 2.10.

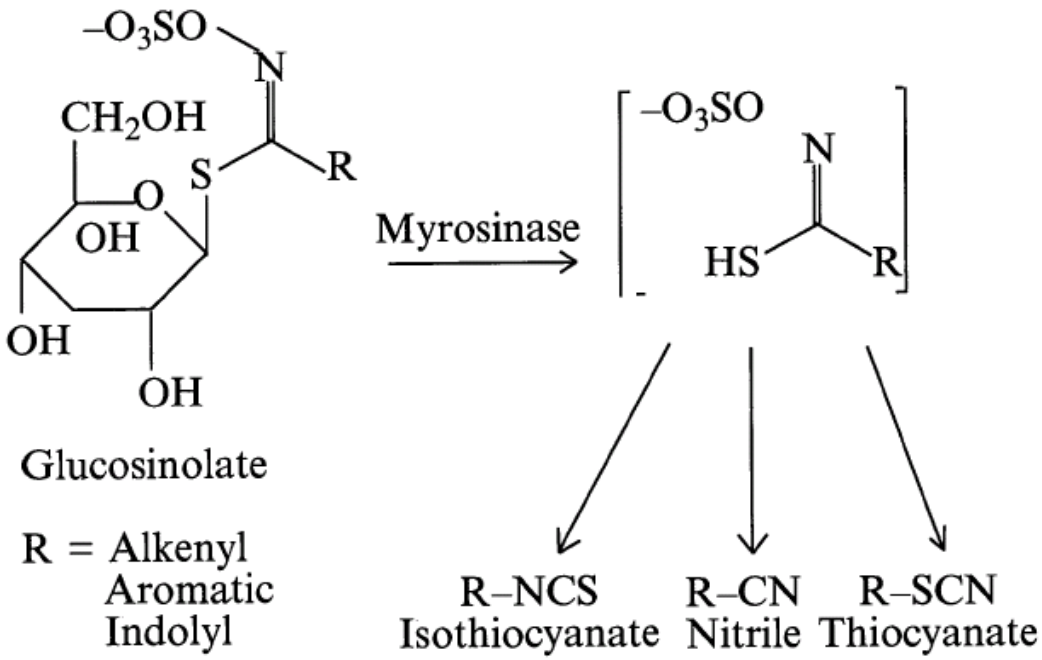

Figure 2.10: The structure of glucosinolates and their breakdown (Bennett & Wallsgrove, 1994). Note a wide variety of glucosinolates exist, where the R group (or side chain) varies.

There is little known about what effects secondary metabolites contained in plant material may have on soil organisms when they are returned to the soil. However, there are some studies that indicate that ITCs in particular are toxic to a range of soil organisms and could influence the activities of soil microbes including the rates of mineralisation/nitrification processes (Bending & Lincoln, 2000; Ryan *et al.*, 2006; Subbarao *et al.*, 2013a). A study by Bending and Lincoln (2000) assessed the potential of several different glucosinolate hydrolysis products to inhibit nitrification, and showed that all products had some impact on nitrification. ITCs,

however, had the largest effect of the products and within ITCs the various R-groups showed varying toxicity.

The mode by which glucosinolate hydrolysis products, such as ITCs, might inhibit nitrification is not entirely clear. The toxicity of ITCs arises from their capacity to bind to proteins, resulting in alteration of the structure of enzymes and thus affecting metabolic processes (Bending & Lincoln, 2000). Kirkegaard *et al.* (1999) states that ITC is a general biocide and may therefore reduce the population of all microbes. The consequent reduction in microbial activity during crop decay might cause an increase in soil mineral N due to reduced immobilisation. However, the same authors also showed a significant difference in soil microbial populations following brassica and wheat crops. In particular, NH_4^+ oxidiser populations were significantly lower following brassicas compared to populations following wheat. It is therefore possible that some hydrolysis products have a targeted effect on nitrifying microorganisms and so may make effective specific nitrification inhibitors. This possibility is also supported by Bending and Lincoln (2000) who demonstrated that ITCs inhibited nitrification by both direct effects on the size of nitrifying bacteria communities, and by reducing their nitrifying activities.

Glucosinolates are hydrolysed into ITC and nitriles upon disruption of plant cells following damage to plant tissues (Cole, 1976; Bones & Rossiter, 1996). The incorporation of plant tissues or residues into soil may, therefore, be one approach that can be utilised to control nitrification and mitigate soil N loss as part of a cropping systems approach (Subbarao *et al.*, 2012). Bending and Lincoln (2000) showed that nitrification was inhibited at concentrations of 0.5 μg ITC g^{-1} (dry weight) soil, and they calculated that this concentration was approximately 1% of that which could potentially be formed following incorporation of brassica crop residues into soil (based on work by Williams *et al.*, 1993). This suggests that incorporation of brassica tissues into soil could be a viable approach for inhibiting nitrification in a cropping system. This incorporation approach was further supported by Brown and Morra (2009) who measured NH_4^+ and NO_2^- accumulation in soil following incorporation of brassica crops into soil, indicating inhibition of nitrification. Kirkegaard *et al.* (1999) and Ryan *et al.* (2006) also both measured greater accumulation of mineral N in soil following growth of brassica (canola) crops than following either cereal, lupin or pea crops. In this case the increase in

mineral N was attributed to both the supply from decomposing root material and other residues, and a reduced rate of nitrification due to the presence of ITCs.

Incorporating plant tissues into soil may inhibit nitrification in cropping systems, however, this approach is less suitable for grazing systems where pastures are generally only renewed every 5-10 years, so there is little opportunity to incorporate plant material. Also, N substrate supplied in the excreta of grazing animals is the main driver of nitrification in these systems. Therefore, an alternative to incorporating plant residues may be to deliver BNI compounds to the soil through the grazing animals. Grazers eliminate unprocessed plant material, and secondary metabolites in excreta (Duncan & Milne, 2007; Estell, 2010). One advantage of this approach is that the inhibitor is delivered with the main N source for N_2O production. For example, Ledgard *et al.* (2008) and Welten *et al.* (2013) showed that urine patches could be individually targeted with a nitrification inhibitor (in this instance DCD) by orally administering the inhibitor to grazing animals that was subsequently excreted in the urine. It is possible that this method could also be used for administering brassica derived inhibitors to urine patches. Luo *et al.* (2015) reported reduced N_2O emissions from urine patches derived from animals fed a brassica crop compared to urine from animals fed perennial pasture. This supports glucosinolate hydrolysis products such as ITCs may be delivered to the soil within the urine of animals fed brassicas, and nitrification may subsequently be inhibited in the urine patch.

2.6 Summary

Review of the literature has identified that ruminant urine patches are the main source of N_2O emissions in grazed agricultural systems. Suppressing nitrification has been recognised as an effective approach for reducing N_2O loss from these systems as it reduces both N_2O produced during nitrification, as well as reducing the N substrate for denitrification which is the main process for N_2O production.

A potentially promising way to reduce nitrification in pastoral agricultural systems is to exploit the BNI capacity of some plants. Secondary metabolites from brassica crops, GLS hydrolysis products, in particular have been suggested as a possible BNI that could be employed in grazed systems. This review has revealed, however,

that there is little research that has examined the potential for these feeds to reduce N_2O emissions. Additionally, most of the research on the BNI potential of GLS hydrolysis products has been carried out under low, or nil (added) N conditions, which does not assess their inhibition potential under high N urine patch conditions.

Therefore, there is a need for further research into glucosinolate hydrolysis products and their potential for inhibiting nitrification and specifically decreasing N_2O emissions from high N environments.

2.7 References

Akiyama, H., Yan, X., & Yagi, K. (2010). Evaluation of effectiveness of enhanced-efficiency fertilizers as mitigation options for N_2O and NO emissions from agricultural soils: meta-analysis. *Global Change Biology, 16*, 1837-1846.

Al-Ansari, A.-M. S., & Abdulkareem, M. A. (2014). Some plant extracts retarde nitrification in soil. *Acta Agriculturae Slovenica, 103*, 5-13.

Alexander, M. (1965). Nitrification. In W. V. Bartholomew & F. E. Clark (Eds.), *Soil Nitrogen* (pp. 307-343). Wisconsin, United States of America: American Society of Agronomy.

Amberger, A. (1989). Research on dicyandiamide as a nitrification inhibitor and future outlook. *Communications in Soil Science and Plant Analysis, 20*, 1933-1955.

Ball, B. C., Parker, J. P., & Scott, A. (1999). Soil and residue management effects on cropping conditions and nitrous oxide fluxes under controlled traffic in Scotland. *Soil and Tillage Research, 52*, 191-201.

Barneze, A. S., Minet, E. P., Cerri, C. C., & Misselbrook, T. (2015). The effect of nitrification inhibitors on nitrous oxide emissions from cattle urine depositions to grassland under summer conditions in the UK. *Chemosphere, 119*, 122-129.

Bateman, E. J., & Baggs, E. M. (2005). Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space. *Biology and Fertility of Soils, 41*, 379-388.

Beever, D. E., Losada, H. R., Cammell, R. T., Evans, R. T., & Haines, M. J. (1986). Effect of forage species and season on nutrient digestion and supply in grazing cattle. *British Journal of Nutrition, 56*, 209-225.

Bending, G. D., & Lincoln, S. D. (2000). Inhibition of soil nitrifying bacteria communities and their activities by glucosinolate hydrolysis products. *Soil Biology and Biochemistry, 32*, 1261-1269.

Bennett, R. N., & Wallsgrove, R. M. (1994). Tansley Review No. 72. Secondary metabolites in plant defence mechanisms. *New Phytologist, 127*, 617-633.

Blažević, I., Montaut, S., Burčul, F., & Rollin, P. (2017). Glucosinolates: Novel sources and biological potential. In J. Merillon & K. G. Ramawat (Eds.), *Glucosinolates* (pp. 15-73). Switzerland: Springer International Publishing.

Bones, A. M., & Rossiter, J. T. (1996). The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiologia Plantarum, 97*, 194-208.

Bouwman, A. F., Beusen, A. H. W., & Billen, G. (2009). Human alteration of the global nitrogen and phosphorus soil balances for the period 1970–2050. *Global Biogeochemical Cycles, 23*, GB0A04.

Bremner, J. M., & McCarty, G. W. (1993). Inhibition of nitrification in soil by allelochemicals derived from plants and plant residues. In J. M. Bollag & G. Stotzky (Eds.), *Soil Biochemistry* (pp. 181-218). New York, United States of America: Marcel Dekker Inc.

Brock, T. D., Madigan, M. T., Martinko, J. M., & Parker, J. (1994). *Biology of Microorganisms (7th edition)*. New Jersey, U.S.: Prentice-Hall International.

Broderick, G. A. (2003). Effects of Varying Dietary Protein and Energy Levels on the Production of Lactating Dairy Cows1. *Journal of Dairy Science, 86*, 1370-1381.

Brown, P. D., & Morra, M. J. (2009). Brassicaceae tissues as inhibbitors of nitrification in soil. *Journal of Agricultural and Food Chemistry, 57*, 7706-7711.

Burford, J. R., & Bremner, J. M. (1975). Relationships between the denitrification capacities of soils and total, water soluble and readily decomposable soil organic matter. *Soil Biology and Biochemistry, 7*, 389-394.

Burke, F., O'Donovan, M. A., Murphy, J. J., O'Mara, F. P., & Mulligan, F. J. (2008). Effect of pasture allowance and supplementation with maize silage and concentrates differing in crude protein concentration on milk production and nitrogen excretion by dairy cows. *Livestock Science, 114*, 325-335.

Butterbach-Bahl, K., Baggs, E. M., Dannenmann, M., Kiese, R., & Zechmeister-Boltenstern, S. (2013). Nitrous oxide emissions from soils: how well do we understand the processes and their controls? *Philosophical Transactions of the Royal Society B: Biological Sciences, 368*, 20130122.

Cameron, K. C. (1992). Nitrogen in soil. In W. A. Nierenberg (Ed.), *Encyclopedia of Earth System Science* (pp. 307-317). San Diego: Academic Press.

Cameron, K. C., Di, H. J., & Moir, J. L. (2013). Nitrogen losses from the soil/plant system: a review. *Annals of Applied Biology, 162*, 145-173.

Carter, M. S. (2007). Contribution of nitrification and denitrificaiton to N_2O emissions from urine patches. *Soil Biology and Biochemistry, 39*, 2091-2102.

Carulla, J. E., Kreuzer, M., Machmüller, A., & Hess, H. D. (2005). Supplementation of Acacia mearnsii tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. *Australian Journal of Agricultural Research, 56*, 961-970.

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

Chapin, F. S. (1980). The Mineral Nutrition of Wild Plants. *Annual Review of Ecology and Systematics, 11*, 233-260.

Choesin, D. N., & Boerner, R. E. J. (1991). Allyl isothiocyanate release and the allelopathic potential of *Brassica napus* (Brassicaceae). *American Journal of Botany, 78*, 1083-1090.

Clough, T. J., Sherlock, R. R., & Kelliher, F. M. (2003). Can liming mitigate N_2O fluxes from a urine-amended soil? *Soil Research, 41*(3), 439-457.

Cole, J. A., & Brown, C. M. (1980). Nitrite reduction to ammonia by fermentative bacteria: A short circuit in the biological nitrogen cycle. *FEMS Microbiology Letters, 7*, 65-72.

Cole, R. A. (1976). Isothiocyanates, nitriles and thiocyanates as products of autolysis of glucosinolates in Cruciferae. *Phytochemistry, 15*, 759-762.

Compton, J. E., Harrison, J. A., Dennis, R. L., Greaver, T. L., Hill, B. H., Jordan, S. J., Walker, H., & Campbell, H. V. (2011). Ecosystem services altered by human changes in the nitrogen cycle: a new perspective for US decision making. *Ecology Letters, 14*, 804-815.

Coyne, M. S. (2018). Denitrification in soil. In R. Lal & B. A. Stewart (Eds.), *Soil nitrogen uses and environmental impacts*. U.S.: CRC Press.

Cuttle, S. P. (2008). Impacts of pastoral grazing on soil quality. In R. W. McDowell (Ed.), *Environmental impacts of pasture-based farming* (pp. 33-74). United Kingdom: CAB International.

Dai, Y., Di, H. J., Cameron, K. C., & He, J.-Z. (2013). Effects of nitrogen application rate and a nitrification inhibitor dicyandiamide on ammonia oxidizers and N_2O emissions in a grazed pasture soil. *Science of the Total Environment, 465*, 125-135.

Davidson, E. A., Keller, M., Erickson, H. E., Verchot, L. V., & Veldkamp, E. (2000). Testing a conceptual model of soil emissions of nitrous and nitric oxides. *BioScience, 50*, 667-680.

Davies, M., Smith, K., & Vinten, A. (2001). The mineralisation and fate of nitrogen following ploughing of grass and grass-clover swards. *Biology and Fertility of Soils, 33*, 423-434.

de Klein, C. A. M., Pinares-Patino, C., & Waghorn, G. C. (2008). Greenhouse gas emissions. In R. W. McDowell (Ed.), *Environmental impacts of pasture-based farming*. United Kingdom: CAB International.

de Klein, C. A. M., Sherlock, R. R., Cameron, K. C., & van der Weerden, T. J. (2001). Nitrous oxide emissions from agricultural soils in New Zealand—A review of current knowledge and directions for future research. *Journal of the Royal Society of New Zealand, 31*, 543-574.

de Klein, C. M., & Ledgard, S. (2005). Nitrous oxide emissions from New Zealand agriculture – key sources and mitigation strategies. *Nutrient Cycling in Agroecosystems, 72*, 77-85.

de Vries, W., Kros, J., Kroeze, C., & Seitzinger, S. P. (2013). Assessing planetary and regional nitrogen boundaries related to food security and adverse environmental impacts. *Current Opinion in Environmental Sustainability, 5*, 392-402.

Dell, C. J., Han, K., Bryant, R. B., & Schmidt, J. P. (2014). Nitrous oxide emissions with enhanced efficiency nitrogen fertilizers in a rainfed system. *Agronomy Journal, 106*, 723-731.

Di, H. J., & Cameron, K. C. (2011). How does the application of different nitrification inhibitors affect nitrous oxide emissions and nitrate leaching from cow urine in grazed pastures? *Soil Use and Management, 28*, 54-61.

Di, H. J., Cameron, K. C., Shen, J. P., Winefield, C. S., O/'Callaghan, M., Bowatte, S., & He, J. Z. (2009). Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature Geosci, 2*(9), 621-624.

Di, H. J., Cameron, K. C., Podolyan, A., Edwards, G. R., de Klein, C. A. M., Dynes, R., & Woods, R. (2016). The potential of using alternative pastures, forage crops and gibberellic acid to mitigate nitrous oxide emissions. *Journal of Soils and Sediments, 16*, 2252-2262.

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

do Carmo, J. B., Neill, C., Garcia-Montiel, D. C., de Cássia Piccolo, M., Cerri, C. C., Steudler, P. A., de Andrade, C. A., Passianoto, C. C., Feigl, B. J., & Melillo, J. M. (2005). Nitrogen dynamics during till and no-till pasture restoration sequences in Rondônia, Brazil. *Nutrient Cycling in Agroecosystems, 71*, 213-225.

Drewry, J. J., Cameron, K. C., & Buchan, G. D. (2008). Pasture yield and soil physical property responses to soil compaction from treading and grazing - a review. *Australian Journal of Agricultural Research, 46*, 237-256.

Duncan, A. J., & Milne, J. A. (2007). Effects of oral administration of brassica secondary metabolites, allyl cyanide, allyl isothiocyanate and dimethyl disulphide, on the voluntary food intake and metabolism of sheep. *British Journal of Nutrition, 70*, 631-645.

Edwards, G. C., De Ruiter, J. M., Dalley, D. E., Pinxterhuis, J. B., Cameron, K. C., Bryant, R. H., Di, H. J., Malcolm, B. J., & Chapman, D. F. (Compiler) (2014). *Urinary nitrogen concentration of cows grazing fodder beet, kale and kale-oat forage systems in winter.* Australian Dairy Science Symposium. Waikato, New Zealand.

Erickson, A. J., Ramsewak, R. S., Smucker, A. J., & Nair, M. G. (2000). Nitrification Inhibitors from the roots of *Leucaena leucocephala*. *Journal of Agricultural and Food Chemistry, 48*, 6174-6177.

Estell, R. E. (2010). Coping with shrub secondary metabolites by ruminants. *Small Ruminant Research, 94*, 1-9.

Firestone, M. K., & Davidson, E. A. (1989). Microbial basis of NO and N_2O production and consumption. In M. O. Andreae & D. S. Schimel (Eds.), *Exchange of trace gases between terrestrial ecosystems and the atmosphere* (pp. 7-21). New York, United States of America: John Wiley and Sons Ltd.

Fowler, D., Coyle, M., Skiba, U., Sutton, M. A., Cape, J. N., Reis, S., Sheppard, L. J., Jenkins, A., Grizzetti, B., Galloway, J. N., Vitousek, P., Leach, A., Bouwman, A. F., Butterbach-Bahl, K., Dentener, F., Stevenson, D., Amann, M., & Voss, M. (2013). The global nitrogen cycle in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences, 368*, 20130164.

Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., Asner, G. P., Cleveland, C. C., Green, P. A., Holland, E. A., Karl, D. M., Michaels, A. F., Porter, J. H., Townsend, A. R., & Vöosmarty, C. J. (2004). Nitrogen cycles: past, present, and future. *Biogeochemistry, 70*, 153-226.

Gimsing, A. L., & Kirkegaard, J. A. (2006). Glucosinolate and isothiocyanate concentration in soil following incorporation of *Brassica* biofumigants. *Soil Biology and Biochemistry, 38*, 2255-2264.

Goh, K. M., & Haynes, R. J. (1986). Nitrogen and Agronomic Practice. In R. J. Haynes (Ed.), *Mineral Nitrogen in the Plant-Soil System* (pp. 379-442). Florida: Academic Press.

Goodroad, L. L., & Keeney, D. R. (1984). Nitrous oxide production in aerobic soils under varying pH, temperature and water content. *Soil Biology and Biochemistry, 16*, 39-43.

Gopalakrishnan, S., Subbarao, G. V., Nakahara, K., Yoshihashi, T., Ito, O., Maeda, I., Ono, H., & Yoshida, M. (2007). Nitrification inhibitors from the root tissues of *Brachiaria humidicola*, a tropical grass. *Journal of Agricultural and Food Chemistry, 55*, 1385-1388.

Granli, T., & Bockman, O. C. (1994). Nitrous oxide from agriculture. *Norwegian Journal of Agricultural Sciences, Supplement No. 12*.

Grave, R. A., Nicoloso, R. d. S., Cassol, P. C., da Silva, M. L. B., Mezzari, M. P., Aita, C., & Wuaden, C. R. (2018). Determining the effects of tillage and nitrogen sources on soil N_2O emission. *Soil and Tillage Research, 175*, 1-12.

Groffman, P., Butterbach-Bahl, K., Fulweiler, R., Gold, A., Morse, J., Stander, E., Tague, C., Tonitto, C., & Vidon, P. (2009). Challenges to incorporating spatially and temporally explicit phenomena (hotspots and hot moments) in denitrification models. *Biogeochemistry, 93*, 49-77.

Gruber, N., & Galloway, J. N. (2008). An earth-system perspective of the global nitrogen cycle. *Nature, 451*, 293-296.

Halkier, B. A., & Du, L. (1997). The biosynthesis of glucosinolates. *Trends in Plant Science, 2*, 425-431.

Hayatsu, M., Tago, K., & Saito, M. (2008). Various players in the nitrogen cycle: Diversity and functions of the microorganisms involved in nitrification and denitrification. *Soil Science & Plant Nutrition, 54*, 33-45.

Haynes, R. J. (1986a). Origin, Distribution and Cycling of Nitrogen in Terrestrial Ecosystems. In R. J. Haynes & F. L. Orlando (Eds.), *Mineral Nitrogen in the Plant-Soil System.* (pp. 1). London,: Academic Press Inc.

Haynes, R. J. (1986b). Uptake and assimilation of mineral nitrogen by plants. In R. J. Haynes & F. L. Orlando (Eds.), *Mineral Nitrogen in the Plant-Soil System* (pp. 303-358). New York: Academic Press,.

Hedley, C. (2015). The role of precision agriculture for improved nutrient management on farms. *Journal of the Science of Food and Agriculture, 95*, 12-19.

Heil, J., Vereecken, H., & Brüggemann, N. (2016). A review of chemical reactions of nitrification intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil. *European Journal of Soil Science, 67*, 23-39.

Herold, M. B., Baggs, E. M., & Daniell, T. J. (2012). Fungal and bacterial denitrification are differently affected by long-term pH amendment and cultivation of arable soil. *Soil Biology and Biochemistry, 54*, 25-35.

Hodge, A., Robinson, D., & Fitter, A. (2000). Are microorganisms more effective than plants at competing for nitrogen? *Trends in Plant Science, 5*, 304-308.

Houlbrooke, D. J., Paton, R. J., Littlejohn, R. P., & Morton, J. D. (2011). Land-use intensitfication in New Zealand: Effects on soil properties and pasture production. *The Journal of Agricultural Science, 149*, 337-349.

Houlbrooke, D. J., Horne, D. J., Hedley, M. J., Hanly, J. A., Scotter, D. R., & Snow, V. O. (2004). Minimising surface water pollution resulting from farm‐dairy effluent application to mole‐pipe drained soils. I. An evaluation of the deferred irrigation system for sustainable land treatment in the Manawatu. *New Zealand Journal of Agricultural Research, 47*, 405-415.

Houlton, B. Z., Morford, S. L., & Dahlgren, R. A. (2018). Convergent evidence for widespread rock nitrogen sources in Earth's surface environment. *Science, 360*, 58-62.

Hu, H.-W., Chen, D., & He, J.-Z. (2015). Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. *FEMS Microbiology Reviews, 39*, 729-749.

Huber, D. M., Warren, H. L., Nelson, D. W., & Tsai, C. Y. (1977). Nitrification inhibitors: New tools for food production. *BioScience, 27*(8), 523-529.

Huhtanen, P., Nousiainen, J. I., Rinne, M., Kytölä, K., & Khalili, H. (2008). Utilization and Partition of Dietary Nitrogen in Dairy Cows Fed Grass Silage-Based Diets. *Journal of Dairy Science, 91*(9), 3589-3599.

IPCC. (2008). IPCC Guidelines for National Greenhouse Gas Inventories - A primer, Prepared by the National Greenhouse Gas Inventories Programme. In H. S. Eggleston, K. Miwa, N. Srivastava & K. Tanabe (Eds.). IGES, Japan.

IPCC. (2014). *Climate change 2014: Mitigation of climate change. Contribution of working group III to the fifth assessment report of the intergovernmental panel on climate change.* Cambridge, United Kingdom and New York, United States of America. 1454p.

Ismande, J., & Touraine, B. (1994). N Demand and the Regulation of Nitrate Uptake. *Plant physiology, 105*, 3-7.

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

Keeney, D. R., Fillery, I. R., & Marx, G. P. (1979). Effect of temperature on the gaseous nitrogen products of denitrification in a silt loam soil. *Soil Science Society of America Journal, 43*, 1124-1128.

Kielland, K. (1994). Amino acid absorption by arctic plants: implications for plant nutrition and nitrogen cycling. *Ecology, 75*, 2373-2383.

Kirkegaard, J. A., & Sarwar, M. (1998). Biofumigation potential of brassicas. *Plant and Soil, 201*, 71-89.

: Kirkegaard, J. A., Howe, G. N., & Mele, P. M. (1999). Enhanced accumulation of mineral-N following Canola. *Australian Journal of Experimental Agriculture, 39*, 587-593.

Knowles, R. (1982). Denitrification. *Microbiological Reviews, 46*, 43-70.

Kunhikrishnan, A., Thangarajan, R., Bolan, N. S., Xu, Y., Mandal, S., Gleeson, D. B., Seshadri, B., Zaman, M., Barton, L., Tang, C., Luo, J., Dalal, R., Ding, W., Kirkham, M. B., & Naidu, R. (2016). Chapter One - Functional Relationships of Soil Acidification, Liming, and Greenhouse Gas Flux. In D. L. Sparks (Ed.), *Advances in Agronomy* (pp. 1-71).

Laughlin, R. J., & Stevens, R. J. (2002). Evidence for Fungal Dominance of Denitrification and Codenitrification in a Grassland Soil. *Soil Science Society of America Journal, 66*, 1540-1548.

Ledgard, S. F., Luo, J., Sprosen, M. S., Wyatt, J. B., Balvert, S. F., & Lindsey, S. B. (2014). Effects of the nitrification inhibitor dicyandiamide (DCD) on pasture production, nitrous oxide emissions and nitrate leaching in Waikato, New Zealand. *New Zealand Journal of Agricultural Research, 57*, 294-315.

Ledgard, S. F., Menneer, J. C., Dexter, M. M., Kear, M. J., Lindsey, S., Peters, J. S., & Pacheco, D. (2008). A novel concept to reduce nitrogen losses from grazed pastures by administering soil nitrogen process inhibitors to ruminant animals: A study with sheep. *Agriculture, Ecosystems & Environment, 125*, 148-158.

Lee, M. R. F., Harris, L. J., Moorby, J. M., Humphreys, M. O., Theodorou, M. K., MacRae, J. C., & Scollan, N. D. (2002). Rumen metabolism and nitrogen flow to the small intestine in steers offered Lolium perenne containing different levels of water-soluble carbohydrate. *Animal Science, 74*, 587-596.

Li, J., Luo, J., Shi, Y., Houlbrooke, D., Wang, L., Lindsey, S., & Li, Y. (2015). Nitrogen gaseous emissions from farm effluent application to pastures and mitigation measures to reduce the emissions: a review. *New Zealand Journal of Agricultural Research, 58*, 339-353.

Li, J., Shi, Y., Luo, J., Zaman, M., Houlbrooke, D., Ding, W., Ledgard, S., & Ghani, A. (2014). Use of nitrogen process inhibitors for reducing gaseous nitrogen losses from land-applied farm effluents. *Biology and Fertility of Soils, 50*, 133-145.

Linn, D. M., & Doran, J. W. (1984). Effect of Water-Filled Pore Space on Carbon Dioxide and Nitrous Oxide Production in Tilled and Nontilled Soils1. *Soil Science Society of America Journal, 48*, 1267-1272.

Lipson, D. A., Bowman, W. D., & Monson, R. K. (1996). Luxury Uptake and Storage of Nitrogen in the Rhizomatous Alpine Herb, Bistorta Bistortoides. *Ecology, 77*, 1277-1285.

Liu, B., Mørkved, P. T., Frostegård, Å., & Bakken, L. R. (2010). Denitrification gene pools, transcription and kinetics of NO, N_2O and N_2 production as affected by soil pH. *FEMS Microbiology Ecology, 72*, 407-417.

Luo, J., Ledgard, S. F., & Lindsey, S. B. (2013). Nitrous oxide and greenhouse gas emissions from grazed pastures as affected by use of nitrification inhibitor and restricted grazing regime. *Science of the Total Environment, 465*, 107-114.

Luo, J., de Klein, C. A. M., Ledgard, S. F., & Saggar, S. (2010). Management options to reduce nitrous oxide emissions from intensively grazed pastures: A review. *Agriculture, Ecosystems & Environment, 136*, 282-291.

Luo, J., Ledgard, S. F., de Klein, C. A. M., Lindsey, S. B., & Kear, M. (2008). Effects of dairy farming intensification on nitrous oxide emissions. *Plant and Soil, 309*, 227-237.

Luo, J., Sun, X. Z., Pacheco, D., Ledgard, S., Lindsey, S., Hoogendoorn, C. J., Wise, B., & Watkins, N. (2015). Nitrous oxide emission factors for urine and dung from sheep fed either fresh forage rape (*Brassica napus* L.) or fresh perennial rygrass (*Lolium perenne* L.). *Animal, 9*, 534-543.

Luo, J., Wyatt, J., van der Weerden, T. J., Thomas, S. M., de Klein, C. A. M., Li, Y., Rollo, M., Lindsey, S., Ledgard, S. F., Li, J., Ding, W., Qin, S., Zhang, N., Bolan, N., Kirkham, M. B., Bai, Z., Ma, L., Zhang, X., Wang, H., Liu, H., & Rys, G. (2017). Chapter Five - Potential Hotspot Areas of Nitrous Oxide Emissions From Grazed Pastoral Dairy Farm Systems. In D. L. Sparks (Ed.), *Advances in Agronomy* (pp. 205-268): Academic Press.

Maag, M., & Vinther, F. P. (1996). Nitrous oxide emission by nitrification and denitrification in different soil types and at different soil moisture contents and temperatures. *Applied Soil Ecology, 4*(1), 5-14.

Maeda, K., Spor, A., Edel-Hermann, V., Heraud, C., Breuil, M.-C., Bizouard, F., Toyoda, S., Yoshida, N., Steinberg, C., & Philippot, L. (2015). N_2O production, a widespread trait in fungi. *Scientific Reports, 5*, 9697.

Malhi, S. S., McGill, W. B., & Nyborg, M. (1990). Nitrate losses in soils: Effect of temperature, moisture and substrate concentration. *Soil Biology and Biochemistry, 22*, 733-737.

Mathieu, O., Hénault, C., Lévêque, J., Baujard, E., Milloux, M. J., & Andreux, F. (2006). Quantifying the contribution of nitrification and denitrification to the nitrous oxide flux using 15N tracers. *Environmental Pollution, 144*(3), 933-940.

Matthews, R. A., Chadwick, D. R., Retter, A. L., Blackwell, M. S. A., & Yamulki, S. (2010). Nitrous oxide emissions from small-scale farmland features of UK livestock farming systems. *Agriculture, Ecosystems & Environment, 136*, 192-198.

Matthiessen, J. N., & Shackleton, M. A. (2005). Biofumigation: environmental impacts on the biological activity of diverse pure and plant-derived isothiocyanates. *Pest Management Science, 61*, 1043-1051.

McDowell, R. W., & Srinivasan, M. S. (2009). Identifying critical source areas for water quality: 2. Validating the approach for phosphorus and sediment losses in grazed headwater catchments. *Journal of Hydrology, 379*, 68-80.

McInnes, K. J., Ferguson, R. B., Kissel, D. E., & Kanemasu, E. T. (1986). Field Measurements of Ammonia Loss from Surface Applications of Urea Solution to Bare Soil. *Agronomy Journal, 78*, 192-196.

McLaren, R. G., & Cameron, K. C. (1990). *Soil science: An introduction to the properties and management of New Zealand soils.* Auckland, New Zealand: Oxford University Press.

McMillan, A. M. S., Pal, P., Phillips, R. L., Palmada, T., Berben, P. H., Jha, N., Saggar, S., & Luo, J. (2016). Can pH amendments in grazed pastures help reduce N_2O emissions from denitrification? - The effects of liming and urine addition on the completion of denitrification in fluvial volcanic soils. *Soil Biology and Biochemistry, 93*, 90-104.

Menneer, J. C., Ledgard, S., McLay, C., & Silvester, W. (2005). Animal treading stimulates denitrification in soil under pasture. *Soil Biology and Biochemistry, 37*, 1625-1629.

Miller, L. A., Moorby, J. M., Davies, D. R., Humphreys, M. O., Scollan, N. D., MacRae, J. C., & Theodorou, M. K. (2001). Increased concentration of water-soluble carbohydrate in perennial ryegrass (Lolium perenne L.): milk production from late-lactation dairy cows. *Grass and Forage Science, 56*, 383-394.

Ministry for Primary Industries. (2014). *Dairy national chemical contaminants programme.* Wellington, New Zealand, Ministry for Primary Industries. from http://www.foodsafety.govt.nz/elibrary/industry/dairy-national-chemical-contam-surveillance-12-13.pdf.

Ministry for the Environment. (2017). *New Zealand's Greenhouse Gas Inventory: 1990-2015.* Wellington, New Zealand. 542p., from:

http://www.mfe.govt.nz/sites/default/files/media/Climate%20Change/FINAL%20GHG%20inventory%20-%2025%20May.pdf.

Misselbrook, T., Del Prado, A., & Chadwick, D. (2013). Opportunities for reducing environmental emissions from forage-based dairy farms. *Agricultural and Food Science, 22*, 15.

Misselbrook, T. H., Powell, J. M., Broderick, G. A., & Grabber, J. H. (2005). Dietary Manipulation in Dairy Cattle: Laboratory Experiments to Assess the Influence on Ammonia Emissions. *Journal of Dairy Science, 88*, 1765-1777.

Misselbrook, T. H., Cardenas, L. M., Camp, V., Thorman, R. E., Williams, J. R., Rollett, A. J., & Chambers, B. J. (2014). An assessment of nitrification inhibitors to reduce nitrous oxide emissions from UK agriculture. *Environmental Research Letters, 9*, 115006.

Moir, J. L., Cameron, K. C., & Di, H. J. (2007). Effects of the nitrification inhibitor dicyandiamide on soil mineral N, pasture yield, nutrient uptake and pasture quality in a grazed pasture system. *Soil Use and Management, 23*, 111-120.

Moorby, J. M., Evans, R. T., Scollan, N. D., MacRae, J. C., & Theodorou, M. K. (2006). Increased concentration of water-soluble carbohydrate in perennial ryegrass (Lolium perenne L.). Evaluation in dairy cows in early lactation. *Grass and Forage Science, 61*, 52-59.

Mori, A., & Hojito, M. (2007). Grassland renovation increases N_2O emission from a volcanic grassland soil in Nasu, Japan. *Soil Science and Plant Nutrition, 53*, 812-818.

Morra, M. J., & Kirkegaard, J. A. (2002). Isothiocyanate release from soil-incorporated Brassica tissues. *Soil Biology and Biochemistry, 34*, 1683-1690.

Mosier, A., Kroeze, C., Nevison, C., Oenema, O., Seitzinger, S., & van Cleemput, O. (1998). Closing the global N_2O budget: nitrous oxide emissions through the agricultural nitrogen cycle. *Nutrient Cycling in Agroecosystems, 52*, 225-248.

Mothapo, N., Chen, H., Cubeta, M. A., Grossman, J. M., Fuller, F., & Shi, W. (2015). Phylogenetic, taxonomic and functional diversity of fungal denitrifiers and associated N_2O production efficacy. *Soil Biology and Biochemistry, 83*, 160-175.

Mothapo, N. V., Chen, H., Cubeta, M. A., & Shi, W. (2013). Nitrous oxide producing activity of diverse fungi from distinct agroecosystems. *Soil Biology and Biochemistry, 66*, 94-101.

Mulligan, F. J., Dillon, P., Callan, J. J., Rath, M., & O'Mara, F. P. (2004). Supplementary Concentrate Type Affects Nitrogen Excretion of Grazing Dairy Cows. *Journal of Dairy Science, 87*, 3451-3460.

Myhre, G., Shindell, D., Breon, F.-M., Collins, W., Fuglestvedt, J., Huang, J., Koch, D., Lamarque, J.-F., Lee, D., Mendoza, B., Nakajima, T., Robock, A., Stephens, G., Tekemura, T., & Zhang, H. (2013). Anthropogenic and natural radiative forcing. In T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex & P. M. Midgley (Eds.), *Climate Change 2013: The physical science basis. Contribution of Working Group I to the fifth assessment report of the Intergovernmental Panel on Climate Change.* (pp. 659-740). Cambridge: Cambridge University Press.

Nasholm, T. (1998). Boreal forest plants take up organic nitrogen. *Nature, 392*, 914-916.

Nie, Z. N., Ward, G. N., & Michael, A. T. (2001). Impact of pugging by dairy cows on pastures and indicators of pugging damage to pasture soil in south-western Victoria. *Australian Journal of Agricultural Research, 52*, 37-43.

Nielsen, N. M., Kristensen, T., Nørgaard, P., & Hansen, H. (2003). The effect of low protein supplementation to dairy cows grazing clover grass during half of the day. *Livestock Production Science, 81*, 293-306.

Parfitt, R. L., Schipper, L. A., Baisden, W. T., & Elliott, A. H. (2006). Nitrogen inputs and outputs for New Zealand in 2001 at national and regional scales. *Biogeochemistry, 80*, 71-88.

Parkin, T. B. (1987). Soil Microsites as a Source of Denitrification Variability1. *Soil Science Society of America Journal, 51*, 1194-1199.

Prasad, R., & Power, J. F. (1995). Nitrification inhibitors for agriculture, health, and the environment. *Advances in Agronomy, Volume 54*, 233-281.

Qiao, C., Liu, L., Hu, S., Compton, J. E., Greaver, T. L., & Li, Q. (2015). How inhibiting nitrification affects nitrogen cycle and reduces environmental impacts of anthropogenic nitrogen input. *Global Change Biology, 21*, 1249-1257.

Ravishankara, A. R., Daniel, J. S., & Portmann, R. W. (2009). Nitrous oxide (N_2O): The dominant ozone-depleting substance emitted in the 21st century. *Science, 326*, 123-125.

Reay, D. S., Davidson, E. A., Smith, K. A., Smith, P., Melillo, J. M., Dentener, F., & Crutzen, P. J. (2012). Global agriculture and nitrous oxide emissions. *Nature Climate Change, 2*, 410-416.

Reinsch, T., Loges, R., Kluß, C., & Taube, F. (2018). Renovation and conversion of permanent grass-clover swards to pasture or crops: Effects on annual N_2O emissions in the year after ploughing. *Soil and Tillage Research, 175*, 119-129.

Robertson, G. P., & Vitousek, P. M. (2009). Nitrogen in agriculture: Balancing the cost of an essential resource. *Annual Review of Environment and Resources, 34*, 97-125.

Robertson, G. P., Bruulsema, T. W., Gehl, R. J., Kanter, D., Mauzerall, C., Rotz, C. A., & Williams, C. O. (2013). Nitrogen-climate interactions in US agriculture. *Biogeochemistry, 114*, 4-70.

Rumberger, A., & Marschner, P. (2003). 2-Phenylethylisothiocyanate concentration and microbial community composition in the rhizosphere of canola. *Soil Biology and Biochemistry, 35*, 445-452.

Rutting, T., Boeckx, P., Muller, C., & Klemedtsson, L. (2011). Assessment of the importance of dissimilatory nitrate reduction to ammonium for the terrestrial nitrogen cycle. *Biogeosciences, 8*, 1779-1791.

Ryan, M. H., Kirkegaard, J. A., & Angus, J. F. (2006). *Brassica* crops stimulate soil mineral N accumulation. *Australian Journal of Soil Research, 44*, 367-377.

Saggar, S., Bolan, N. S., Bhandral, R., Hedley, C. B., & Luo, J. (2004). A review of emissions of methane, ammonia, and nitrous oxide from animal excreta deposition and farm effluent application in grazed pastures. *New Zealand Journal of Agricultural Research, 47*, 513-544.

Schils, R. L. M., Eriksen, J., Ledgard, S. F., Vellinga, T. V., Kuikman, P. J., Luo, J., Petersen, S. O., & Velthof, G. L. (2013). Strategies to mitigate nitrous oxide emissions from herbivore production systems. *Animal, 7*(Supplements1), 29-40.

Schimel, J. P., & Bennett, J. (2004). Nitrogen Mineralization: Challenges of a Changing Paradigm. *Ecology, 85*, 591-602.

Schreiber, F., Wunderlin, P., Udert, K. M., & Wells, G. F. (2012). Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: Biological pathways, chemical reactions and novel technologies. *Frontiers in Microbiology, 3*.

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

Selbie, D. R., Lanigan, G. J., Laughlin, R. J., Di, H. J., Moir, J. L., Cameron, K. C., Clough, T. J., Watson, C. J., Grant, J., Somers, C., & Richards, K. G. (Compiler) (2015b). *Confirmation of co-denitrification in grazed grassland*. *Scientific reports*, 5, 17361.

Senbayram, M., Chen, R., Budai, A., Bakken, L., & Dittert, K. (2012). N_2O emission and the $N_2O/(N_2O+N_2)$ product ratio of denitrification as controlled by available carbon substrates and nitrate concentration. *Agriculture, Ecosystems & Environment, 147*, 4-12.

Shoun, H., Kim, D.-H., Uchiyama, H., & Sugiyama, J. (1992). Denitrification by fungi. *FEMS Microbiology Letters, 94*, 277-281.

Singh, S. N., & Verma, A. (2007). The potential of nitrification inhibitors to manage the pollution effect of nitrogen fertilizers in agricultural and other soils: A review. *Environmental Practice, 9*, 266-279.

Smith, K., Dobbie, K., Thorman, R., Watson, C., Chadwick, D., Yamulki, S., & Ball, B. (2012). The effect of N fertilizer forms on nitrous oxide emissions from UK arable land and grassland. *Nutrient Cycling in Agroecosystems, 93*, 127-149.

Smits, N., Bobbink, R., Laanbroek, H., Paalman, A., & Hefting, M. (2010). Repression of potential nitrification activities by matgrass sward species. *Plant and Soil, 337*, 435-445.

Snyder, A. J., Johnson-Maynard, J. L., & Morra, M. J. (2010). Nitrogen mineralization in soil incubated with 15N-labeled Brassicaceae seed meals. *Applied Soil Ecology, 46*, 73-80.

Sommer, S. G., & Jensen, E. S. (1991). Foliar Absorption of Atmospheric Ammonia by Ryegrass in the Field. *Journal of Environmental Quality, 20*, 153-156.

Steffen, W., Richardson, K., Rockström, J., Cornell, S. E., Fetzer, I., Bennett, E. M., Biggs, R., Carpenter, S. R., de Vries, W., de Wit, C. A., Folke, C., Gerten, D., Heinke, J., Mace, G. M., Persson, L. M., Ramanathan, V., Reyers, B., & Sörlin, S. (2015). Planetary boundaries: Guiding human development on a changing planet. *Science, 347*.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., & de Haan, C. (2006). Livestock's long shadow: Environmental issues and options. *Renewable Resources Journal, 24*, 15-17.

Steinshamn, H., Höglind, M., Garmo, T. H., Thuen, E., & Brenøe, U. T. (2006). Feed nitrogen conversion in lactating dairy cows on pasture as affected by concentrate supplementation. *Animal Feed Science and Technology, 131*, 25-41.

Stevenson, F. J. (1965). Origin and distribution of nitrogen in soil. In W. V. Bartholomew & F. E. Clark (Eds.), *Soil nitrogen* (pp. 1-17). Wisconsin, United States of America: American Society of Agronomy.

Subbarao, G. V., Rao, I. M., Nakahara, K., Sahrawat, K. L., Ando, Y., & Kawashima, T. (2013a). Potential for biological nitrification inhibition to reduce nitrification and N_2O emissions in pasture crop-livestock systems. *Animal : an international journal of animal bioscience, 7 Suppl 2*, 322-332.

Subbarao, G. V., Rondon, M., Ito, O., Ishikawa, T., Rao, I. M., Nakahara, K., Lascano, C., & Berry, W. (2007). Biological nitrification inhibition - is it a widespread phenomenon? *Plant Soil, 294*, 5-18.

Subbarao, G. V., Yoshihashi, T., Worthington, M., Nakahara, K., Ando, Y., Sahrawat, K. L., Rao, I. M., Lata, J. C., Kishii, M., & Braun, H. J. (2015). Suppression of soil nitrification by plants. *Plant Science, 233*, 155-164.

Subbarao, G. V., Rao, I. M., Nakahara, K., Ando, Y., Sahrawat, K. L., Tesfamariam, T., Lata, J. C., Boudsocq, S., Miles, J. W., Ishitani, M., & Peters, M. (2013b). Nitrogen management in grasslands and forage-based production systems - Role of biological nitrification inhibition (BNI). *Tropical Grasslands - Forajes Tropicales, 1*, 168-174.

Subbarao, G. V., Sahrawat, K. L., Nakahara, K., Ishikawa, T., Kishii, M., Rao, I. M., Hash, C. T., George, T. S., Srinivasa Rao, P., Nardi, P., Bonnett, D., Berry, W., Suenaga, K., & Lata, J. C. (2012). Chapter six - Biological nitrification inhibition—A novel strategy to regulate nitrification in agricultural systems. In L. S. Donald (Ed.), *Advances in Agronomy* (pp. 249-302): Academic Press.

Sun, X., Henderson, G., Cox, F., Molano, G., Harrison, S. J., Luo, D., Janssen, P. H., & Pacheco, D. (2015). Lambs Fed Fresh Winter Forage Rape (Brassica napus L.) Emit Less Methane than Those Fed Perennial Ryegrass (Lolium perenne L.), and Possible Mechanisms behind the Difference. *PLOS ONE, 10*, e0119697.

Sutton, M. A., Oenema, O., Erisman, J. W., Leip, A., van Grinsven, H., & Winiwarter, W. (2011). Too much of a good thing. *Nature, 472*, 159-161.

Taylor, A. E., Giguere, A. T., Zoebelein, C. M., Myrold, D. D., & Bottomley, P. J. (2017). Modeling of soil nitrification responses to temperature reveals thermodynamic differences between ammonia-oxidizing activity of archaea and bacteria. *The ISME Journal, 11*, 896-908.

Thomas, S. M., Beare, M. H., Francis, G. S., Barlow, H. E., & Hedderley, D. I. (2008). Effects of tillage, simulated cattle grazing and soil moisture on N_2O emissions from a winter forage crop. *Plant and Soil, 309*, 131.

Tiedje, J. M. (1994). Denitrifiers. In S. H. Mickelson (Ed.), *Methods of Soil Analysis: Part 2 Microbiological and Biochemical Properties* (pp. 245-268). USA: Soil Science Society of America.

Tischner, R. (2000). Nitrate uptake and reduction in higher and lower plants. *Plant, Cell & Environment, 23*, 1005-1024.

Ussiri, D., & Lal, R. (2013). *Soil emission of nitrous oxide and its mitigation*. Dordrecht, NLD: Springer.

van der Weerden, T. J., & Styles, T. M. (2012). Reducing nitrous oxide emissions from grazed winter forage crops. *Poceedings of the New Zealand Grassland Association, 14, 57-62.*

van der Weerden, T. J., Luo, J., de Klein, C. A. M., Hoogendoorn, C. J., Littlejohn, R. P., & Rys, G. J. (2011). Disaggregating nitrous oxide emission factors for ruminant urine and dung deposited onto pastoral soils. *Agriculture, Ecosystems & Environment, 141*, 426-436.

Velthof, G. L., Jarvis, S. C., Stein, A., Allen, A. G., & Oenema, O. (1996). Spatial variability of nitrous oxide fluxes in mown and grazed grasslands on a poorly drained clay soil. *Soil Biology and Biochemistry, 28*, 1215-1225.

Webb, J., Anthony, S. G., Brown, L., Lyons-Visser, H., Ross, C., Cottrill, B., Johnson, P., & Scholefield, D. (2005). The impact of increasing the length of the cattle grazing season on emissions of ammonia and nitrous oxide and on nitrate leaching in England and Wales. *Agriculture, Ecosystems & Environment, 105*, 307-321.

Weier, K. L., & Gilliam, J. W. (1986). Effect of acidity on denitrificaiton and nitrous oxide evolution from atlantic coastal-plain soils. *Soil Science Society of America Journal, 50*, 1202-1205.

Weier, K. L., Doran, J. W., Power, J. F., & Walters, D. T. (1993). Denitrification and the Dinitrogen/Nitrous Oxide Ratio as Affected by Soil Water, Available Carbon, and Nitrate. *Soil Science Society of America Journal, 57*, 66-72.

Weil, R. R., & Brady, N. C. (2016). The nature and properties of soils; 15th edition. Pearson, United States.

Welten, B. G., Ledgard, S. F., Schipper, L. A., & Judge, A. A. (2013). Effect of amending cattle urine with dicyandiamide on soil nitrogen dynamics and leaching of urinary-nitrogen. *Agriculture, Ecosystems & Environment, 167*, 12-22.

Whitehead, D. C. (1995a). *Grasses: Uptake of Nitrogen and Effects on Morphology and Physiology.* Grassland Nitrogen. Wallingford, UK: CAB International.

Whitehead, D. C. (1995b). *Soil N: Amounts, Sources and Fractionation.* Grassland Nitrogen. Wallingford, UK: CAB International.

Williams, L., III, Morra, M., Brown, P., & McCaffrey, J. (1993). Toxicity of allyl isothiocyanate-amended soil to Limonius californicus (Mann.) (Coleoptera: Elateridae) wireworms. *Journal of Chemical Ecology, 19*, 1033-1046.

Wittstock, U., & Halkier, B. A. (2002). Glucosinolate research in the Arabidopsis era. *Trends in Plant Science, 7*, 263-270.

Wrage, N., Velthof, G. L., Oenema, O., & Laanbroek, H. J. (2004). Acetylene and oxygen as inhibtors of nitrous oxide production in *Nitrosomonas europaea* and *Nitrosopira briensis*: A cautionary tale. *FEMS Microbiology Ecology, 47*, 13-18.

Zerulla, W., Barth, T., Dressel, J., Erhardt, K., Horchler von Locquenghien, K., Pasda, G., Rädle, M., & Wissemeier, A. (2001). 3,4-Dimethylpyrazole phosphate (DMPP) – a new nitrification inhibitor for agriculture and horticulture. *Biology and Fertility of Soils, 34*, 79-84.

Zhang, J., Müller, C., & Cai, Z. (2015a). Heterotrophic nitrification of organic N and its contribution to nitrous oxide emissions in soils. *Soil Biology and Biochemistry, 84*, 199-209.

Zhang, M., Fan, C. H., Li, Q. L., Li, B., Zhu, Y. Y., & Xiong, Z. Q. (2015b). A 2-yr field assessment of the effects of chemical and biological nitrification inhibitors on nitrous oxide emissions and nitrogen use efficiency in an intensively managed vegetable cropping system. *Agriculture, Ecosystems & Environment, 201*, 43-50.

Zhu, X., Burger, M., Doane, T. A., & Horwath, W. R. (2013). Ammonia oxidation pathways and nitrifier denitrification are significant sources of N_2O and NO under low oxygen availability. *Proceedings of the National Academy of Sciences, 110*, 6328-6333.

Zuluaga, D., van Ommen Kloeke, A. E. E., Verkerk, R., Röling, W. M., Ellers, J., Roelofs, D., & Aarts, M. M. (2015). Biofumigation using a wild Brassica oleracea accession with high glucosinolate content affects beneficial soil invertebrates. *Plant and Soil, 394*, 155-163.

Chapter 3

Do glucosinolate hydrolysis products reduce nitrous oxide emissions from urine affected soil?

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Do glucosinolate hydrolysis products reduce nitrous oxide emissions from urine affected soil?

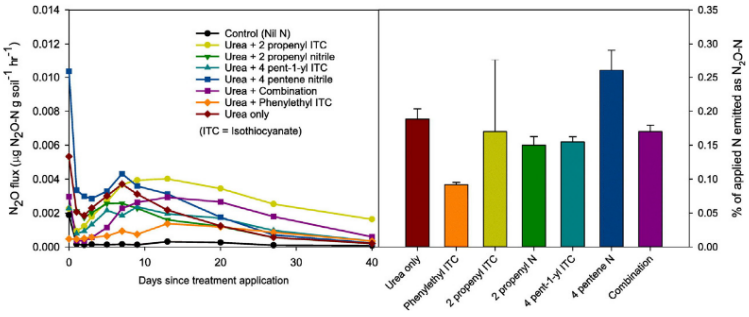
S.F. Balvert [a,b,*], J. Luo [a], L.A. Schipper [b]

[a] AgResearch Limited, Ruakura Research Centre, Private Bag 3123, Hamilton 3240, New Zealand
[b] University of Waikato, Faculty of Science and Engineering, Private Bag 3105, Hamilton 3240, New Zealand

HIGHLIGHTS

- The effectiveness of glucosinolate hydrolysis products at inhibiting N_2O emissions in a high N environment was tested.
- Laboratory and field trials measuring N_2O production and soil mineral N cycling were conducted.
- Some glucosinolate hydrolysis products reduced nitrification rates.
- Where inhibition occurred it was short lived.
- Glucosinolate hydrolysis products with different R groups did not inhibit soil nitrogen processes to the same degree.

GRAPHICAL ABSTRACT



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ABSTRACT

New Zealand agriculture is predominantly comprised of pastoral grazing systems and deposition of animal excreta during grazing has been identified as a major source of nitrous oxide (N_2O) emissions. Nitrification inhibitors have been shown to significantly reduce nitrous oxide emissions from grazing pastoral systems, and some plants have been identified as having nitrification inhibiting properties. Brassica crops are one such example as they contain the secondary metabolite glucosinolate (GLS) whose hydrolysis products are thought to slow soil nitrogen cycling. Forage brassicas have been increasingly used to supplement the diet of grazing animals. The aim of this study was to determine if GLS hydrolysis products (phenylethyl isothiocyanate, 4-pent-1-yl isothiocyanate, 2-propenyl nitrile, 2 propenyl isothiocyanate, 4-pentene nitrile) produced in brassica crops reduced N_2O emissions from soil amended with urea or animal urine. In the laboratory, some GLS hydrolysis products added with urea to soil were found to decrease N_2O emissions and the most effective product (phenylethyl isothiocyante) reduced N_2O emissions by 51% during the study. There was some evidence that the reduction in N_2O emissions found in the lab could be attributed to inhibition of nitrification. Results suggest that the inhibition by GLS hydrolysis products was short-lived and, if considered for use, multiple applications may be necessary to achieve effective inhibition of N_2O emissions. This reduction, however, was not observed under field conditions. Further investigation is required to test more GLS hydrolysis products to fully understand their impact on N_2O emissions from urine affected soil.

* Corresponding author at: AgResearch Limited, Ruakura Research Centre, Private Bag 3123, Hamilton 3240, New Zealand.
E-mail addresses: sheree.balvert@agresearch.co.nz (S.F. Balvert), Jiafa.luo@agresearch.co.nz (J. Luo), louis.schipper@waikato.ac.nz (L.A. Schipper).

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1. Introduction

Global food production needs to increase because the population is expected to reach 9 billion by the middle of the century (Godfray et al., 2010). At the same time, it is important that we consider the environmental effects of increased production. New Zealand agriculture is predominantly comprised of pastoral grazing systems where high levels of nitrogen (N) are lost to potentially sensitive environments (e.g. Ledgard et al., 2000). This is due to the inefficient use of dietary N by ruminants where 70 to 95% of ingested N is excreted in urine and dung (Oenema et al., 2005). N loading rates from urine can be as high as 1000 kg N ha^{-1} (average 613 kg N ha^{-1}; Selbie et al., 2015), depending on plant and dietary content. This loading exceeds the N requirements of the pasture or forage in the vicinity (Haynes & Williams, 1993) and so the excess N is available for loss via nitrate (NO_3^-) leaching, or emissions of gaseous N such as ammonia (NH_3), nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2) (Luo et al., 2010). N_2O is produced during the biological transformation of urinary N in soil, particularly, the microbial processes of nitrification and denitrification (Firestone & Davidson, 1989). Losses of N_2O are of particular concern as it is a potent greenhouse gas with a global warming potential 298 times that of CO_2 (Myhre et al., 2013), and because they contribute significantly to the depletion of stratospheric ozone (Ravishankara et al., 2009). In New Zealand, about 11% of the national greenhouse gas production can be attributed to N_2O emissions derived from deposited urine patches (Ministry for the Environment, 2015). Identifying approaches that decrease N_2O production is a key strategy for reducing greenhouse gas emissions in New Zealand and in other countries with grazed pastures.

Over the last decade, there has been a significant amount of research on the use of the nitrification inhibitors to reduce N_2O emissions from urine patches. Much of this work was carried out using the inhibitor dicyandiamide (DCD) which proved to be effective at reducing N_2O emissions from urine patches by an average of 57% (de Klein et al., 2011; Di & Cameron, 2011; Li et al., 2015; Luo et al., 2013). However, use of DCD was temporarily halted in 2013, waiting for the establishment of standards by the Codex Committee for Food. Inhibition of nitrification remains a promising tool for mitigation of both N_2O emissions and nitrate leaching and so alternative 'biological' inhibitors have been investigated. Biological nitrification inhibitors (BNIs) are secondary metabolites that are produced in, and are released to the soil by plants, and inhibit nitrification. The term BNIs is used to distinguish them from synthetic nitrification inhibitors (Subbarao et al., 2007). A range of plants have been shown to release compounds that inhibit nitrification e.g. *Sorghum*, tropical grass *Brachiaria* (Gopalakrishnan et al., 2007; Ishikawa et al., 2003) and some pines (Lodhi & Killingbeck, 1980). Research suggests that the grazing of certain pasture and forage species may reduce N_2O emissions because secondary metabolites contained in these plants are excreted in urine patches and deposited onto soil, thus inhibiting N cycling and microbial processes (e.g. Prasad & Power, 1995; Ryan et al., 2006).

Brassica crops, in particular, may reduce N_2O emissions as they contain secondary metabolites called glucosinolates (GLS), whose hydrolysis products (isothiocyanates (ITCs), thiocyanates and nitriles) have been shown to inhibit nitrification processes in soil incubated with GLS hydrolysis products (Bending & Lincoln, 2000) and soil incubated with brassica seed meals that also contain GLSs (Reardon et al., 2013). Brassica plants have also been shown to inhibit soil N cycling in situ (Kirkegaard et al., 1999; Ryan et al., 2006), which has been attributed to GLS hydrolysis products. In soil incubation studies, Bending and Lincoln (2000) demonstrated a reduction in nitrifying bacteria population following application of several different GLS hydrolysis products. The mode by which GLS hydrolysis products such as ITCs might inhibit nitrification is not entirely clear, however, it appears that ITCs in particular are toxic to a range of soil organisms (Bending & Lincoln, 2000; Choesin & Boerner, 1991; Kawakishi & Kaneko, 1987; Subbarao et al., 2013). These studies measured the impact of GLS hydrolysis products on N cycling in soils with relatively low N inputs. However, in grazed pasture the majority of N_2O emissions arise from high N inputs such as those from a urine patch. Utilising the potential of forage crops to reduce N loss from pastoral grazing systems requires understanding how GLSs might function in the presence of high N inputs. Additionally, none of these previous studies examined the impact of GLS hydrolysis products on N_2O production. The objective of this study was to test the impact of brassica derived GLS hydrolysis products on N_2O production from soil. We hypothesise that GLS hydrolysis products will inhibit nitrification and N_2O production from urine patches. We initially tested the efficacy of GLS hydrolysis products in a laboratory screening trial and subsequently promising candidate products were tested in a field trial.

2. Materials and methods

2.1. Experiment 1 – laboratory incubation

2.1.1. Trial set-up

A Bruntwood silt loam soil (Typic Impeded Allophanic Soil; Hewitt, 1998) of 0–7.5 cm depth, from under a mixed perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) pasture, was collected for an incubation study. General soil properties are presented in Table 1.

The soil was sieved to 4 mm, homogenised and allowed to pre-incubate for 24 h to allow for the effects of soil disturbance to subside. Moist soil (140 g oven dry equivalent) was weighed into preserving jars (1 L) and amended with N (as urea 600 μg N g^{-1} soil) plus one of five GLS hydrolysis products (phenylethyl ITC, 4 pent-1-yl ITC, 2 propenyl nitrile, 2 propenyl ITC, 4 pentene nitrile) at 2 rates. Other treatments included: A combination of all hydrolysis products, Urea Only, and a nil N control (Table 2). Each treatment was replicated 6 times. The GLS hydrolysis products were applied at either 30 or 60 μg g^{-1} soil. The addition rates of hydrolysis products was determined based on Kirkegaard and Sarwar (1998) who estimated that upon breakdown of brassica green manures incorporated into soil GLS was released at 135 μg g^{-1} soil. This equated to about 20 μg g^{-1} soil of ITC cation, and 30 and 60 μg g^{-1} soil of hydrolysis products contain about 10 and 20 μg g^{-1} soil of ITC or nitrile cation respectively. Urea was applied as a solution and the GLS hydrolysis products were dispersed in water by sonicating for 30 min. The soil was placed in a randomised block design in a constant temperature room where temperature was maintained at 20 °C. Jars were covered in perforated parafilm™ to allow for normal gas exchange yet minimising evaporation loss. During subsequent incubation, soil moisture content was maintained at 70% of water holding capacity with distilled water following weighing to determine water loss.

A matching set of soils and treatments was established to allow for measurement of mineral N transformations. Soil (140 g) was weighed into plastic bags and the same treatments applied. These bags were also stored in a constant temperature room at 20 °C. The bags were stored closed but opened regularly to allow for gas exchange as per the jars and soil moisture monitored and adjusted through time.

2.1.2. Gas flux measurement

Nitrous oxide and CO_2 fluxes were measured 11 times over the 40 days of incubation (0, 1, 2, 3, 5, 7, 9, 13, 20, 27 and 40 days following treatment application). This time length was chosen because N_2O flux had generally returned to background by this time. Sampling was

Table 1
General physical and chemical properties of the soil (Bruntwood silt loam, 0–7.5 cm) used for the incubation trial.

Soil texture (%)			Total-N (%)	Total-C (%)	Olsen P (mg kg^{-1})	pH
Sand	Silt	Clay				
17	61	22	0.61	6.0	34	5.9

Table 2
Treatments and concentrations of products used in laboratory incubation trial.

Treatment name	Rate of urea-N (μg g^{-1} soil)	GLS hydrolysis product	Rate of GLS hyd. product (μg g^{-1} soil)
Control	–	–	–
Urea Only	600	–	–
Ph-ITC 30	600	Phenylethyl isothiocyanate	30
Ph-ITC 60	600	Phenylethyl isothiocyanate	60
Pl-ITC 30	600	4 pent-1-yl isothiocyanate	30
Pl-ITC 60	600	4 pent-1-yl isothiocyanate	60
Pp-Nit 30	600	2 propenyl nitrile	30
Pp-Nit 60	600	2 propenyl nitrile	60
Pp-ITC 30	600	2 propenyl isothiocyanate	30
Pp-ITC 60	600	2 propenyl isothiocyanate	60
Pt-Nit 30	600	4 pentene nitrile	30
Pt-Nit E 60	600	4 pentene nitrile	60
Combination 30	600	All above	150
Combination 60	600	All above	300

more frequent during the first 14 days to capture the initial flush. To measure gas production, jars were sealed with gas tight lids with a septum. Accumulated N_2O was measured following 1 h (i.e. samples taken at T_0 and T_{60} minutes) and CO_2 was measured following 16 h (samples taken at T_0 and T_{16} hours). Preliminary work demonstrated that N_2O accumulation in the jar was linear over 1 h with this soil to headspace ratio. Headspace samples (12 mL) were collected and stored in pre-evacuated glass vials (6 mL) containing a rubber septum. Samples were analysed using a SRI 8610 automated gas chromatograph equipped with a ^{63}Ni-electron capture detector (310 °C), a HayesepD column (40 °C) and N_2 as carrier gas. Hourly N_2O fluxes were calculated for each jar from the increase in headspace concentration. The hourly fluxes were integrated to estimate daily emissions, total emission and % of applied N emitted as N_2O over the study period, where the % of applied N emitted was calculated as follows:

$$\%\text{of applied N emitted as } N_2O = \frac{N_2O\text{-N total (treatment)} - N_2O\text{-N total (control)}}{\text{Urea N applied} + \text{hydrolysis product N applied}} \times 100$$

CO_2 samples were analysed using an infrared gas analyser (IRGA, LI-COR® LI-6262). Similar to N_2O, average hourly fluxes were calculated from the increase in headspace CO_2 concentration over 16 h.

2.1.3. N transformations

The soil bags were subsampled for soil mineral N and pH on the same days as the gas measurements. For NH_4^+-N and $NO_2^- + NO_3^-$-N, soil (5 g oven dry equivalent) was extracted with 2 M KCl (50 ml). Soil pH was measured in water using a 1:2.5 ratio. Soil moisture was also determined on a subsample by oven drying the moist soil at 105 °C for 24 h. Concentrations of NH_4^+-N and NO_3^--N (plus nitrite – NO_2^--N) in soil extracts were measured colorimetrically using a Skaler SAN^{++} segmented flow analyser (Skalar Analytical B.V., The Netherlands) (Blakemore et al., 1987).

2.1.4. Microbial community quantification

On days 1, 9 and 27, the soil in the incubation bags from Control, Urea Only, Ph-ITC, Pl-ITC and Pp-NIT, (60 μg g^{-1} soil only) were also subsampled for determination of *amoA* and *16S* rRNA gene abundance to determine whether addition of GLS hydrolysis products decreased abundance of ammonia oxidising bacteria (AOB) and the whole microbial population. Soil samples were stored at −80 °C prior to extraction. Total soil genomic DNA was extracted using MoBio Powersoil™ DNA isolation kits (MoBio Laboratories, GeneWorks, Australia) according to the manufacturer's instructions. PCR primer pairs amoA1F/amoA2R and BACT1369F/PROK1492R were used to amplify regions of the bacterial *amoA* and *16S* rRNA genes respectively (Rotthauwe et al., 1997; Suzuki et al., 2000). 16 μl reactions contained 8 μl SYBR®Premix Ex Taq™ (Takara, Japan), 0.4 μl of each primer (10 μM concentration), 1.5 μl DNA (diluted 1:10 to reduce potential PCR inhibition), and 5.7 μl milli-Q water. Real time qPCR analysis was performed on a Rotor-Gene™ 6000 (Corbett Life Science, Australia). Raw data analysis was carried out using Rotor-Gene™ 6000 series software version 2.1. Melting curve analysis was performed following each run to confirm PCR product specificity. Standard curves for qPCR analysis were developed by cloning *amoA* and *16SrRNA* gene amplicons into pGEM® –T Easy vectors (Promega, USA) and transforming TOP10 *E. coli* (Thermo Fisher, USA). Selected clones were incubated overnight in Luria Broth liquid medium and the plasmid DNA was subsequently extracted using a PureLink™ Quick Plasmid MiniPrep Kit (Thermo Fisher, USA). Plasmid DNA concentration was measured on a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA) and standard curves were subsequently generated by dilution series. Only AOB were enumerated as Di et al. (2009) demonstrated that AOB as opposed to AOA are largely responsible for ammonia oxidation in pastoral soils and under high N loads.

2.2. Experiment 2 – field plot study

2.2.1. Trial set-up

A small plot trial was carried out on the AgResearch Ruakura farm located in Hamilton, New Zealand (37°46′S, 175°3′E; 40 m above sea level). This trial was based on the results of the laboratory trial and measured N_2O emissions and N mineralisation from field plots amended with inhibitors that were most successful in the lab trial. Two trial areas were set up on two different soil types: a well-drained Horotiu (Typic Orthic Allophanic) and a poorly drained Te Kowhai silt loam (Typic Orthic Gley) (Hewitt, 1998). Both plots contained a permanent mixed perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) pasture. The pasture had been excluded from grazing for 3 months prior to the study and maintained by regular mowing to remove any effect of excreta deposits from previous grazings. General soil properties prior to treatment application are presented in Table 3.

Treatments included a nil N control, N only control, N plus dicyandiamide (DCD), N plus one of three GLS hydrolysis products identified as most effective in the above laboratory study (phenylethyl ITC, 4 pent-1-yl ITC and 2 propenyl nitrile), and N plus a combination of two GLS combined at two rates of application (Table 4). Treatments (5 replicates for each treatment) were laid out in a randomised block design which included a 1 m^2 plot adjacent to a N_2O sampling chamber. N was applied in the form of artificial cow urine at a rate of 600 kg N ha^{-1} and 10 L m^2 urine. The artificial urine was adapted from de Klein et al. (2003) and contained 11.69 g L^{-1} urea, 4.99 g L^{-1} potassium chloride, 1.36 g L^{-1} potassium sulphate, 13.8 g L^{-1} potassium bicarbonate and 2.92 g L^{-1} glycine. The GLS hydrolysis products were applied at a rate of 60 kg ha^{-1} and were mixed with artificial urine prior to application. The Combination treatments were applied at rates of both 60 kg ha^{-1} of each product (Combination 120) and 60 kg ha^{-1} in total (Combination 60). DCD was applied at a rate of 10 kg ha^{-1} and was also mixed with the artificial urine. Treatments were applied mid-June 2016.

Table 3
General physical and chemical properties of the soils (Horotiu silt loam and Te Kowhai silt loam, 0–7.5 cm) used for the field trial.

	Soil texture (%)			Bulk density (g cm^3)	Total-N (%)	Total-C (%)	Olsen P (mg kg^{-1})	pH
	Sand	Silt	Clay					
Horotiu	34	48	18	0.71	0.82	8.0	36	5.8
Te Kowhai	10	55	35	0.78	0.54	5.3	36	5.8

Table 4
Treatments and concentrations of products used in field trial.

Treatment	Rate of urine-N (kg N ha^{-1})	GLS hyd. product	Rate of GLS hyd. Product (kg ha^{-1})
Control	–	–	–
Urea	600	–	–
DCD 10	600	–	10
Ph-ITC 60	600	Phenylethyl isothiocyanate	60
Pl-ITC 60	600	4 pent-1-yl isothiocyanate	60
Pp-Nit 60	600	2 propenyl nitrile	60
Combination 60	600	Ph-ITC 30 + Pp-NIT 30	60
Combination 120	600	Ph-ITC 60 + Pp-NIT 60	120

2.2.2. Climate data monitoring

Daily rainfall and air temperature were obtained from a weather station situated adjacent to the site. Soil temperature (10 cm depth) was measured at each site using HOBO temperature loggers (UA-001-08; Onset, US). Water filled pore space (WFPS) was calculated by dividing volumetric water content by porosity (Linn & Doran, 1984).

2.2.3. Gas flux measurement

A soil chamber technique was used to measure N_2O emissions and was based on previous studies measuring emissions from urine patches (e.g. Luo et al., 2015b). Stainless steel chamber bases (236 mm diameter) were inserted into the soil to a depth of 12 cm, 7 days prior to treatment application. The chamber bases had water channels on the upper rim that accommodated insulated aluminium chamber lids (about 5.8 L) and formed a gas tight seal. The chamber bases remained in place for the duration of the trial. On each sampling day, the chambers were closed for 60 min commencing at approximately 10:30 am. Headspace gas samples were taken by syringe at 0 (T_0), 30 (T_{30}) and 60 (T_{60}) minutes for the first 3 samplings when emission rates were shown to be linear over this time period. For subsequent samplings, headspace gas samples were taken at T_0 and T_{60} only. As with the laboratory study, N_2O samples (12 mL) were collected and stored in pre-evacuated glass vials (6 mL) containing a rubber septum. Samples were analysed as described above. Hourly fluxes were calculated from the increase in chamber concentration over 60 min. Sampling occurred twice weekly for the first 6 weeks, then weekly until background N_2O levels were reached. Hourly N_2O fluxes were calculated for each plot from the increase in headspace concentration during the hour. The hourly fluxes were integrated to estimate daily emissions and total emission over the study period. The emission factor for urine (designated EF_3 by the Intergovernmental Panel for Climate Change) was calculated for each treatment according to the IPCC methodology, as described in the following equation:

$$EF_3 = \frac{N_2O \text{ total (treatment)} - N_2O \text{ total (control)}}{\text{Urine N applied} + \text{hydrolysis product N applied}} \times 100$$

2.2.4. N transformation measurement

On the same day as gas sampling, soil samples were taken from each adjacent plot (75 mm × 25 mm cores; 3 cores bulked) for soil mineral N and soil moisture determination. The core holes were sealed with PVC tubes immediately following sampling in order to minimise the effects of soil aeration. On the same day as collection, soil samples were returned to the laboratory, sieved to 4 mm and homogenised prior to analysis of NH_4^+-N, NO_2^- + NO_3^--N and moisture content as described above.

2.3. Statistical analysis

All data was log transformed prior to analysis to achieve a normal distribution. One-way ANOVA was carried out on the % of applied N emitted as N_2O (lab incubation) or EF_3 (field study), total emissions and microbial community data to determine the significance of the difference between treatments. For daily N_2O flux and mineral N comparisons, significance was determined by a Duncan pair-wise test which allows for multiple comparisons. All analyses were carried out using the 'r' statistical package (Version 3.3.2; R Core Team, 2016).

3. Results

3.1. Experiment 1 – laboratory incubation

3.1.1. Gas flux and % of applied N emitted as N_2O

All treatments, including the Control, showed an initial high N_2O flux immediately following treatment application. Subsequently, for all individual GLS hydrolysis product treatments and Urea Only, peak N_2O fluxes were observed 7 days following application of urea (Fig. 1). For the Combination treatment, however, peak N_2O emissions occurred 13 days following urea application. In general, addition of hydrolysis products decreased N_2O fluxes but the differences were not always statistically significant. Ph-ITC at the 60 μg g^{-1} rate had a significantly lower ($p < 0.001$) peak N_2O flux than the Urea Only treatment for all sampling days. The Combination treatment (both 30 and 60 μg g^{-1} rates) also had significantly lower ($p < 0.001$) N_2O flux than Urea Only for the first 3 days of measurement. No other treatments had a significant effect on N_2O flux.

Relative to the Urea Only treatment (0.95 μg N_2O-N g^{-1} soil), total N_2O emissions in the initial 20 days post urea application were significantly lower ($p < 0.001$) for the Ph-ITC treatments, both 30 and 60 μg g^{-1} rates (0.54 and 0.29 μg N_2O-N g^{-1} soil, respectively), and initially for the Combination treatment 30 μg rate (0.55 μg N_2O-N g^{-1} soil) (Table 5). However, this effect subsided with time and only Ph-ITC (30 μg g^{-1} soil) had significantly lower total emissions (0.74 μg N_2O-N g^{-1} soil) than the Urea Only over the full 40 days (1.3 μg N_2O-N g^{-1} soil). The N_2O loss as % of applied N was significantly reduced, compared to Urea Only, by 48% (Ph-ITC 30 μg), 78% (Ph-ITC 60 μg) and by 49% (Combination 30 μg) at 20 days post urea application ($p < 0.001$). Similar to total N_2O emissions, this effect was reduced by day 40 and only Ph-ITC (60 μg) showed a significant reduction (51% reduction; $p < 0.01$).

For all treatments, soil respiration increased compared with the control and CO_2 flux peaked (0.019–0.032 μg C g^{-1} soil $hour^{-1}$) immediately following treatment addition (data not shown). However, respiration dropped to that of the control soils (0.005 μg C g^{-1} soil $hour^{-1}$) by day 2 and remained no different from the control until the end of the measurement period. Ph-ITC, Pl-ITC, Pt-NIT and Combination had significantly higher ($p < 0.001$) CO_2 flux than the Urea Only treatment immediately following treatment addition. No significant difference was measured between any treatments following day zero.

3.1.2. N transformations

Soil NH_4^+-N peak was observed 1 day following application of urea in all treatments except Ph-ITC where NH_4^+-N peak was observed on day 7 (Fig. 2). From Day 5, soil NH_4^+-N concentration was significantly higher in the Ph-ITC, Pl-ITC, Pp-ITC and Combination treatments, both rates for all, than in the urea only treatment. Soil NO_3^--N peak was observed 27 days following urea application in all treatments and was highest in the Urea Only treatment (671 μg N g^{-1} soil; Fig. 3). Soil NO_3^--N concentration was significantly reduced ($p < 0.001$) relative to Urea Only until day 13 in the Pl-ITC and Pp-ITC treatments (30 and 60 μg) and until day 20 in the Ph-ITC and Combination treatments (30 and 60 μg). Beyond day 20 there was no significant difference between treatments. Pp-NIT and Pt-NIT had no detectable effect on soil NH_4^+-N or NO_3^-N concentration.

3.1.3. Microbial community

In comparison to the control, there was no effect of any treatments, including Urea Only, on the *16S* rRNA gene copy numbers on all the days

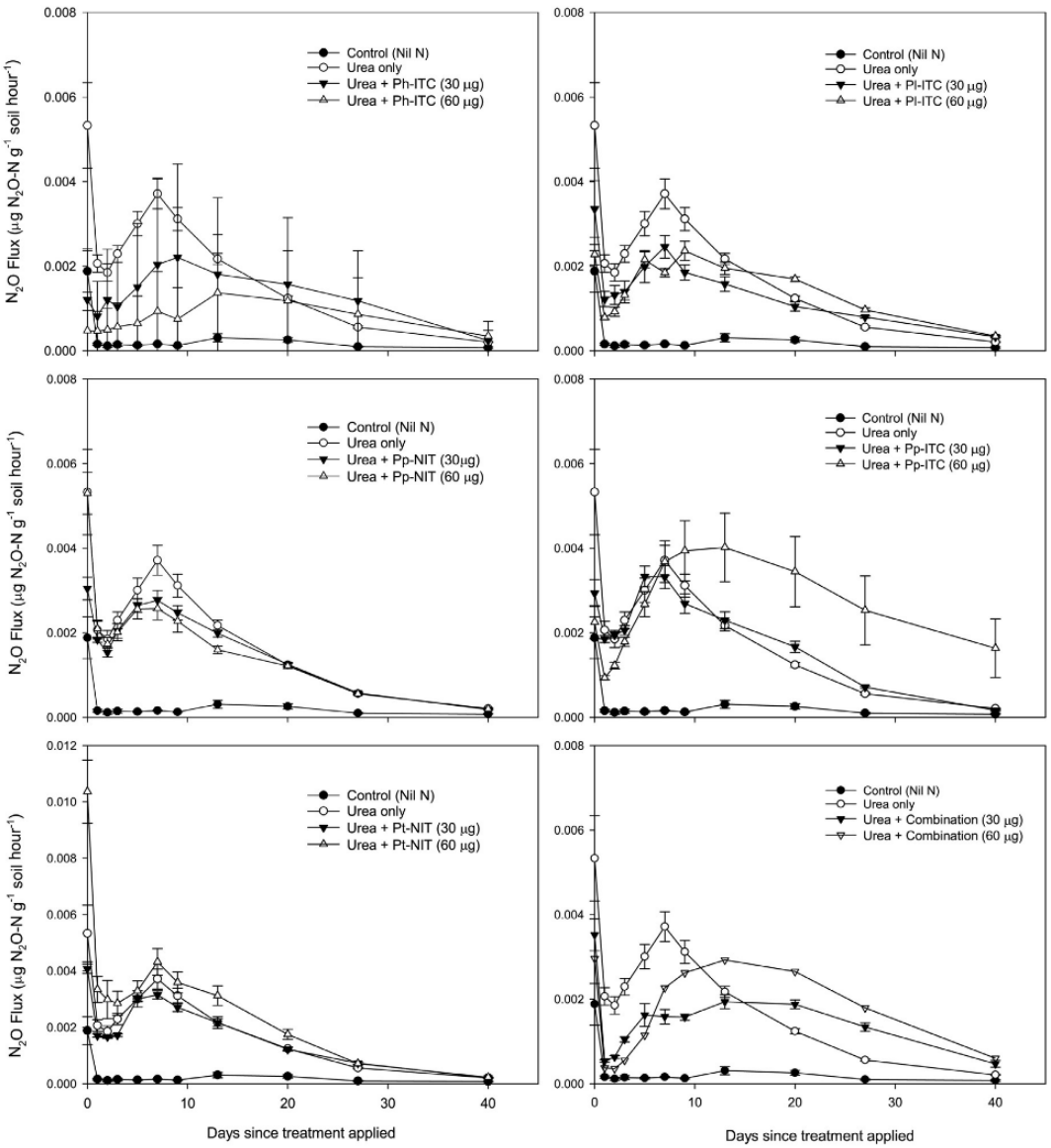

Fig. 1. Nitrous oxide fluxes from incubated soils following application of urea and one of five glucosinolate hydrolysis products: phenylethyl isothiocyanate (Ph-ITC), 4 pent-1-yl isothiocyanate (Pl-ITC), 2 propenyl nitrile (Pp-NIT), 2 propenyl isothiocyanate (Pp-ITC), and 4 pentene nitrile (Pt-NIT). Error bars represent the standard error of the mean ($n = 6$). Note different scales on the Y axis for Pt-NIT.

measured (data not presented). AOB gene copy numbers increased following the addition of urea, with an average increase of 8.9×10^5 greater than the Control for the 3 time points measured. For days 1 and 9, there was a general reduction in AOB abundance in the hydrolysis product treatments relative to Urea Only. However, this was statistically significant for Pl-ITC and on day 1 only ($p < 0.02$). No difference in AOB abundance was observed between Urea Only and any of the hydrolysis product treatments on Day 27.

3.2. Experiment 2 – field plot study

3.2.1. Climate

Heavy rainfall (average 12 mm day^{-1} over first 10 days) occurred immediately following treatment application (Fig. 4) and rainfall for the first month after treatments were applied was much higher than the 45 year average (144 mm vs 119 mm, data not presented). Soil water filled pore space (WFPS) was relatively stable for both soils and remained between 60% and 75% until day 50 of the trial. The Te Kowhai soil reduced to 55% over the last 10 days due to decreased rainfall. Soil temperature fluctuated between 5 and 15 °C in both soil types.

3.2.2. Gas flux and emission factor

As with the laboratory incubation, all treatments (including the Control) on both soils showed an initial high N_2O flux immediately following treatment application (data not presented). Both soils also showed 2 main peaks in N_2O flux following urine application. These occurred on days 5 and 17 from the Horotiu soil and days 20 and 31 from the Te Kowhai soil. N_2O fluxes from the Te Kowhai soil were generally higher than those from the Horotiu soil. The largest N_2O flux from the Te Kowhai soil reached 54 g N_2O-N ha^{-1} day^{-1} on day 20 whereas that

Table 5
Laboratory incubation results: Total N_2O emissions and percent of applied N emitted as N_2O for the periods of 20 and 40 days, as affected by application of urea and one of five glucosinolate hydrolysis products: phenylethyl isothiocyanate (Ph-ITC), 4 pent-1-yl isothiocyanate (Pl-ITC), 2 propenyl nitrile (Pp-NIT), 2 propenyl isothiocyanate (Pp-ITC), and 4 pentene nitrile (Pt-NIT). Significant differences between Urea Only and hydrolysis product treatments are identified with their p values ($n = 6$).

	Total emission (μg N_2O-N g^{-1} soil)				% of applied N emitted as N_2O					
	To day 20	p	To day 40	p	To day 20	Reduction by hyd. product (%)	p	To day 40	Reduction by hyd. product (%)	p
Control (Nil N)	0.10		0.19							
Urea Only	0.95		1.31		0.14			0.19		
Urea + Ph-ITC (30 μg)	0.54	<0.001	1.08	NS	0.07	48	<0.001	0.15	21	NS
Urea + Ph-ITC (60 μg)	0.29	<0.001	0.74	<0.001	0.03	78	<0.001	0.09	51	0.01
Urea + Pl-ITC (30 μg)	0.62	NS	1.04	NS	0.09	39	NS	0.14	25	NS
Urea + Pl-ITC (60 μg)	0.62	NS	1.17	NS	0.09	40	NS	0.16	14	NS
Urea + Pp-NIT (30 μg)	0.77	NS	1.13	NS	0.11	22	NS	0.16	17	NS
Urea + Pp-NIT (60 μg)	0.77	NS	1.13	NS	0.11	23	NS	0.15	18	NS
Urea + Pp-ITC (30 μg)	0.88	NS	1.33	NS	0.13	9	NS	0.19	0	NS
Urea + Pp-ITC (60 μg)	0.72	NS	1.25	NS	0.10	28	NS	0.17	7	NS
Urea + Pt-NIT (30 μg)	0.85	NS	1.24	NS	0.12	13	NS	0.17	8	NS
Urea + Pt-NIT (60 μg)	1.29	NS	1.78	<0.001	0.20	−38	NS	0.26	−38	NS
Urea + Combination (30 μg)	0.55	<0.001	1.24	NS	0.07	49	<0.001	0.17	10	NS
Urea + Combination (60 μg)	0.67	NS	1.60	NS	0.09	38	NS	0.22	−17	NS

from the Horotiu soil only reached 13 g N_2O-N ha^{-1} day^{-1} on Day 17 (excluding the initial peak). In the Horotiu soil, N_2O fluxes returned to background levels approximately 40 days following treatment application. Those for the Te Kowhai soil returned to background much later at approximately 60 days following treatment application. No hydrolysis product treatments had any significant impact on daily N_2O fluxes compared to the Urine Only treatment for either soil.

In both soils, no significant reduction in total N_2O emissions over the measurement period, or emission factor, was observed as a result of the addition of the inhibitors. Surprisingly this included the treatment of the benchmark inhibitor DCD (Table 6).

3.2.3. N transformations

Results showed no significant response of soil NH_4^+-N or soil NO_3^--N to any of the hydrolysis products relative to the Urine Only treatment in either soil (data not shown). Soil NH_4^+-N was high immediately following application of urine in both soil types and in all treatments. Soil NO_3^--N peak was significantly delayed in treatments containing hydrolysis products. For the Urine Only treatment, soil NO_3^--N peak was observed at 13 and 16 days following urine application in the Horotiu and Te Kowhai soils respectively. NO_3^--N peak was observed on day 26 in all treatments containing hydrolysis products and on both soil types.

4. Discussion

4.1. Evidence for inhibition of nitrification

The hypothesis tested was that GLS hydrolysis products would delay nitrification and hence reduce N_2O emissions through a lack of NO_3^- as a substrate for N_2O production. In this laboratory study, nitrification and N_2O emissions were generally lower when hydrolysis products were added but not for all products at the test rates. This supports observations by Bending and Lincoln (2000) who also measured reduced nitrification rates following addition of GLS hydrolysis compounds. N_2O fluxes increased immediately following N application and N_2O flux had peaked and returned to background levels within 6 weeks. This is in agreement with a number of other studies which also show elevated N_2O flux which subsides over time following application of a high rate of N. (e.g. Di et al., 2010; Ledgard et al., 2014; Luo et al., 2008; Rochette et al., 2014). The rate of GLS hydrolysis product applied did not significantly influence the results, so the effects of hydrolysis products are discussed irrespective of rate used.

The addition of Ph-ITC and the Combination treatment reduced the size of the N_2O peak and total N_2O emissions resulting from the addition of N. The Combination treatment also delayed the onset of the peak of N_2O flux. This reduction in the size of the N_2O peak was similar to the response of N_2O flux following addition of synthetic nitrification inhibitors such as DCD (e.g. Bell et al., 2015; Di et al., 2010; Luo et al., 2015a). N_2O emissions following addition of Pl-ITC and Pp-NIT were also reduced but this reduction was not significant. The addition of Pp-ITC and Pt-NIT, however, resulted in an increase in either the height or duration of the N_2O peak (Pt-NIT significant only).

The soil N transformation data also provided evidence some of the GLS hydrolysis compounds inhibited or delayed nitrification. A successful nitrification inhibitor would result in more NH_4^+-N and less NO_3^--N in the soil as the inhibitor slowed the conversion of NH_4^+ to NO_2^- and NO_3^- (Amberger, 1989). Three out of the five GLS hydrolysis products tested, as well as the Combination treatment, showed significantly higher soil NH_4^+-N concentration and corresponding lower NO_3^--N concentration, in comparison to the Urea Only treatment on all sampling days. Pt-NIT, which showed greater N_2O production than Urea Only, did not show correspondingly higher soil NO_3^--N. The reason for this is not clear. GLS hydrolysis products may also be inhibiting another pathway in N_2O formation.

Other evidence for the effectiveness of some GLS products on inhibiting nitrification was changes in populations of AOB. AOB abundance, which increased following addition of urea N, was generally lower when GLS hydrolysis products were added, suggesting that nitrification inhibition had occurred. Bending and Lincoln (2000) also demonstrated inhibition of nitrifying bacteria by two GLS derived ITCs. However, this relationship is not straight forward as Ph-ITC, which showed lower soil NO_3^--N and lower N_2O emissions, did not have significantly lower AOB gene abundance. In contrast, Pl-ITC reduced AOB gene abundance but not N_2O emissions.

While there was good evidence that some of these products (e.g. Ph-ITC and Pl-ITC) inhibit nitrification and reduce N_2O emissions, the observed reduction of efficacy suggests the effect of hydrolysis products on N_2O production is short-lived. By the end of the trial only Ph-ITC showed a significant reduction (51%) in total N_2O emissions and % of applied N emitted as N_2O-N. Differences in volatility of each GLS hydrolysis product may alter efficacy as losses to the atmosphere will determine the length of time GLS products will inhibit nitrifying organisms. Choesin and Boerner (1991) demonstrated that many GLS hydrolysis products have high volatilisation losses after application to soil. Additionally, Rumberger and Marschner (2003) found that ITCs are rapidly degraded in soil with only traces remaining 44 h after application. If suitable GLSs are identified for inhibiting N_2O emissions then multiple applications may be necessary for effective long term inhibition of N_2O emission. Multiple applications through the year have also been suggested for other nitrification inhibitors to achieve maximum impact (Ledgard et al., 2014). Additionally, the incubations in this study were

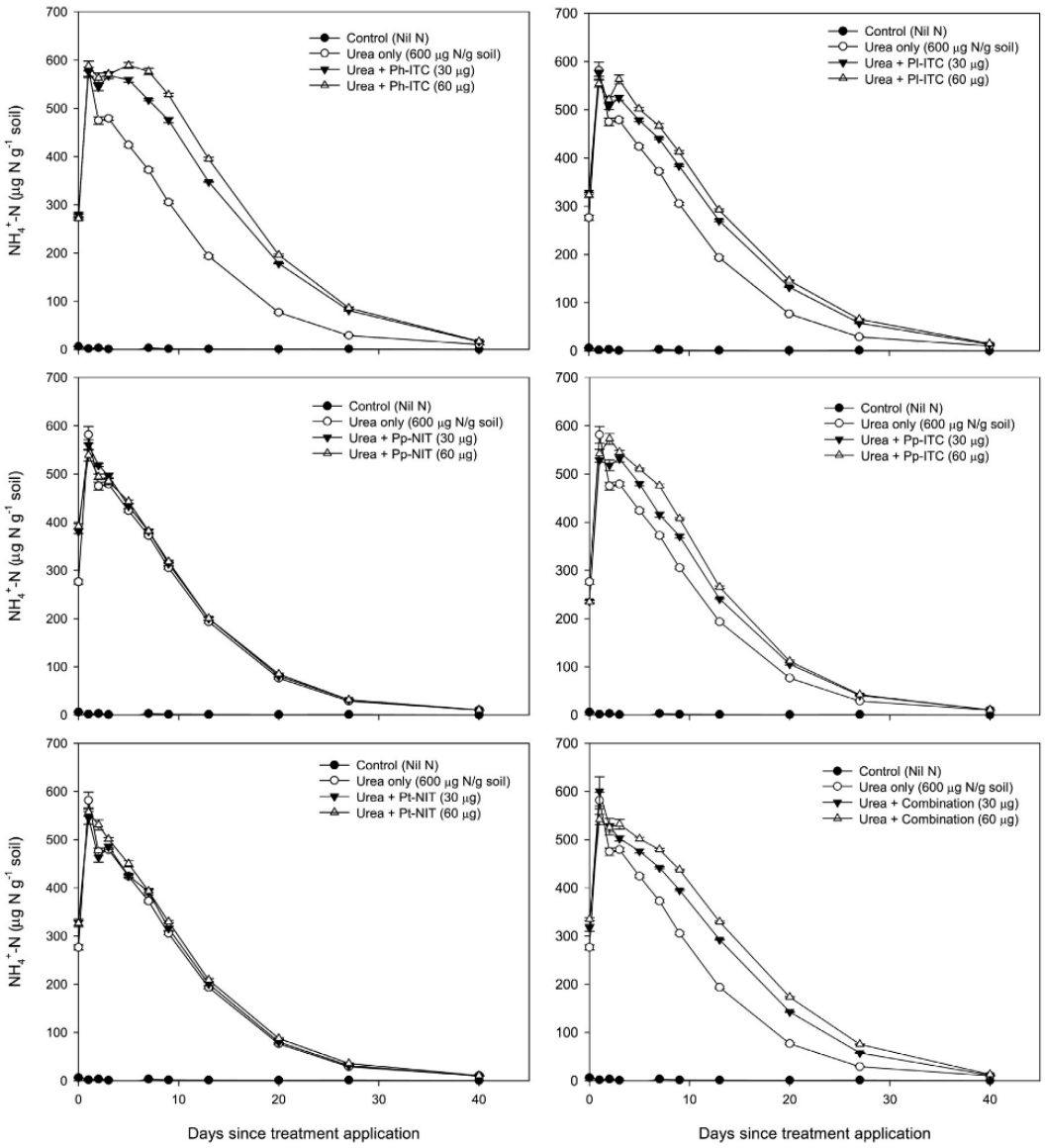

Fig. 2. Soil ammonium N (NH_4^+-N) concentrations in incubated soils following application of urea and one of five glucosinolate hydrolysis products: phenylethyl isothiocyanate (Ph-ITC), 4 pent-1-yl isothiocyanate (Pl-ITC), 2 propenyl nitrile (Pp-NIT), 2 propenyl isothiocyanate (Pp-ITC), and 4 pentene nitrile (Pt-NIT). Error bars represent the standard error of the mean ($n = 6$).

conducted at 20 °C which is relatively high and might encourage increased microbial degradation of the hydrolysis products decreasing their efficiency at inhibiting N_2O production. Other compounds such as DCD and 3,4-dimethylpyrazol phosphate (DMPP) are also short-lived at higher incubation temperatures (Prasad & Power, 1995). Higher concentrations of hydrolysis product may result in a longer lasting inhibition, although we found no additional reduction with increased rates of application (30 μg g^{-1} soil versus 60 μg g^{-1} soil).

The apparent recovery of the AOB community after day 9 also supported the idea that the inhibitive effect of some of the hydrolysis products was short lived and multiple applications may be required to be effective in cumulative reductions in N_2O losses. Additionally, nitrate concentration was significantly lower up until day 13 for Pl-ITC and Pp-NIT treatments and until day 20 for Ph-ITC and Combination treatments. This further indicated that the impact GLS hydrolysis products had on soil N transformations had dissipated by this time.

The differing levels of inhibition of N_2O and NO_3^- production suggested that although GLS hydrolysis products impacted on soil nitrogen processes, the effect was not consistent. The amount of N added with the hydrolysis products only accounted for 0.9–1.8% (at the 60 μg rate) of the total N applied and so this additional N was unlikely to cause differences in N_2O emission. Calculations for determining any reduction based on the % of applied N that was emitted as N_2O-N also accounted for the differences in N contents of the hydrolysis products. These differences in N_2O inhibition were more likely due to the nature of GLSs themselves. GLSs consist of a side chain (designated R) that distinguishes one GLS from another. The hydrolysis products of a particular GLS will contain the same R chain as the parent GLS. More than 100 different GLSs have been identified (Brown & Morra, 1997; Fahey et al., 2001) and a range of hydrolysis products with differing R chain structures were chosen for this study to cover a broad range of hydrolysis products and their likely chemistry. Based on our results, it was possible

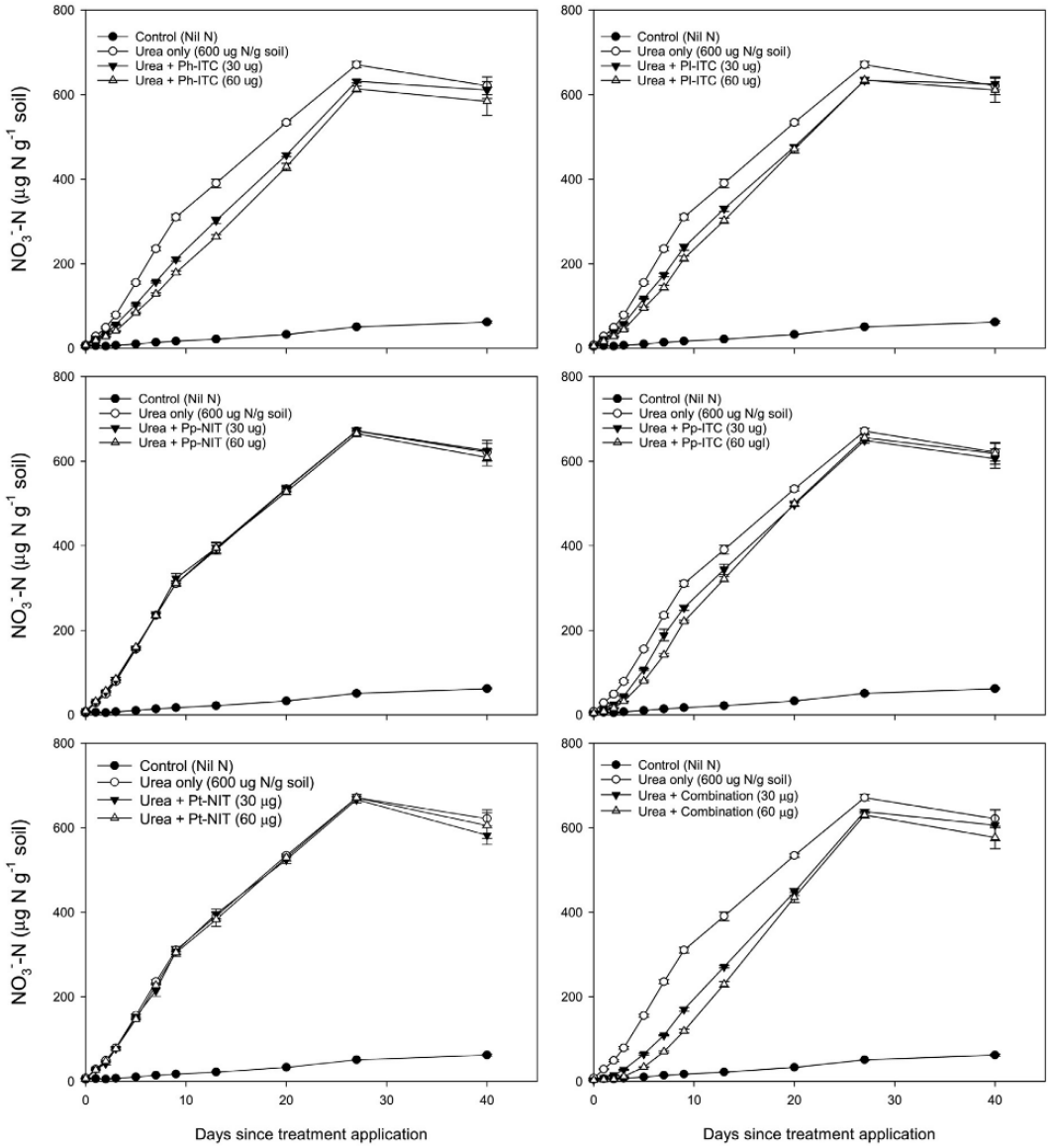

Fig. 3. Soil nitrate N (NO_3 -N) concentrations in incubated soils following application of urea and one of five glucosinolate hydrolysis products: phenylethyl isothiocyanate (Ph-ITC), 4 pent-1-yl isothiocyanate (Pl-ITC), 2 propenyl nitrile (Pp-NIT), 2 propenyl isothiocyanate (Pp-ITC), and 4 pentene nitrile (Pt-NIT). Error bars represent the standard error of the mean ($n = 6$).

that the aromatic structure of Ph-ITC may contribute to its efficacy (Fahey et al., 2001). Understanding the mechanisms by which different R chains of hydrolysis products inhibit microbial processes would allow better targeting of useful inhibitors.

GLS are found in brassica tissues and are hydrolysed upon breakdown of tissues. There are many studies that have demonstrated the toxic effects of brassica tissues on soil microfauna when incorporated into soil (Kirkegaard & Sarwar, 1998; Ryan et al., 2006) which have been attributed to the GLS hydrolysis products and their capacity to alter enzyme structure and inhibit metabolic processes (Kawakishi & Kaneko, 1987). This general inhibition of soil processes has led GLS hydrolysis products to be considered as general biocides. Bending and Lincoln (2000), however, demonstrated that GLS hydrolysis products could specifically inhibit nitrifying organisms rather than acting as a general biocide. Our *16S* rRNA data, and lack of change in soil respiration data, do not support the hypothesis that GLS and their hydrolysis products are general biocides. If GLS hydrolysis products were general biocides we would expect a large flush of CO_2 relative to the Urea Only treatment caused by decomposition of soil microbes. The increase in CO_2 in the treatments containing hydrolysis products that was observed in this study is relatively small and likely due to the additional carbon added with the hydrolysis products.

4.2. Field evaluation

Although the GLS hydrolysis products selected were identified in the lab study as inhibitors of N_2O production, they did not demonstrate any inhibition in this field study. There are many environmental and experimental factors that mean field efficacy is difficult to demonstrate, particularly with only a single season's data. DCD which was used as a benchmark inhibitor also did not inhibit N_2O emissions, which is in contrast to a wide range of reports of DCD efficacy in the field (de Klein

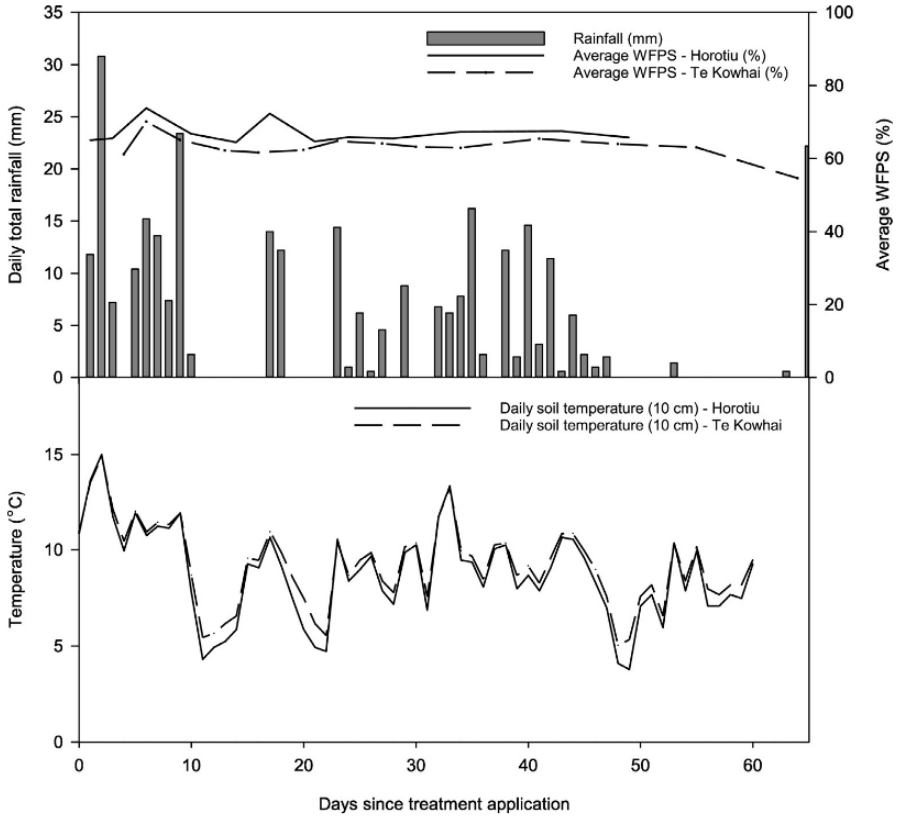

Fig. 4. Rainfall, soil temperature (10 cm depth) and water-filled pore space (WFPS) (0–7.5 cm) during the field experimental period.

et al., 2011). However, there have been studies demonstrating that DCD can be much less effective under warm and wet conditions (Kelliher et al., 2008; Shepherd et al., 2014) as occurred in this field trial.

Total N_2O emissions from the Horotiu soil were much lower than from the Te Kowhai soil, which was expected as urine applied to free draining soils generally result in lower N_2O emissions than from urine applied to poorly draining soils (de Klein et al., 2003). The N_2O flux pattern was erratic, particularly from the Te Kowhai soil, with multiple peaks which might be attributed to fluctuating soil moisture status, but this seems unlikely as soil moisture for both soils remained relatively stable throughout. Very heavy rain occurred immediately after treatment application and there was consistent rain throughout the trial. Generally, higher N_2O emissions are expected from urine applied to continuously wet soils due to lower oxygen levels that enhance denitrification (Saggar et al., 2004), however, very low emissions were observed in this study. High N_2O emissions were potentially not observed here as the high rainfall shortly following treatment application may have moved the majority of the NO_3^--N to the subsoil where it was spatially separated from the denitrifying microbes in the topsoil responsible for N_2O production (Luo et al., 1998). The wet conditions may also have resulted in complete denitrification and loss of N as N_2.

Although we did not measure any effect of individual GLS hydrolysis products on N_2O emissions or nitrification in the field, previous studies have reported inhibition of nitrification and/or accumulation of NH_4^+-N in situ by brassica crops which they have attributed to GLSs and their hydrolysis products (Brown & Morra, 2009; Kirkegaard et al., 1999; Ryan et al., 2006). Potentially, we did not measure nitrification inhibition because generally examples of biological nitrification inhibition are likely associated with plant tissues that are composed of a combination of nitrification inhibitors (or the full complement of GLS hydrolysis products). These inhibitors may all have different modes of inhibitory effect on enzymatic pathways of nitrifying bacteria and instead synergistically inhibit the nitrification process (Subbarao et al., 2015). In their laboratory study, Bending and Lincoln (2000) reported different levels of nitrification inhibition when different combinations of GLS hydrolysis products were used. They suggested the greater nitrification

Table 6
Field study results: Total N_2O emissions and emission factors (EF_3) as affected by application of urea and glucosinolate hydrolysis products (Ph-ITC, PI-ITC, Pp-NIT). Least significant differences (LSD; 5%) are presented ($n = 5$).

	Total emission (μg N_2O-N g^{-1} soil)	Emission factor (EF_3)	EF_3 reduction by hyd. product (%)
Horotiu			
Control	0.01		
Urine Only	0.19	0.029	
Urine + Ph-ITC	0.15	0.022	24
Urine + PI-ITC	0.18	0.027	6
Urine + Pp-NIT	0.10	0.015	49
Urine + Combination 120	0.22	0.033	−14
Urine + Combination 60	0.11	0.016	46
Urine + DCD	0.15	0.023	21
LSD	0.09	0.015	
Te Kowhai			
Control	0.04		
Urine Only	0.57	0.088	
Urine + Ph-ITC	0.72	0.113	−29
Urine + PI-ITC	0.52	0.079	10
Urine + Pp-NIT	0.64	0.100	−12
Urine + Combination 120	0.70	0.108	−22
Urine + Combination 60	0.64	0.100	−13
Urine + DCD	0.33	0.049	45
LSD	0.40	0.07	

inhibition was a result of the different hydrolysis products acting on different metabolic processes. The laboratory study did not show increased inhibition from the Combination treatment so these synergistic responses may rely on specific combinations. In a field study, Luo et al. (2015b) observed reduced N_2O emissions from urine of sheep fed on a brassica crop compared to urine from sheep fed ryegrass. As brassicas contain a wide range of GLS compounds, the urine from animals fed on brassicas will likely contain several hydrolysis products. Their observations may be a result of the combined effect of the different GLS hydrolysis products which assists our understanding of the differences between these results and ours.

5. Conclusions

Overall, this study provided evidence that addition of some GLS hydrolysis products reduced nitrification as soil NO_3^--N, nitrifying bacteria and N_2O emissions were all reduced. The mechanisms behind this, however, were not fully understood. Where inhibition occurred, the results suggested the impact was short lived and may require multiple applications of GLS hydrolysis products to achieve a meaningful reduction in N_2O emissions. There was evidence that GLS hydrolysis products with different R groups did not inhibit soil nitrogen processes to the same degree. Additionally, it is possible that certain GLS hydrolysis products act synergistically as they inhibit different enzymatic pathways. This requires further investigation. This study only examined 3 GLS hydrolysis products, and additional compounds could be tested to determine whether they might be utilised for reducing N_2O emissions from urine affected soil. Demonstrating effectiveness of GLS hydrolysis products as nitrification inhibitors in the field remained a challenge and deserves further investigation, particularly over multiple seasons.

Acknowledgements

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References

Amberger, A., 1989. Research on dicyandiamide as a nitrification inhibitor and future outlook. Commun. Soil Sci. Plant Anal. 20 (19–20), 1933–1955.
Bell, M.J., Rees, R.M., Cloy, J.M., Topp, C.F.E., Bagnall, A., Chadwick, D.R., 2015. Nitrous oxide emissions from cattle excreta applied to a Scottish grassland: effects of soil and climatic conditions and a nitrification inhibitor. Sci. Total Environ. 508, 343–353.
Bending, G.D., Lincoln, S.D., 2000. Inhibition of soil nitrifying bacteria communities and their activities by glucosinolate hydrolysis products. Soil Biol. Biochem. 32, 1261–1269.
Blakemore, L.C., Searle, P.L., Daley, B.K., 1987. Methods for chemical analysis of soils. New Zealand Soil Bureau Scientific Report No. 80. New Zealand Soil Bureau, Wellington, New Zealand.
Brown, P.D., Morra, M.J., 1997. Control of soil-borne plant pests using glucosinolate-containing plants. Adv. Agron. 61, 167–231.
Brown, P.D., Morra, M.J., 2009. Brassicaceae tissues as inhibitors of nitrification in soil. J. Agric. Food Chem. 57, 7706–7711.
Choesin, D.N., Boerner, R.E.J., 1991. Allyl isothiocyanate release and the allelopathic potential of *Brassica napus* (Brassicaceae). Am. J. Bot. 78 (8), 1083–1090.
de Klein, C.A.M., Barton, L., Sherlock, R.R., Li, Z., Littlejohn, R.P., 2003. Estimating a nitrous oxide emission factor for animal urine from some New Zealand pastoral soils. Soil Res. 41 (3), 381–399.
de Klein, C.A.M., Cameron, K.C., Di, H.J., Rys, G., Monaghan, R.M., Sherlock, R.R., 2011. Repeated annual use of the nitrification inhibitor dicyandiamide (DCD) does not alter its effectiveness in reducing N2O emissions from cow urine. Anim. Feed Sci. Technol. 166–167, 480–491.
Di, H.J., Cameron, K.C., 2011. How does the application of different nitrification inhibitors affect nitrous oxide emissions and nitrate leaching from cow urine in grazed pastures? Soil Use Manag. 28 (1), 54–61.
Di, H.J., Cameron, K.C., Shen, J.P., Winefield, C.S., O'Callaghan, M., Bowatte, S., He, J.Z., 2009. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. Nat. Geosci. 2 (9), 621–624.
Di, H.J., Cameron, K.C., Sherlock, R.R., Shen, J.-p., He, J.-z., & Winefield, C. S., 2010. Nitrous oxide emissions from grazed grassland as affected by a nitrification inhibitor, dicyandiamide, and relationships with ammonia-oxidizing bacteria and archaea. J. Soils Sediments 10 (5), 943–954.
Fahey, J.W., Zalcmann, A.T., Talalay, P., 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 56 (1), 5–51.
Firestone, M.K., Davidson, E.A., 1989. Microbial basis of NO and N_2O production and consumption. In: Andreae, M.O., Schimel, D.S. (Eds.), Exchange of Trace Gases between Terrestrial Ecosystems and the Atmosphere. John Wiley and Sons Ltd., New York, United States of America, pp. 7–21.
Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M., Toulmin, C., 2010. Food security: the challenge of feeding 9 billion people. Science 327 (5967), 812–818.
Gopalakrishnan, S., Subbarao, G.V., Nakahara, K., Yoshihashi, T., Ito, O., Maeda, I., Ono, H., Yoshida, M., 2007. Nitrification inhibitors from the root tissues of *Brachiaria humidicola*, a tropical grass. J. Agric. Food Chem. 55 (4), 1385–1388.
Haynes, R.J., Williams, P.H., 1993. Nutrient cycling and soil fertility in the grazed pasture ecosystem. In: Donald, L.S. (Ed.), Advances in Agronomy. vol. 49. Academic Press, pp. 119–199.
Hewitt, A.E., 1998. New Zealand Soil Classification - Landcare Research Science Series No. 1. Manaaki Whenua Press, Lincoln, Canterbury, New Zealand.
Ishikawa, T., Subbarao, G.V., Ito, O., Okada, K., 2003. Suppression of nitrification and nitrous oxide emission by the tropical grass *Brachiaria humidicola*. Plant Soil 255 (1), 413–419.
Kawakishi, S., Kaneko, T., 1987. Interaction of proteins with allyl isothiocyanate. J. Agric. Food Chem. 35, 85–88.
Kelliher, F.M., Clough, T.J., Clark, H., Rys, G., Sedcole, J.R., 2008. The temperature dependence of dicyandiamide (DCD) degradation in soils: a data synthesis. Soil Biol. Biochem. 40 (7), 1878–1882.
Kirkegaard, J.A., Howe, G.N., Mele, P.M., 1999. Enhanced accumulation of mineral-N following canola. Aust. J. Exp. Agric. 39, 587–593.
Kirkegaard, J.A., Sarwar, M., 1998. Biofumigation potential of brassicas. Plant Soil 201 (1), 71–89.
Ledgard, S.F., De Klein, C.A.M., Crush, J.R., Thorrold, B.S., 2000. Dairy farming, nitrogen losses and nitrate-sensitive areas. New Zealand Society of Animal Production. vol. 60, pp. 256–260 (Hamilton, New Zealand).
Ledgard, S.F., Luo, J., Sprosen, M.S., Wyatt, J.B., Balvert, S.F., Lindsey, S.B., 2014. Effects of the nitrification inhibitor dicyandiamide (DCD) on pasture production, nitrous oxide emissions and nitrate leaching in Waikato, New Zealand. N. Z. J. Agric. Res. 57 (4), 294–315.
Li, J., Luo, J., Shi, Y., Houlbrooke, D., Wang, L., Lindsey, S., Li, Y., 2015. Nitrogen gaseous emissions from farm effluent application to pastures and mitigation measures to reduce the emissions: a review. N. Z. J. Agric. Res.
Linn, D.M., Doran, J.W., 1984. Effect of water-filled pore space on carbon dioxide and nitrous oxide production in tilled and nontilled soils1. Soil Sci. Soc. Am. J. 48 (6), 1267–1272.
Lodhi, M.A.K., Killingbeck, K.T., 1980. Allelopathic inhibition of nitrification and nitrifying bacteria in a ponderosa pine (*Pinus ponderosa* Dougl.) community. Am. J. Bot. 67 (10), 1423–1429.
Luo, J., de Klein, C.A.M., Ledgard, S.F., Saggar, S., 2010. Management options to reduce nitrous oxide emissions from intensively grazed pastures: a review. Agric. Ecosyst. Environ. 136 (3–4), 282–291.
Luo, J., Ledgard, S., Wise, B., Welten, B.G., Lindsey, S., Judge, A., Sprosen, M.S., 2015a. Effect of dicyandiamide (DCD) delivery method, application rate, and season on pasture urine patch nitrous oxide emissions. Biol. Fertil. Soils 51, 453–464.
Luo, J., Ledgard, S.F., de Klein, C.A.M., Lindsey, S.B., Kear, M., 2008. Effects of dairy farming intensification on nitrous oxide emissions. Plant Soil 309 (1–2), 227–237.
Luo, J., Ledgard, S.F., Lindsey, S.B., 2013. Nitrous oxide and greenhouse gas emissions from grazed pastures as affected by use of nitrification inhibitor and restricted grazing regime. Sci. Total Environ. 465 (0), 107–114.
Luo, J., Sun, X.Z., Pacheco, D., Ledgard, S., Lindsey, S., Hoogendoorn, C.J., Wise, B., Watkins, N., 2015b. Nitrous oxide emission factors for urine and dung from sheep fed either fresh forage rape (*Brassica napus* L.) or fresh perennial ryegrass (*Lolium perenne* L.). Animal 9 (3), 534–543.
Luo, J., Tillman, R.W., White, R.E., Ball, P.R., 1998. Variation in denitrification activity with soil depth under pasture. Soil Biol. Biochem. 30 (7), 897–903.
Ministry for the Environment, 2015. New Zealand's Greenhouse Gas Inventory 1990–2013. Wellington, New Zealand http://www.mfe.govt.nz/publications/climate-change/new-zealands-greenhouse-gas-inventory-1990-2013 (485 p.).
Myhre, G., Shindell, D., Breon, F.-M., Collins, W., Fuglestvedt, J., Huang, J., Koch, D., Lamarque, J.-F., Lee, D., Mendoza, B., Nakajima, T., Robock, A., Stephens, G., Tekemura, T., Zhang, H., 2013. Anthropogenic and natural radiative forcing. In: Stocker, T.F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M. (Eds.), Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, pp. 659–740.
Oenema, O., Wrage, N., Velthof, G.L., Van Groenigen, J.W., Dolfing, J., Kuikman, P.J., 2005. Trends in global nitrous oxide emissions from animal production systems. Nutr. Cycl. Agroecosyst. 46, 119–199.
Prasad, R., Power, J.F., 1995. Nitrification inhibitors for agriculture, health, and the environment. Adv. Agron. 54, 233–281.
R Core Team, 2016. R: A Language and Environment for Statistical Computing.
Ravishankara, A.R., Daniel, J.S., Portmann, R.W., 2009. Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century. Science 326 (5949), 123–125.

Reardon, C.L., Strauss, S.L., Mazzola, M., 2013. Changes in available nitrogen and nematode abundance in response to *Brassica* seed meal amendment of orchard soil. Soil Biol. Biochem. 57, 22–29.

Rochette, P., Chantigny, M.H., Ziadi, N., Angers, D.A., Bélanger, G., Charbonneau, É., Pellerin, D., Liang, C., Bertrand, N., 2014. Soil nitrous oxide emissions after deposition of dairy cow excreta in Eastern Canada. J. Environ. Qual. 43 (3), 829–841.

Rotthauwe, J.H., Witzel, K.P., Liesack, W., 1997. The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63 (12), 4704–4712.

Rumberger, A., Marschner, P., 2003. 2-Phenylethylisothiocyanate concentration and microbial community composition in the rhizosphere of canola. Soil Biol. Biochem. 35 (3), 445–452.

Ryan, M.H., Kirkegaard, J.A., Angus, J.F., 2006. *Brassica* crops stimulate soil mineral N accumulation. Aust. J. Soil Res. 44, 367–377.

Saggar, S., Bolan, N.S., Bhandral, R., Hedley, C.B., Luo, J., 2004. A review of emissions of methane, ammonia, and nitrous oxide from animal excreta deposition and farm effluent application in grazed pastures. N. Z. J. Agric. Res. 47 (4), 513–544.

Selbie, D.R., Buckthought, L.E., Shepherd, M.A., 2015. The challenge of the urine patch for managing nitrogen in grazed pasture systems. Adv. Agron. 129, 229–292.

Shepherd, M., Welten, B., Wyatt, J., Balvert, S., 2014. Precipitation but not soil texture alters effectiveness of dicyandiamide to decrease nitrate leaching from dairy cow urine. Soil Use Manag. 30 (3), 361–371.

Subbarao, G.V., Rao, I.M., Nakahara, K., Sahrawat, K.L., Ando, Y., Kawashima, T., 2013. Potential for biological nitrification inhibition to reduce nitrification and N_2O emissions in pasture crop-livestock systems. Animal 7 (Suppl. 2), 322–332.

Subbarao, G.V., Rondon, M., Ito, O., Ishikawa, T., Rao, I.M., Nakahara, K., Lascano, C., Berry, W., 2007. Biological nitrification inhibition - is it a widespread phenomenon? Plant Soil 294, 5–18.

Subbarao, G.V., Yoshihashi, T., Worthington, M., Nakahara, K., Ando, Y., Sahrawat, K.L., Rao, I.M., Lata, J.C., Kishii, M., Braun, H.J., 2015. Suppression of soil nitrification by plants. Plant Sci. 233, 155–164.

Suzuki, M.T., Taylor, L.T., DeLong, E.F., 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. Appl. Environ. Microbiol. 66 (11), 4605–4614.

Chapter 4

Can incorporating brassica tissues into soil reduce nitrification rates and nitrous oxide emissions?

This chapter has been submitted to an international peer-reviewed journal and is presented in this thesis in manuscript format:

Please refer to Appendix B for the contribution of each of the authors.

Journal of Environmental Quality TECHNICAL REPORTS
ATMOSPHERIC POLLUTANTS AND TRACE GASES

Can Incorporating Brassica Tissues into Soil Reduce Nitrification Rates and Nitrous Oxide Emissions?

S. F. Balvert,* J. Luo, and L. A. Schipper

Abstract

New Zealand agriculture is composed predominantly of pastoral grazing systems; however, forage crops have been increasingly used to supplement the diet of grazing animals. Excreta from grazing animals has been identified as a main contributor of N_2O emissions. Some forage crops, such as brassicas (*Brassica* spp.), contain secondary metabolites that have been identified to inhibit soil N cycling processes, and nitrification in particular. Our objective was to determine if secondary metabolites released from brassica tissues inhibited nitrification and reduced N_2O emissions when incorporated into soil, which was amended with a large amount of urea N (such as derived from urine patches deposited during grazing). Three brassica tissues (kale [*Brassica oleracea* L.], turnip [*Brassica rapa* L.] bulb, and turnip leaf and stem) and ryegrass (*Lolium perenne* L.) tissue were incorporated into soil with and without urea solution, and N_2O, NO_3^-, and NH_4^+ were measured during a 52-d incubation. All brassica tissues reduced urea-derived N_2O emissions relative to ryegrass tissues when incorporated into soil. According to the mineral N and microbial community data, this reduction, however, could not be attributed to inhibition of nitrification. Although there was less N_2O from urea in the brassica treatments, total N_2O emissions increased after incorporation of all tissue residues into soil, so this tradeoff must be explored if brassica tissues are to be considered as a tool for N_2O reduction.

Core Ideas

- Brassicas have been postulated to inhibit nitrification and N_2O emissions.
- Incorporating brassica tissues into soil reduced N_2O emissions from added urea.
- There was no evidence that tissue incorporation inhibited nitrification.
- However, addition of ryegrass and brassica tissues increased total N_2O emissions.
- N_2O emission tradeoffs between tissue inputs and inhibition need to be explored.

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*Corresponding author (shereebalvert@gmail.com).

With the growing global population and improving standards of living, there is a demand for increased food production. Concomitantly, there is a need to mitigate the environmental costs of this increased production, such as agricultural greenhouse gas (GHG) emissions (Smith, 2013). In New Zealand, pastoral agriculture contributes ~5% of gross domestic product and 48% of the country's total GHG inventory, and soil-derived N_2O emissions makes up 11% of total GHG emissions (Ministry for the Environment, 2015). Nitrous oxide is a particularly potent GHG, having a global warming potential 265 to 298 times that of CO_2 (Myhre et al., 2013), and it also plays a role in stratospheric ozone depletion (Ravishankara et al., 2009). Identifying approaches that decrease N_2O emissions from pastoral agriculture is essential for reducing New Zealand's national GHG emissions.

In grazed pastoral agriculture, the main source of N_2O production is urine patches, where N_2O is produced during the biological transformation of urinary N in soil, in particular the soil microbial processes related to nitrification and denitrification (Butterbach-Bahl et al., 2013; van Groenigen et al., 2015). Due to the inefficient use of dietary N by grazing ruminants, where 70 to 95% of ingested N is excreted (Oenema et al., 2005), a urine patch delivers a very high rate of N to the soil (average = 613 kg N ha^{-1}), which is well above plant requirements (Selbie et al., 2015). The high N concentration in urine patches also leads to NO_3^- leaching losses that can contribute to indirect N_2O emissions (Butterbach-Bahl et al., 2013; Fowler et al., 2013).

Inhibitors of soil inorganic N transformations have been extensively researched, with nitrification inhibitors in particular identified by the Intergovernmental Panel for Climate Change (IPCC) as a significant opportunity for reducing N_2O loss from pastoral agriculture (IPCC, 2014). This focus is because nitrification has been estimated to indirectly contribute up to 80% of soil N_2O emissions (Hu et al., 2015) through the loss of N_2O during the conversion of the nitrification intermediary NH_2OH, the denitrification of NO_2^- by nitrifiers, and more significantly, due to the provision of NO_3^- as a substrate for classical

S.F. Balvert and J. Luo, AgResearch Limited, Ruakura Research Centre, Private Bag 3123, Hamilton 3240, New Zealand; S.F. Balvert and L.A. Schipper, Univ. of Waikato, Faculty of Science and Engineering, Private Bag 3105, Hamilton 3240, New Zealand. Assigned to Associate Editor Kate Congreves.

Abbreviations: AOB, ammonia-oxidizing bacteria; BNI, biological nitrification inhibition; DCD, dicyandiamide; GHG, greenhouse gas; GLS, glucosinolate; HP, hydrolysis product; qPCR, quantitative polymerase chain reaction; PCR, polymerase chain reaction; TGA, trace gas analyzer.

denitrification (Firestone and Davidson, 1989; Wrage et al., 2005; Butterbach-Bahl et al., 2013; Saggar et al., 2013).

The majority of nitrification inhibitor studies have examined synthetic, organic compounds such as dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (Di and Cameron, 2011; Li et al., 2014; Barneze et al., 2015). The DCD compound, for example, was shown to be effective at reducing N_2O emissions by an average of 57% (de Klein et al., 2011; Di and Cameron, 2011). However, there is now an interest in finding nonsynthetic inhibitors. Some plants have been shown to naturally inhibit nitrification via the production of secondary metabolites (Subbarao et al., 2015). This is termed biological nitrification inhibition (BNI), and there are many documented examples, particularly among tropical grasses (Subbarao et al., 2007). Nitrification is generally inhibited by the exudation of secondary metabolites from roots (Subbarao et al., 2015). However, changes in N transformation rates have also been observed after incorporation of crop tissues into soil; for example, brassica (*Brassica* spp.) crops (Brown and Morra, 2009; Reardon et al., 2013). Furthermore, many studies have reported higher residual soil mineral N concentrations following brassica crops compared with those following other crops such as wheat (*Triticum aestivum* L.) (Kirkegaard et al., 1999; Ryan et al., 2006; O'Sullivan et al., 2016), which also suggests the potential for these plants to alter nitrification and denitrification rates. This inhibition by brassicas has been attributed to the hydrolysis products (HPs) of the secondary metabolites called glucosinolates (GLSs), which are present in brassica tissues (Blažević et al., 2017). Upon tissue disruption, GLSs come into contact with the enzyme myrosinase and are hydrolyzed to isothiocyanates, thiocyanates, and nitriles (Collett et al., 2014). Bending and Lincoln (2000) demonstrated the specific inhibition of soil nitrifying bacteria by these GLS HPs, indicating that nitrification in particular may be inhibited. Balvert et al. (2017) also demonstrated that some pure GLS HPs could inhibit nitrification in vitro (e.g., phenylethyl isothiocyanate and 2-propenyl nitrile); however, these effects were not evident in field trials.

Although consistent observation of BNI is still lacking, the inclusion of pastures and forages that exhibit BNI potential in pastoral agricultural systems is being considered as a useful tool for reducing N_2O emissions (Di et al., 2016; Gardiner et al., 2016; Balvert and Luo, 2017; Luo et al., 2018). In New Zealand pastoral agriculture, diverse pastures and forages are also being increasingly used for additional benefits such as drought tolerance or to meet feed deficits in winter or summer (Hoogendoorn et al., 2016). Brassica crops are one such common example. Demonstrating whether N_2O emissions are reduced by the use of these forages in agricultural systems will allow for better farm management decisions with potential dual benefits of increased food production and decreased N_2O emissions.

Brassica crops are generally break fed daily, and stocking density may be very high when grazing occurs (1000–1400 cows ha^{-1}; Monaghan et al., 2013). To date, studies of BNI have focused on root exudates (Zakir et al., 2008; Subbarao et al., 2009, 2013); however, during grazing, damaged plants can be trampled into soil representing an alternative pathway for inputs of GLS HPs into soil. There have been very few published studies on brassica forage crop residues or wastage (Bastiman and Slade, 1978; Gowers and Armstrong, 1994), and none investigating a current New Zealand system, yet crop residue or wastage may represent a significant input of BNI. Additionally, incorporating brassica crops directly into soil as a green manure may also provide a biological option for inhibiting nitrification and N_2O production.

As mentioned above, previous studies have investigated the impact of brassica crops on soil N cycling (Kirkegaard et al., 1999; Velthof et al., 2002; Ryan et al., 2006; Brown and Morra, 2009); however, these were in relatively low-N-input management systems and did not include grazing animals or urine patches. Using forage crops to reduce N losses from pastoral grazing systems requires an understanding of how such crops affect N_2O emissions. Therefore, the objective of this study was to determine whether the incorporation of brassica tissues reduced N_2O emissions under high-N-input conditions. We hypothesized that brassica tissues would reduce nitrification rates and thus N_2O production from a urine patch. To test this hypothesis, we incubated soil amended with three brassica tissues and a urea solution while measuring soil mineral N concentrations and N_2O emissions over time. We also measured ammonia-oxidizing bacteria (AOB) population numbers to determine whether any difference in N transformations was a result of nitrification inhibition. A treatment with ryegrass (*Lolium perenne* L.), the dominant feed in New Zealand pastures, was also included for comparison.

Materials and Methods

Experimental Setup

A Bruntwood silt loam soil (typic impeded allophanic soil; Hewitt, 1998) from under a mixed perennial ryegrass and white clover (*Trifolium repens* L.) pasture was collected (0–7.5 cm) for an incubation study. General soil properties were pH 5.9, 0.61% total N, 6.0% total C, and 17, 61, and 22% sand, silt, and clay, respectively. The collected soil was sieved (4 mm) and homogenized before being preincubated for 24 h to allow the effects of soil disturbance to subside. A jar incubation was set up to permit N_2O flux determinations. Moist soil (140 g oven dry equivalent) was weighed into preserving jars (1 L) and amended with one of four crop tissues (ryegrass and three brassica: kale [*Brassica oleracea* L.], turnip [*Brassica rapa* L.] leaf and stem, and turnip bulb), with or without the addition of N. Soils without added crop tissues (with and without N) were also included as controls. The soil was packed to achieve a soil bulk density of 0.75 g cm^{-3}, which was standardized across all treatments. This bulk density is similar to the bulk density of this soil in the field (Singleton, 1991). Each treatment was replicated four times. Urea was applied as a solution at 600 μg N g^{-1} soil to match concentrations expected in a urine patch in the field (Selbie et al., 2015). Freshly collected crop tissues were applied as a pulp at 0.008 g dry matter g^{-1} soil to achieve 20 μg GLS HP g^{-1} soil (based on GLS content being about 1% of dry matter in brassica tissues; Fahey et al., 2001), and since Balvert et al. (2017) observed a reduction in N_2O production at this rate. Ryegrass tissues were added at the same dry matter rate to approximately match both the N and C addition of the brassica treatments. Carbon and N concentrations of each plant are presented in Table 1. The moisture content of the soil in the jars was adjusted to 65% of water-holding capacity (including moisture from the plant tissues), and the jars were placed in a randomized block format in a constant temperature room (20 ± 2°C). Between sampling, jars were covered with perforated Parafilm allowing normal gas exchange while minimizing

Table 1. Carbon, N, C/N ratio, and tissue C and N additions of each of the crop tissues used in the incubation trial.

Parameter	Ryegrass	Kale	Turnip leaf and stem	Turnip bulb
C (%)	42.7	42.6	38.5	37.9
N (%)	3.50	2.80	4.84	2.24
C/N	12.2	15.2	7.95	16.9
Tissue C added (g g^{-1} soil)	0.0034	0.0034	0.0031	0.0030
Tissue N added (μg g^{-1} soil)	280	224	387	179
Urea N added (μg g^{-1} soil)	600	600	600	600

evaporative losses. Soil moisture content was maintained during the subsequent incubation by weighing the jars and then adding distilled water to replenish any water loss.

A matching set of soils and treatments were established to allow for measurement of mineral N transformations. Moist soil (300 g oven dry equivalent) was weighed into plastic bags, and the same treatments as for the gas measurements were applied. These bags were also stored in a constant temperature room at 20°C. The bags were stored closed but opened regularly to allow for gas exchange as per the jars, and soil moisture was monitored and adjusted through time.

Gas Flux Measurement

Nitrous oxide fluxes were measured 13 times over a 52-d incubation period (1, 2, 3, 5, 7, 10, 13, 17, 21, 28, 36, 43, and 52 d after treatment application). This time length was chosen because N_2O flux had generally returned to background by this time. Sampling was more frequent early in the incubation to capture the initial flush. Gas production was measured by headspace accumulation using a trace gas analyzer (TGA; Innova 1412i-2, Lumasense Technologies). Jars were sealed with gas-tight lids fitted with ports and Teflon tubes for attachment to the TGA. Prior to entering the TGA, samples were drawn through a soda-lime filter to minimize interference by CO_2. The TGA corrected for interference from water vapor and any remaining CO_2. The N_2O concentration in the headspace was measured following 1 h of accumulation (i.e., samples taken at 0 and 60 min). Preliminary work demonstrated that, at this soil to headspace ratio, N_2O accumulation in the jar was linear over 1 h. Hourly N_2O fluxes were calculated for each jar using the increase in headspace N_2O concentration. The hourly fluxes were integrated to estimate daily emissions, total emission, and the percentage of applied N emitted as N_2O-N over the study period. The percentage of applied urea N emitted as N_2O-N was calculated as follows:

$$\%\ \text{Applied urea N emitted as } N_2O = \left(\frac{\text{Total } N_2O_{\text{Plant + 600N}} - \text{Total } N_2O_{\text{Plant + 0N}}}{\text{Urea N}}\right) 100$$

where Total $N_2O_{\text{Plant + 600N}}$ was the N_2O emissions for each crop tissue plus urea addition, and Total $N_2O_{\text{Plant + 0N}}$ was the N_2O emissions for the crop tissue only. This approach, however, assumes that the C/N ratio of the plant + 600N amendment does not influence N_2O production.

For measurement of CO_2 production, the jars were sealed with gas-tight lids containing a septum, and CO_2 was measured after accumulation over 16 h (samples taken at 0 and 16 h). Headspace samples (12 mL) were collected and stored over pressurized in pre-evacuated (0.1 kPa) glass vials (6 mL). The CO_2 concentration was determined using an infrared gas analyzer (IRGA, LI-COR LI-6262). Similar to N_2O, CO_2 fluxes were calculated from the increase in headspace CO_2 concentration over the 16-h period.

Nitrogen Transformations

On the same days as the gas measurements, the soils in the incubation bags were sampled for measurement of soil mineral N, pH, and moisture. For NH_4^+–N and NO_2^- + NO_3^-–N, soil (3 g oven dry equivalent) was extracted with 2 M KCl (30 mL). Soil pH was measured in water using a 1:2.5 ratio, and soil moisture was determined by oven drying a subsample of the moist soil at 105°C for 24 h. Concentrations of NH_4^+–N and NO_2^- + NO_3^-–N in the KCl extracts were measured colorimetrically using a Skalar SAN^{2+} segmented flow analyzer (Skalar Analytical) (Blakemore et al., 1987).

Microbial Community Quantification

Soils from the soil bags were also sampled on Days 5 and 21 for *amoA* gene abundance to determine whether incorporation of crop tissues decreased the abundance of AOB in the soils. Only AOB were enumerated, as Di et al. (2009) demonstrated that AOB, as opposed to ammonia-oxidizing archaea, are largely responsible for ammonia oxidation in pastoral soils and under high N loads. Soil samples were stored at −80°C between collection and DNA extraction. Total soil genomic DNA was extracted using MoBio Powersoil DNA isolation kits (MoBio Laboratories, GeneWorks) according to the manufacturer's instructions. The polymerase chain reaction (PCR) primer pair amoA1F/amoA2R was used to amplify regions of the bacterial *amoA* gene (Rotthauwe et al., 1997). Reactions (16 μL) contained 8 μL SYBR Premix Ex Taq (Takara Bio), 0.4 μL of each primer (10 μM concentration), 1.5 μL DNA (diluted 1:10 to reduce potential PCR inhibition), and 5.7 μL Milli-Q water. Real-time quantitative PCR (qPCR) analysis was performed on a Rotor-Gene 6000 (Corbett Life Science). Raw data analysis was performed using Rotor-Gene 6000 series software version 2.1 (Qiagen, 2012). Melting curve analysis was performed after each run to confirm PCR product specificity. Standard curves for qPCR analysis were developed by cloning *amoA* gene amplicons into pGEM–T Easy vectors (Promega) and transforming TOP10 *E. coli* (Thermo Fisher). Selected clones were incubated overnight in Luria broth liquid medium, and the plasmid DNA was subsequently extracted using a PureLink Quick Plasmid MiniPrep Kit (Thermo Fisher). Plasmid DNA concentration was measured on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and a standard curve was subsequently generated by dilution series.

Data Analysis

Daily N_2O flux and mineral N data were analyzed using the mixed model smoothing program FLEXI (Upsdell, 1994), which allows for comparison of multiple curves. Both treatment and time since treatment application were included as both random and fixed effects. Spline curves were fitted against time since treatment application, which allows for repeat measure analysis. Analysis of variance, using the statistical software package Genstat for Windows 18th edition (VSN International, 2015), was performed on the total emissions, the percentage of applied urea N emitted as N_2O data, and the microbial community data to determine the significance of the difference between treatments. Microbial community data were log transformed prior to analysis.

Results

Nitrous Oxide Flux and Total Emissions

All treatments except those receiving ryegrass tissues showed a high N_2O flux immediately after treatment addition, including the treatments that did not receive any urea N (Fig. 1). For the ryegrass treatments, peak N_2O flux for both the 0N and 600N treatments occurred 2 and 7 d after plant addition, respectively. The peaks from the ryegrass treatments were not as high as those from the brassica treatments; however, the peaks from the ryegrass treatments remained higher ($p < 0.05$) than the other treatments for ~10 d in the 0N treatments and for the entire study period in the 600N treatments.

In the 0N treatments, total N_2O emissions (Table 2) from turnip bulb addition (4.19 μg N_2O-N g^{-1} soil) were higher ($p < 0.05$) than from the other treatments (0.18– 2.61 μg N_2O-N g^{-1} soil). In the 600N treatments, total N_2O emissions from both turnip bulb (5.70 μg N_2O-N g^{-1} soil) and ryegrass (4.99 μg N_2O-N g^{-1} soil) addition were higher ($p < 0.001$) than from the other treatments. There was no difference in total N_2O emissions between 600N turnip bulb and 600N ryegrass treatments. All 600N treatments that received plant tissues had higher ($p < 0.05$) total N_2O emissions than the 600N no-plant treatment (1.33 μg N_2O-N g^{-1} soil).

Production of N_2O from urea alone was determined by subtracting the N_2O derived from the added plant material (treatment + 0N) from N_2O derived from both added plant and urea N (treatment + 600N). The ryegrass treatment had both the highest urea-derived emissions and the highest percentage of applied urea N emitted as N_2O (3.82 μg N_2O-N g^{-1} soil and 0.64%, respectively; $p < 0.05$). The brassica treatments reduced the percentage of applied N emitted as N_2O by up to 62% relative to the ryegrass treatment.

Soil Respiration

Soil respiration increased immediately after treatment addition in all treatments that received plant tissues (Fig. 2). Carbon dioxide flux peaked (5.4–7.9 μg C g^{-1} soil h^{-1}) within 3 d of treatment addition and dropped near to that of the control by

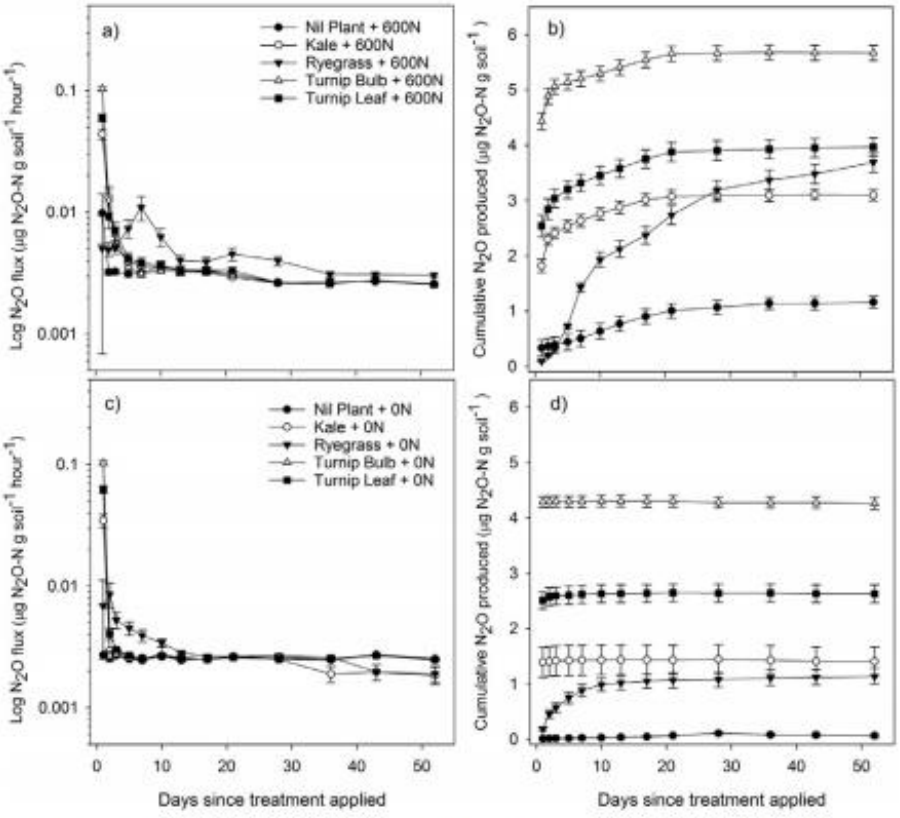

Fig. 1. Daily and cumulative N_2O fluxes from incubated soils after addition of urea and plant tissue (ryegrass, kale, turnip bulb, or turnip leaf): (a) N_2O flux for treatments with 600 μg N g^{-1} soil (600N); (b) cumulative N_2O produced for 600N treatments; (c) N_2O flux for no-N (0N) treatments; and (d) cumulative N_2O produced for 0N treatments. Note that the flux values are displayed on a log-scale axis. Error bars represent the SEM ($n = 4$).

Table 2. Total N_2O emissions, urea-derived N_2O emissions, and percentage of applied urea N emitted as N_2O, as affected by the addition of urea and either ryegrass, kale, turnip bulb, or turnip leaf plant tissue. The SEMs are included in parentheses, and the LSD ($p < 0.05$) for determination of significance is displayed ($n = 4$).

Treatment	Total N_2O emissions		Urea-derived N_2O emissions†	Applied urea N emitted as N_2O	Change cf. ryegrass ‡
	0N	600N			
	μg N_2O–N g^{-1} soil			%	
No plant	0.18 (0.11)a§	1.33 (0.19)a	1.14 (0.22)a	0.19 (0.03)a	−70
Ryegrass	1.17 (0.30)b	4.99 (0.45)de	3.82 (0.36)b	0.64 (0.08)b	
Kale	1.44 (0.53)b	3.13 (0.20)bc	1.69 (0.21)a	0.28 (0.03)a	−56
Turnip bulb	4.19 (0.19)d	5.70 (0.27)e	1.51 (0.28)a	0.25 (0.04)a	−60
Turnip leaf	2.61 (0.35)c	4.05 (0.36)cd	1.44 (0.33)a	0.24 (0.06)a	−62
LSD (5%)	0.964	0.964	0.798	0.16	
P value	<0.001	<0.001	<0.001	<0.001	

† Calculated as the difference between plant + 600 μg N g^{-1} soil (600N) and plant + no N (0N).

‡ Calculated as the percentage difference between brassica crop percentage of applied urea N emitted as N_2O and ryegrass percentage of applied urea N emitted as N_2O: [1 − (brassica crop + 600N)/(ryegrass + 600N)] × 100.

§ Different letters in the same column indicate significant differences.

the end of the measurement period. The treatments containing brassica tissues all had higher peak fluxes (although not statistically significant) than the ryegrass treatments; however, the ryegrass treatment fluxes remained high for a longer period.

Nitrogen Transformations

The impacts of the incorporation of the different crop tissues on mineral N transformations are shown in Fig. 3. For all treatments, soil NH_4^+–N concentrations peaked 1 d after treatment application and then gradually decreased. There was no significant difference in response of soil NH_4^+–N to any of the treatments over the first 13 d; however, the ryegrass 600N treatment tended to be higher. On Days 17 and 21, soil NH_4^+–N was significantly higher ($p < 0.05$) in the ryegrass 600N treatment compared with the other 600N treatments. There was no difference in soil NH_4^+–N for the remainder of the trial period. Soil NO_3^-–N concentrations increased throughout the trial period in all treatments except the turnip bulb 0N treatment, which showed a sharp decrease 2 d after treatment application. In the 0N treatments, ryegrass produced a higher ($p < 0.05$) soil NO_3^-–N concentration compared with

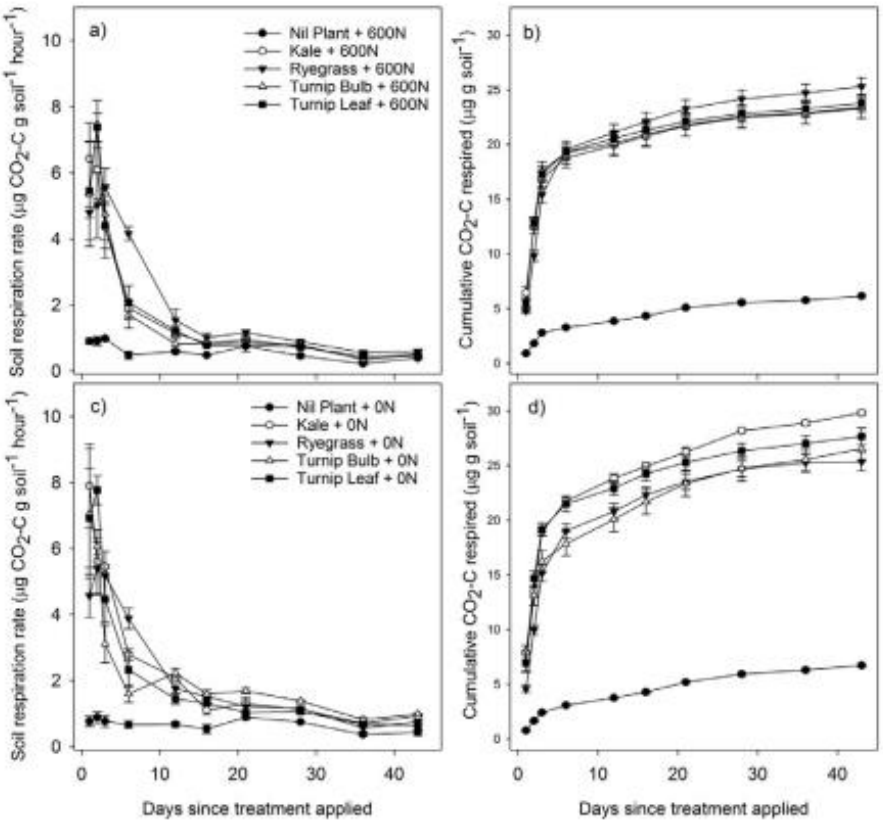

Fig. 2. Soil respiration rates (CO_2 flux) and cumulative C respired from incubated soil after addition of urea and plant tissue (ryegrass, kale, turnip bulb, or turnip leaf): (a) soil respiration rate in treatments with 600 μg N g^{-1} soil (600N) ; (b) cumulative C respired for 600N treatments; (c) soil respiration rate for no-N (0N) treatments; and (d) cumulative C respired for 0N treatments. Error bars represent the SEM ($n = 4$).

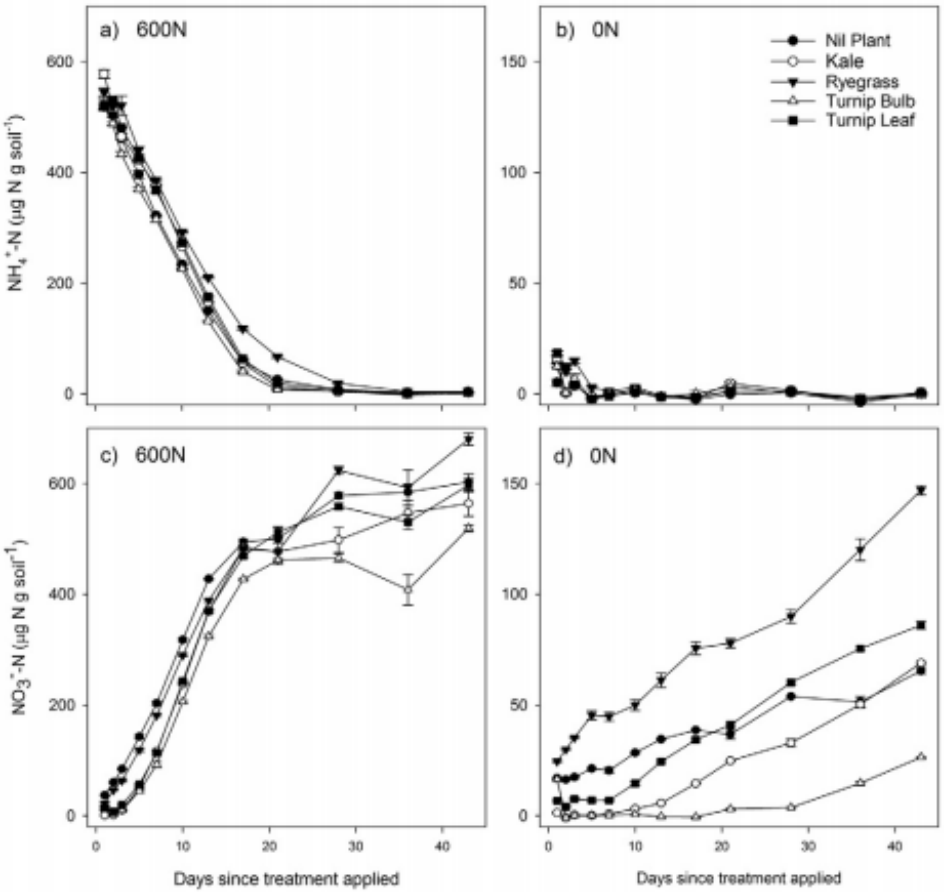

Fig. 3. Soil mineral N concentrations in incubated soils after application of urea and plant tissues: (a) soil NH_4^+–N concentrations for treatments with 600 µg N g^{-1} soil (600N); (b) soil NH_4^+–N concentrations for no-N (0N) treatments; (c) soil NO_3^-–N concentrations for 600N treatments; and (d) soil NO_3^-–N concentrations for 0N treatments. Error bars represent the SEM (n = 4). Note the different y axis scales between the 0N and 600N treatments.

the other treatments for the whole trial period, and turnip bulb induced lower soil NO_3^-–N concentration from Day 17 onward. When these data are expressed as per unit N applied (accounting for differences in plant N content) the same pattern was observed (data not shown). There was no significant difference between all 600N treatments.

Microbial Community

The addition of plant tissues without urea did not significantly affect AOB *amoA* gene abundance. The addition of the urea solution significantly increased the AOB *amoA* gene abundance in all treatments, except turnip leaf, relative to plant tissues only (Fig. 4), and on both sampling days (Day 5 and 21) measured. The average increase, as a result of urea addition, was 5.9 × 10^5 on Day 5 and decreased to 1.8 × 10^5 on Day 21. The addition of brassica tissues did not affect AOB *amoA* gene abundance relative to the ryegrass treatment on either of the days sampled.

Discussion

Nitrous Oxide Emissions Derived from Urea Nitrogen

We were able to distinguish N_2O emissions derived from urea N and those derived from added plant N by calculating the difference between emissions from the combined urea and plant treatment (ryegrass 600N, kale 600N, turnip leaf 600N, and turnip bulb 600N) and the plant-only treatment (ryegrass 0N, kale 0N, turnip leaf 0N, and turnip bulb 0N). The addition of plants, without urea, also increased N_2O emissions, presumably because increased labile C and N from plant cells provided substrate for nitrification and denitrification (Reddy et al., 1982; Baggs et al., 2000; Kuzyakov and Bol, 2006; Qiu et al., 2016).

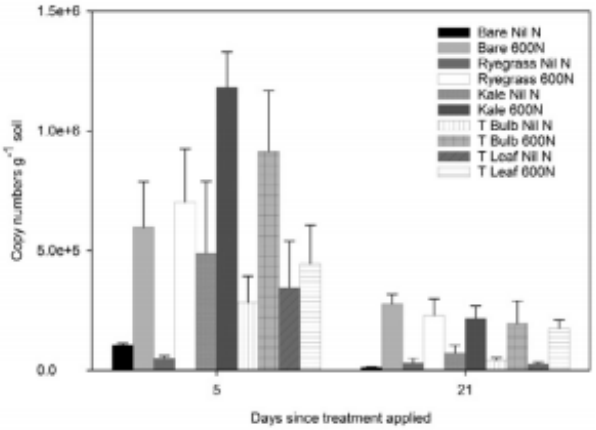

Fig. 4. Ammonia-oxidizing bacteria *amoA* gene copy numbers as affected by incorporation of urea and plant tissues (ryegrass, kale, turnip [T] bulb, or turnip [T] leaf). Error bars represent the SEM (n = 4).

However, this addition of C alone does not explain the significant difference in emissions between the ryegrass treatment and other tissue treatments, as roughly similar C was added for all plant treatments. It is likely that the quality of C provided by the decomposing tissues is important in explaining the observed differences, and this is discussed further below.

In this study, crop tissues were incorporated on a consistent dry matter basis rather than on a percentage N basis, resulting in slightly different N additions among the different treatments. This small difference in percentage N might be expected to generate differences in emissions in the 0N treatments but would not be expected to affect treatments where urea was added, as available soil N would be in excess. Additionally, previous research on a similar soil type (de Klein et al., 2014), found that the percentage of applied N emitted as N_2O (or emission factor) was independent of N application rate between 200 and 1400 kg N ha^{-1} (approximately equivalent to 200–1400 μg N g^{-1} soil).

Our hypothesis was that brassica tissues would reduce nitrification rates and thus N_2O production from a urine patch, and the finding that lower N_2O emissions derived from urea in the presence of brassicas in comparison with ryegrass supports this hypothesis. One potential explanation for this observed difference was inhibition of N transformation processes by GLS HPs derived from GLSs present in brassica tissues. Balvert et al. (2017) reported a reduction in N_2O emissions after incorporation of some GLS HPs into soil receiving 600 μg g^{-1} soil. The mechanism by which this reduction occurred was attributed to the potential inhibition of soil nitrification by these compounds (Bending and Lincoln, 2000; Brown and Morra, 2009; Balvert et al., 2017).

Inhibition of nitrification would result in a decrease in the rate of soil NO_3^- formation in the brassica tissue treatments relative to the ryegrass treatments. In this current study, lower soil NO_3^- was measured in the 0N brassica treatments relative to ryegrass, and generally there was less NO_3^- in the urea brassica treatments (but these differences were not significant). This reduction suggested some inhibition of nitrification in the 0N treatments (which has been observed in other studies; Brown and Morra, 2009) that was perhaps swamped in the presence of high concentrations of N such as in the urea treatments. There was, however, no corresponding increase in soil NH_4^+ concentrations as would be expected if nitrification was being inhibited. It is possible that nitrification inhibition did occur, but that there was microbial uptake of NH_4^+–N perhaps as a result of the supply of C from the plant tissues. Enumeration of the AOB community, however, did not show evidence of inhibition of nitrification (Di and Cameron, 2016). The AOB community showed a significant increase after the addition of urea, as expected, but there was no observable difference between the ryegrass treatment and the brassica treatments to indicate inhibition of nitrification in the presence of GLS HPs. It is therefore unlikely that the observed reduction in urea-derived N_2O emissions was due to inhibition of nitrification by GLS HPs. Although there was no strong evidence for inhibition of nitrification by GLS HPs, it is possible that the GLS HPs were responsible for the observed reduction in N_2O emissions through another inhibitory mechanism. For example, GLS HPs are known to have broad biocidal activity, including fungicidal activity (Angus et al., 1994), and fungal contributions to N_2O production (Laughlin and Stevens, 2002) may have been significant in this instance. Plant tissues were incorporated to achieve an assumed level of 20 μg HP g^{-1} soil (Fahey et al., 2001), as Balvert et al. (2017) showed a reduction in N_2O production at that rate. However, Morra and Kirkegaard (2002) measured HP release from brassica tissues incorporated into soil and could only account for ~30% of the potentially available HP based on the original GLS content of the plants. Although the brassica tissues used in this study were blended into a slurry with the aim of achieving maximum cellular disruption, it is also possible that some of the GLSs remained unhydrolyzed and the intended concentration was not fully achieved. Before brassicas might be considered as a possible approach for reducing N_2O emissions, concentrations of GLSs in plant matter need to be determined along with rates and extent of hydrolysis once added into soil.

Total Nitrous Oxide Emissions

Because denitrification is generally C limited (Coyne, 2018), the addition of C-rich plant tissues to soil would increase the rate of denitrification. Total N_2O production was significantly higher from the turnip bulb treatment, both with and without the addition of urea N, than in other treatments. This was likely a result of the added C driving denitrification. This greater production, however, cannot be simply explained by the addition of C in plant tissue, as neither the C/N ratio nor total C of the tissues corresponded with N_2O production. In contrast, other studies have found relationships between CO_2 and N_2O emissions (Millar and Baggs, 2004) or between N_2O production and the C/N ratio of plant material being incorporated, with generally higher emissions from plants with low C/N ratios (Kaiser et al., 1998; Baggs et al., 2000). Qiu et al. (2016), however, suggested that the C/N ratio was not a good predictor of N_2O emissions if available soil N was not limited, as was the case in the current study with the addition of urea.

As discussed earlier, crop tissues were incorporated on a consistent dry matter basis rather than on a percentage N basis, resulting in differences in the level of N added to each treatment. Interestingly, although turnip leaf tissues had the highest percentage N (almost double that of the other brassica treatments), this treatment did not have the highest total N_2O emissions. Differences in total tissue C additions, however, were minimal. So the explanation for the observed difference in N_2O between turnip and other treatments is not fully clear. The addition of C in the plant tissues would likely have resulted in some priming of the soil microbial community, which could also have affected N_2O production (Kuzyakov and Bol, 2006). The added C may also have driven more complete denitrification and/or altered the N_2/N_2O product ratios; however, this could not be determined by the methodology used in this study. Also, other biogeochemical processes such as assimilation and immobilization of added N would likely have been affected by the addition of plant tissues and may contribute to the observed differences.

Pattern of Nitrous Oxide Emissions

The N_2O production (proportion of applied N emitted as N_2O-N) measured in this study was similar to that found in other studies using a similar incubation method (Clough et al., 2003; van Groenigen et al., 2005).

The high initial peak in N_2O flux after tissue incorporation was in line with other studies of this nature (Velthof et al., 2002;

Baggs et al., 2003) and was likely due to rapid stimulation (or priming) of microbial decomposition and provision of C and N substrate supply. Additionally, the slurry form in which the residues were applied may have initially created anaerobic microsites in the soil due to moisture addition and increased microbial respiration, temporarily increasing N_2O production via denitrification (Tiedje et al., 1984). This initial peak was significantly higher in those treatments that received urea solution, as would be expected after a large supply of a N substrate, as well as due to anaerobic conditions resulting from CO_2 produced after hydrolysis of HCO_3^- (Kelliher et al., 2014).

The peak in N_2O in response to N addition was immediate in all of the brassica treatments as well as the bare soil treatment; however, it was delayed in the ryegrass treatment. This peak was likely from the breakdown of the plant tissues that also supplied excess labile C as an energy source for denitrification and N_2O production, and so was driven by the biodegradability of C compounds of the different plant tissues. As mentioned earlier, not only is the quantity of C added important, but the quality of C will also play a major role in determining the availability of C for soil microorganisms. Brassica tissues contain a higher proportion of soluble sugars than ryegrass (DairyNZ, 2017), which are a readily degradable source of C. Ryegrass tissues, on the other hand, contain a higher proportion of lignified fiber and degrade much more slowly (Bonanomi et al., 2013), potentially resulting in a delay in the supply of labile C to the microbial pool and a corresponding delay in N_2O production. This delay was also supported by the soil respiration data where the bare soil treatments did not show a significant increase in soil respiration after treatment addition, and the soil respiration peak was delayed in the ryegrass treatment relative to the other tissue treatments. However, it appeared that residue C was not exclusively responsible for differences in N_2O production.

Potential Future Work Consideration

Future studies, such as using an isotope tracer technique, are required to fully explain the processes and factors affecting N_2O emissions after the addition of urea and brassica plant tissues to soil. In particular, future studies should be designed to investigate the degree of priming resulting from the addition of urea and/or brassica tissues and the effect this may have on N_2O emissions. Also, addition of brassica plant tissues would change the soil environment that affects soil N transformation processes. This could consequently enhance N_2O production or consume N_2O, potentially affecting the interpretation of results on N_2O emissions from the application of urea and on the inhibitory effects of GLS HPs. Other factors including soil moisture level would also potentially influence the effect of GLS HPs on N_2O production. Therefore, the N_2O production processes in soils amended with brassica plant tissues and/or urea, under different environmental conditions, need to be further explored

Conclusion

Incorporation of all brassica tissue types reduced urea-derived N_2O emissions relative to the ryegrass treatment. Mineral N data and microbial community analyses, however, showed that inhibition of nitrification by GLS HPs could not explain the difference in urea-derived N_2O emissions. The mechanism for reduced N_2O emissions from urea was not elucidated in this study. Although incorporation of brassica tissues decreased N_2O released from urea, suggesting there may be scope to use brassicas as a N_2O management tool, these tissues also supported increased N_2O emissions, presumably due to the provision of labile C and N substrates for nitrification and denitrification.

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References

Angus, J.F., P.A. Gardner, J.A. Kirkegaard, and J.M. Desmarchelier. 1994. Biofumigation: Isothiocyanates released from *Brassica* roots inhibit the growth of the take-all fungus. Plant Soil 162:107–112. doi:10.1007/BF01416095

Baggs, E.M., R. Rees, K. Smith, and A. Vinten. 2000. Nitrous oxide emission from soils after incorporating crop residues. Soil Use Manage. 16:82–87. doi:10.1111/j.1475-2743.2000.tb00179.x

Baggs, E.M., M. Stevenson, M. Pihlatie, A. Regar, H. Cook, and G. Cadisch. 2003. Nitrous oxide emissions following application of residues and fertiliser under zero and conventional tillage. Plant Soil 254:361–370. doi:10.1023/A:1025593121839

Balvert, S., and J. Luo. 2017. Dietary manipulation as a tool for mitigating nitrous oxide emissions. In: J. Luo and Y. Li, editors, Greenhouse gas emissions and nitrogen losses from grazed dairy and animal housing systems. NOVA Sci. Publ., New York. p. 61–70.

Balvert, S.F., J. Luo, and L.A. Schipper. 2017. Do glucosinolate hydrolysis products reduce nitrous oxide emissions from urine affected soil? Sci. Total Environ. 603–604:370–380. doi:10.1016/j.scitotenv.2017.06.089

Barneze, A.S., E.P. Minet, C.C. Cerri, and T. Misselbrook. 2015. The effect of nitrification inhibitors on nitrous oxide emissions from cattle urine depositions to grassland under summer conditions in the UK. Chemosphere 119:122–129. doi:10.1016/j.chemosphere.2014.06.002

Bastiman, B., and C.F.R. Slade. 1978. The utilisation of forage crops- a review of recent work undertaken by Agricultural Development and Advisory Service (ADAS). ADAS Quarterly Rev. 28. Agric. Dev. Advisory Serv., Wolverhapton, UK.

Bending, G.D., and S.D. Lincoln. 2000. Inhibition of soil nitrifying bacteria communities and their activities by glucosinolate hydrolysis products. Soil Biol. Biochem. 32:1261–1269. doi:10.1016/S0038-0717(00)00043-2

Blakemore, L.C., P.L. Searle, and B.K. Daley. 1987. Methods for chemical analysis of soils. Sci. Rep. 80. N. Z. Soil Bureau, Wellington, New Zealand.

Blažević, I., S. Montaut, F. Burčul, and P. Rollin. 2017. Glucosinolates: Novel sources and biological potential. In: J. Merillon and K.G. Ramawat, editors, Glucosinolates. Springer Int. Publ., Cham, Switzerland. p. 15–73.

Bonanomi, G., G. Incerti, F. Giannino, A. Mingo, V. Lanzotti, and S. Mazzoleni. 2013. Litter quality assessed by solid state ^{13}C NMR spectroscopy predicts decay rate better than C/N and lignin/N ratios. Soil Biol. Biochem. 56:40–48. doi:10.1016/j.soilbio.2012.03.003

Brown, P.D., and M.J. Morra. 2009. Brassicaceae tissues as inhibitors of nitrification in soil. J. Agric. Food Chem. 57:7706–7711. doi:10.1021/jf901516h

Butterbach-Bahl, K., E.M. Baggs, M. Dannenmann, R. Kiese, and S. Zechmeister-Boltenstern. 2013. Nitrous oxide emissions from soils: How well do we understand the processes and their controls? Philos. Trans. R. Soc. Lond. B Biol. Sci. 368:20130122. doi:10.1098/rstb.2013.0122

Clough, T.J., R.R. Sherlock, and F.M. Kelliher. 2003. Can liming mitigate N_2O fluxes from a urine-amended soil? Soil Res. 41:439–457. doi:10.1071/SR02079

Collett, M.G., B.L. Stegelmeier, and B.A. Tapper. 2014. Could nitrile derivatives of turnip (*Brassica rapa*) glucosinolates be hepato- or cholangiotoxic in cattle? J. Agric. Food Chem. 62:7370–7375. doi:10.1021/jf500526u

Coyne, M.S. 2018. Denitrification in soil. In: R. Lal and B.A. Stewart, editors, Soil nitrogen uses and environmental impacts. CRC Press, Boca Raton, FL. p. 95–139.

DairyNZ. 2017. Feed values. DairyNZ, Hamilton, New Zealand. https://www.dairynz.co.nz/feed/supplements/feed-values/ (accessed 13 Nov. 2017).

de Klein, C.A.M., K.C. Cameron, H.J. Di, G. Rys, R.M. Monaghan, and R.R. Sherlock. 2011. Repeated annual use of the nitrification inhibitor dicyandiamide (DCD) does not alter its effectiveness in reducing N_2O emissions from cow urine. Anim. Feed Sci. Technol. 166–167:480–491. doi:10.1016/j.anifeedsci.2011.04.076

de Klein, C.A.M., J. Luo, K.B. Woodward, T. Styles, B. Wise, S. Lindsey, and N. Cox. 2014. The effect of nitrogen concentration in synthetic cattle urine on nitrous oxide emissions. Agric. Ecosyst. Environ. 188:85–92. doi:10.1016/j.agee.2014.02.020

Di, H.J., and K.C. Cameron. 2011. How does the application of different nitrification inhibitors affect nitrous oxide emissions and nitrate leaching from cow urine in grazed pastures? Soil Use Manage. 28:54–61. doi:10.1111/j.1475-2743.2011.00373.x

Di, H.J., and K.C. Cameron. 2016. Inhibition of nitrification to mitigate nitrate leaching and nitrous oxide emissions in grazed grassland: A review. J. Soils Sediments 16:1401–1420. doi:10.1007/s11368-016-1403-8

Di, H.J., K.C. Cameron, A. Podolyan, G.R. Edwards, C.A.M. de Klein, R. Dynes, and R. Woods. 2016. The potential of using alternative pastures, forage crops and gibberellic acid to mitigate nitrous oxide emissions. J. Soils Sediments 16:2252–2262. doi:10.1007/s11368-016-1442-1

Di, H.J., K.C. Cameron, J.P. Shen, C.S. Winefield, M. O'Callaghan, S. Bowatte, and J.Z. He. 2009. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. Nat. Geosci. 2:621–624. doi:10.1038/ngeo613

Fahey, J.W., A.T. Zalcmann, and P. Talalay. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 56:5–51. doi:10.1016/S0031-9422(00)00316-2

Firestone, M.K., and E.A. Davidson. 1989. Microbial basis of NO and N_2O production and consumption. In: M.O. Andreae and D.S. Schimel, editors, Exchange of trace gases between terrestrial ecosystems and the atmosphere. John Wiley & Sons, New York. p. 7–21.

Fowler, D., M. Coyle, U. Skiba, M.A. Sutton, J.N. Cape, S. Reis, et al. 2013. The global nitrogen cycle in the twenty-first century. Philos. Trans. R. Soc. Lond. B Biol. Sci. 368:20130164. doi:10.1098/rstb.2013.0164

Gardiner, C.A., T.J. Clough, K.C. Cameron, H.J. Di, G.R. Edwards, and C.A.M. de Klein. 2016. Potential for forage diet manipulation in New Zealand pasture ecosystems to mitigate ruminant urine derived N_2O emissions: A review. N. Z. J. Agric. Res. 59:301–317.

Gowers, S., and S.D. Armstrong. 1994. A comparison of the yield and utilisation of six kale cultivars. N. Z. J. Agric. Res. 37:481–485. doi:10.1080/00288233.1994.9513087

Hewitt, A.E. 1998. New Zealand soil classification. Landcare Res. Sci. Ser. 1. Manaaki Whenua Press, Lincoln, New Zealand.

Hoogendoorn, C.J., J. Luo, C.M. Lloyd-West, B.P. Devantier, S.B. Lindsey, S. Sun, et al. 2016. Nitrous oxide emission factors for urine from sheep and cattle fed forage rape (*Brassica napus* L.) or perennial ryegrass/white clover pasture (*Lolium perenne* L./*Trifolium repens*). Agric. Ecosyst. Environ. 227:11–23. doi:10.1016/j.agee.2016.04.029

Hu, H.-W., D. Chen, and J.-Z. He. 2015. Microbial regulation of terrestrial nitrous oxide formation: Understanding the biological pathways for prediction of emission rates. FEMS Microbiol. Rev. 39:729–749. doi:10.1093/femsre/fuv021

IPCC. 2014. Climate change 2014: Mitigation of climate change. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge Univ. Press, Cambridge, UK.

Kaiser, E.A., K. Kohrs, M. Kücke, E. Schnug, O. Heinemeyer, and J.C. Munch. 1998. Nitrous oxide release from arable soil: Importance of N-fertilization, crops and temporal variation. Soil Biol. Biochem. 30:1553–1563. doi:10.1016/S0038-0717(98)00036-4

Kelliher, F.M., Z. Li, and A.D.L. Nobel. 2014. Nitrogen application rate and nitrous oxide flux from a pastoral soil. N. Z. J. Agric. Res. 57:370–376. doi:10.1080/00288233.2014.927772

Kirkegaard, J.A., G.N. Howe, and P.M. Mele. 1999. Enhanced accumulation of mineral-N following Canola. Aust. J. Exp. Agric. 39:587–593. doi:10.1071/EA98163

Kuzyakov, Y., and R. Bol. 2006. Sources and mechanisms of priming effect induced in two grassland soils amended with slurry and sugar. Soil Biol. Biochem. 38:747–758. doi:10.1016/j.soilbio.2005.06.025

Laughlin, R.J., and R.J. Stevens. 2002. Evidence for fungal dominance of denitrification and codenitrification in a grassland soil. Soil Sci. Soc. Am. J. 66:1540–1548. doi:10.2136/sssaj2002.1540

Li, J., Y. Shi, J. Luo, M. Zaman, D. Houlbrooke, W. Ding, et al. 2014. Use of nitrogen process inhibitors for reducing gaseous nitrogen losses from land-applied farm effluents. Biol. Fertil. Soils 50:133–145. doi:10.1007/s00374-013-0842-2

Luo, J., S.F. Balvert, B. Wise, B. Welten, S.F. Ledgard, C.A.M. de Klein, et al. 2018. Using alternative forage species to reduce emissions of the greenhouse gas nitrous oxide from cattle urine deposited onto soil. Sci. Total Environ. 610–611:1271–1280. doi:10.1016/j.scitotenv.2017.08.186

Millar, N., and E.M. Baggs. 2004. Chemical composition, or quality, of agroforestry residues influences N_2O emissions after their addition to soil. Soil Biol. Biochem. 36:935–943. doi:10.1016/j.soilbio.2004.02.008

Ministry for the Environment. 2015. New Zealand's greenhouse gas inventory 1990–2013. Minist. Environ., Wellington, New Zealand. http://www.mfe.govt.nz/publications/climate-change/new-zealands-greenhouse-gas-inventory-1990-2013 (accessed 1 May 2017).

Monaghan, R.M., L.C. Smith, and C.A.M. de Klein. 2013. The effectiveness of the nitrification inhibitor dicyandiamide (DCD) in reducing nitrate leaching and nitrous oxide emissions from a grazed winter forage crop in southern New Zealand. Agric. Ecosyst. Environ. 175:29–38. doi:10.1016/j.agee.2013.04.019

Morra, M.J., and J.A. Kirkegaard. 2002. Isothiocyanate release from soil-incorporated *Brassica* tissues. Soil Biol. Biochem. 34:1683–1690. doi:10.1016/S0038-0717(02)00153-0

Myhre, G., D. Shindell, F.-M. Breon, W. Collins, J. Fuglestvedt, J. Huang, et al. 2013. Anthropogenic and natural radiative forcing. In: T.F. Stocker, et al., editors, Climate change 2013: The physical science basis. Contribution of Working Group I to the Fifth Assessment report of the Intergovernmental Panel on Climate Change. Cambridge Univ. Press, Cambridge, UK. p. 659–740.

Oenema, O., N. Wrage, G.L. Velthof, J.W. Van Groenigen, J. Dolfing, and P.J. Kuikman. 2005. Trends in global nitrous oxide emissions from animal production systems. Nutr. Cycl. Agroecosyst. 46:119–199.

O'Sullivan, C.A., E.G. Duncan, K. Whisson, K. Treble, M.M. Roper, and M.B. Peoples. 2016. Changes in N cycling in the rhizosphere of canola lead to decreased N requirements in a following wheat crop. In: Proceedings of the 7th International Nitrogen Initiative Conference: Solutions to improve nitrogen use efficiency for the world, Melbourne, Australia. http://www.ini2016.com/pdf-papers/INI2016_Osullivan_Cathryn2.pdf (accessed 19 May 2017).

Qiagen. 2012. Rotor-Gene Q user manual. Version 2. Qiagen, Hilden, Germany.

Qiu, Q., L. Wu, Z. Ouyang, B. Li, and Y. Xu. 2016. Effects of different forms of plant derived organic matter on nitrous oxide emissions. Environ. Sci. Process. Impacts 18:854–862.

Ravishankara, A.R., J.S. Daniel, and R.W. Portmann. 2009. Nitrous oxide (N_2O): The dominant ozone-depleting substance emitted in the 21st century. Science 326:123–125. doi:10.1126/science.1176985

Reardon, C.L., S.L. Strauss, and M. Mazzola. 2013. Changes in available nitrogen and nematode abundance in response to *Brassica* seed meal amendment of orchard soil. Soil Biol. Biochem. 57:22–29. doi:10.1016/j.soilbio.2012.10.011

Reddy, K.R., P.S.C. Rao, and R.E. Jessup. 1982. The effect of carbon mineralisation on denitrification kinetics in mineral and organic soils. Soil Sci. Soc. Am. J. 46:62–68. doi:10.2136/sssaj1982.03615995004600010011x

Rotthauwe, J.H., K.P. Witzel, and W. Liesack. 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63:4704–4712.

Ryan, M.H., J.A. Kirkegaard, and J.F. Angus. 2006. *Brassica* crops stimulate soil mineral N accumulation. Aust. J. Soil Res. 44:367–377. doi:10.1071/SR05143

Saggar, S., N. Jha, J. Deslippe, N.S. Bolan, J. Luo, D.L. Giltrap, et al. 2013. Denitrification and N_2O:N_2 production in temperate grasslands: Processes, measurements, modelling and mitigating negative impacts. Sci. Total Environ. 465:173–195. doi:10.1016/j.scitotenv.2012.11.050

Selbie, D.R., L.E. Buckthought, and M.A. Shepherd. 2015. The challenge of the urine patch for managing nitrogen in grazed pasture systems. Adv. Agron. 129:229–292. doi:10.1016/bs.agron.2014.09.004

Singleton, P.L. 1991. Soils of Ruakura: A Window on the Waikato. DSIR Land Resources, Lower Hutt, New Zealand.

Smith, P. 2013. Delivering food security without increasing pressure on land. Glob. Food Secur. 2:18–23. doi:10.1016/j.gfs.2012.11.008

Subbarao, G.V., K. Nakahara, M.P. Hurtado, H. Ono, D.E. Moreta, A.F. Salcedo, et al. 2009. Evidence for biological nitrification inhibition in *Brachiaria* pastures. Proc. Natl. Acad. Sci. USA 106:17302–17307. doi:10.1073/pnas.0903694106

Subbarao, G.V., K. Nakahara, T. Ishikawa, H. Ono, M. Yoshida, T. Yoshihashi, et al. 2013. Biological nitrification inhibition (BNI) activity in sorghum and its characterization. Plant Soil 366:243–259. doi:10.1007/s11104-012-1419-9

Subbarao, G.V., M. Rondon, O. Ito, T. Ishikawa, I.M. Rao, K. Nakahara, et al. 2007. Biological nitrification inhibition- is it a widespread phenomenon? Plant Soil 294:5–18. doi:10.1007/s11104-006-9159-3

Subbarao, G.V., T. Yoshihashi, M. Worthington, K. Nakahara, Y. Ando, K.L. Sahrawat, et al. 2015. Suppression of soil nitrification by plants. Plant Sci. 233:155–164. doi:10.1016/j.plantsci.2015.01.012

Tiedje, J.M., A.J. Sextone, T.B. Parkin, N.P. Revsbech, and D.R. Shelton. 1984. Anaerobic processes in soil. Plant Soil 76:197–212. doi:10.1007/BF02205580

Upsdell, M.P. 1994. Bayesian smoothers as an extension of nonlinear regression. N. Z. Stat. 29:66–81.

van Groenigen, J.W., D. Huygens, P. Boeckx, T.W. Kuyper, I.M. Lubbers, T. Rütting, and P.M. Groffman. 2015. The soil N cycle: New insights and key challenges. Soil 1:235–256. doi:10.5194/soil-1-235-2015

van Groenigen, J.W., G.L. Velthof, F.J.E. van der Bolt, A. Vos, and P.J. Kuikman. 2005. Seasonal variation in N_2O emissions from urine patches: Effects of urine concentration, soil compaction and dung. Plant Soil 273:15–27. doi:10.1007/s11104-004-6261-2

Velthof, G., P. Kuikman, and O. Oenema. 2002. Nitrous oxide emission from soils amended with crop residues. Nutr. Cycl. Agroecosyst. 62:249–261. doi:10.1023/A:1021259107244

VSN International. 2015. GenStat for Windows 18th ed. VSN Int., Hemel Hempstead, UK.

Wrage, N., J.W.v. Groenigen, O. Oenema, and E.M. Baggs. 2005. A novel dual-isotope labelling method for distinguishing between soil sources of N_2O. Rapid Commun. Mass Spectrom. 19:3298–3306. doi:10.1002/rcm.2191

Zakir, H.A.K.M., G.V. Subbarao, S.J. Pearse, S. Gopalakrishnan, O. Ito, T. Ishikawa, et al. 2008. Detection, isolation and characterization of a root-exuded compound, methyl 3-(4-hydroxyphenyl) propionate, responsible for biological nitrification inhibition by sorghum (*Sorghum bicolor*). New Phytol. 180:442–451. doi:10.1111/j.1469-8137.2008.02576.x

Chapter 5

Are nitrous oxide emissions from soil less when amended with urine from brassica fed cows than pasture fed cows?

This chapter has been submitted to an international peer-reviewed journal and is presented in this thesis in manuscript format:

Please refer to Appendix B for the contribution of each of the authors.

Title: Are nitrous oxide emissions from soil less when amended with urine from brassica fed cows than pasture fed cows?

Authors: S.F. Balvert*[a,b], J. Luo[a], L.A. Schipper[b]

[a]*AgResearch Limited, Ruakura Research Centre, Private Bag 3123, Hamilton 3240, New Zealand*

Email: shereebalvert@gmail.com*;* Jiafa.luo@agresearch.co.nz

[b]*University of Waikato, Faculty of Science and Engineering, Private Bag 3105, Hamilton 3240, New Zealand*

Email: louis.schipper@waikato.ac.nz

**Corresponding author.* shereebalvert@gmail.com

Abstract

New Zealand agriculture is comprised predominantly of year-round outdoor grazing of pasture and excreta from grazing cows and sheep has been identified as the single largest source of emissions of nitrous oxide (N_2O), a potent greenhouse gas (GHG). Nitrification inhibitors can significantly reduce N_2O emissions, and some secondary metabolites of brassica crops have been shown to act as biological nitrification inhibitors (BNIs). The aims of this study were to 1) determine if the BNI capacity of brassica plants (specifically kale) was transferred into the urine of animals grazing those crops and subsequently reduced N_2O emissions from the deposited urine patch, and 2) determine if soil growing brassica crops contained BNI compounds that decreased N_2O emissions following addition of urine. In a laboratory study, urine from animals fed a predominantly kale diet and urine from animals fed a predominantly ryegrass pasture diet, was applied to either soil collected from under pasture or kale crop. Soils were incubated for 60 days and the following parameters were measured: Soil mineral N, N_2O and carbon dioxide emissions, and ammonia oxidising bacteria populations. Kale urine did not decrease nitrification rate or N_2O emissions compared to pasture urine when applied to soil. N_2O emissions were higher from the kale cropped soil than the pasture soil, likely

due to the higher fertility status of this crop soil. Overall, these results provided no evidence that feeding grazing animals kale reduced N_2O production following urine inputs to soil.

Key words: Nitrogen, Urine, Nitrous oxide, BNI, Glucosinolate.

Introduction

In New Zealand, agriculture is predominantly comprised of grazed pastoral systems dominated by ryegrass/clover swards. Excreta deposited by grazing animals has been identified as the main contributor to emissions of nitrous oxide (N_2O). N_2O is a particularly potent greenhouse gas (GHG) with a global warming potential 265-298 times that of carbon dioxide (Myhre *et al.*, 2013). Additionally, N_2O contributes to the degradation of stratospheric ozone (Ravishankara *et al.*, 2009). The main source of N_2O production in New Zealand is from urine patches, where due to the inefficient use of dietary nitrogen (N) by grazing animals, very high loads of N are delivered to the soil (Selbie *et al.*, 2015). This load of N exceeds plant requirements and leads to losses of gaseous N such as N_2O as a result of microbial processes related to nitrification and denitrification. These high N loads also lead to losses of nitrate (NO_3^-) by leaching which contributes to indirect N_2O emissions (Butterbach-Bahl *et al.*, 2013; Fowler *et al.*, 2013).

Forage crops such as brassicas, are widely used in NZ pastoral systems in order to meet feed deficits due to their tolerance to drought, fast growth rates, and high nutritional value. Due to their high dry matter yields, forage crops are generally break fed resulting in very high stocking densities (1000-1400 cows ha^{-1}) (Monaghan *et al.*, 2013). Additionally, the grazed soil will remain fallow with no plant uptake of N until a new pasture is established. Some research has shown that the grazing of forage crops in this way may result in N_2O losses of more than twice those from grazing of traditional pastures (Smith *et al.*, 2008).

However, multiple studies have shown an increase in soil mineral N concentrations following brassica crops compared to that following other non-brassica crops (Kirkegaard *et al.*, 1999; O'Sullivan *et al.*, 2016; Reardon *et al.*, 2013; Ryan *et al.*,

2006), indicating brassica crops may influence soil N cycling. This alteration of the N cycle has been attributed to plant secondary metabolites called glucosinolates (GLSs) (Blažević *et al.*, 2017), found in brassica crops and whose hydrolysis products have been identified as having the potential to alter soil N cycling (Brown & Morra, 2009; Kirkegaard *et al.*, 1999; O'Sullivan *et al.*, 2016). In particular, GLS hydrolysis products have been identified as inhibiting soil nitrifying bacteria (Balvert *et al.*, 2017; Bending & Lincoln, 2000), indicating nitrification (the conversion of soil NH_4^+ to NO_3^-), specifically, may be inhibited. Nitrification has been estimated to indirectly contribute up to 80% of soil N_2O emissions (Hu *et al.*, 2015), largely due to the provision of NO_3^- as a substrate for denitrification (Butterbach-Bahl *et al.*, 2013; Firestone & Davidson, 1989; Wrage *et al.*, 2005), and inhibiting nitrification has been shown to be effective at reducing N_2O emissions from urine patches (e.g. Di & Cameron, 2011; Luo *et al.*, 2013b). This suggests that brassica crops, when selected instead of other forage crops, may provide a useful N_2O mitigation option for some farm systems.

Glucosinolate hydrolysis occurs following disruption of plant tissues when GLSs come into contact with the enzyme myrosinase (Collett *et al.*, 2014), which is likely to occur during grazing when crops are pulled and trampled. Glucosinolate hydrolysis products are thus likely released to soil during grazing. Glucosinolate hydrolysis may also occur during chewing and digestion of grazing animals. Some studies have demonstrated that some plant secondary metabolites, including GLS hydrolysis products, are excreted in the urine of animals following grazing (Duncan & Milne, 2007; Estell, 2010). If the efficacy of the GLS hydrolysis products as nitrification inhibitors remains when excreted, this would provide the additional benefit of the inhibitors being delivered directly to the urine patch with the urine N where they are required. This approach has been verified with the nitrification inhibitor dicyandiamide (DCD), where DCD fed to cows was excreted in urine and consequently reduced N2O emissions when the urine was applied to soil (Luo *et al.*, 2015a).

Luo *et al.* (2015b) observed reduced N_2O emission factors (EF; the percent of applied N emitted as N_2O-N) from the urine of sheep fed forage rape compared to those fed on a pasture diet, and they suggested this difference may be due to GLS hydrolysis products ingested by the sheep being delivered to the urine patch. van der Weerden *et al.* (2017) also reported a discrepancy between measured soil

mineral N concentrations and N_2O emissions from a plot study, where real kale fed urine was applied to N_2O chambers and artificial urine was applied to the soil plots. They also suggested this difference may be due to GLS hydrolysis products and their absence in the artificial urine that was applied to the soil plots. Hoogendoorn *et al.* (2016), however, reported higher EFs from urine of forage rape fed animals compared to pasture fed animals. This indicates that there is still uncertainty as to whether a reduction in N_2O production occurs from brassica crops or the urine derived during grazing for different soils.

The objective of this laboratory study was to determine whether there were lower N_2O emissions from urine derived from a predominantly kale diet than urine from animals fed on a predominantly pasture diet. A secondary objective was to assess whether there was a compounding effect of applying brassica fed urine to soil collected from beneath growing brassica crops. We hypothesised that urine from kale-fed animals and soil collected from beneath a kale crop would reduce nitrification and N_2O production relative to ryegrass fed urine and soil from beneath pasture.

Materials and methods

Experimental set-up

A well-drained Ngakuru loam soil (Typic Orthic Allophanic soil; Hewitt, 1998) of 0-7.5 cm depth, from under both a mixed perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) pasture, and a kale (*Brassica oleracea* L.) crop, was collected for an incubation study. The pasture and kale crop paddocks were adjacent and both had been under permanent ryegrass/clover pasture for about 5 years. The kale crop had been sown into one paddock approximately 5 months prior to soil collection. General soil properties for each soil are presented in Table 1. The collected soil was sieved (4 mm) and homogenised (pasture and crop soils separately) and pre-incubated at 20 ºC for 5 days in order to allow the effects of soil disturbance to dissipate. Although there were slight differences in soil fertility status of the two soils, no nutrient additions were made to the pasture soil in order to avoid any priming of soil microbial activity.

Table 1: General soil properties (0-75 mm) of the pasture and crop soils used.

Soil type	Total Nitrogen %	Total Carbon %	Organic Matter %	C:N ratio	Olsen Phosphorus µg ml^{-1}	pH	Bulk Density g cm^{-3}	Water Holding Capacity g g^{-1} soil
Pasture soil	0.9	9.6	16.5	10.7	58	6.3	0.82	1.43
Crop Soil	1.04	10.8	18.6	10.4	82	5.9	0.63	1.75

Urine from lactating dairy cows fed on a predominantly pasture diet (ryegrass/white clover mix; 14 kg DM day^{-1} plus maize silage 4 kg DM day^{-1}), and urine from non-lactating dairy cows fed on a predominantly kale diet (14 kg DM day^{-1} plus pasture bailage 3 kg DM day^{-1}) were collected. Both groups had been receiving this diet for at least 10 days prior to urine collection. Urine was stored chilled for 48 hours prior to use while awaiting analysis of N content.

Moist soil (140 g oven dry equivalent) was weighed into preserving jars (1 litre) and amended with either distilled water (Nil N control) or one of the urine types. Pasture urine was applied at a rate of 600 µg N g^{-1} soil and kale urine was applied at 400 µg N g^{-1} soil in line with average urine patch N loading rates under the different diets (Hoogendoorn *et al.*, 2016; Selbie *et al.*, 2015; van der Weerden *et al.*, 2017). A treatment where pasture urine was diluted with distilled water to match the N content of the kale urine was also included. The soil moisture contents of the jars were adjusted to 65% of water holding capacity. This resulted in slightly different moisture (0.88 and 1.1 g H_2O g^{-1} soil for the pasture and crop soils respectively) contents between the two soil types. The jars were placed in a randomised block format and incubated at 20 ºC. Jars were covered in perforated parafilm™ while being incubated to minimise evaporation loss and to allow for normal gas exchange. Soil moisture content was monitored by weight and maintained throughout the 60 day incubation period.

An additional duplicate set of soils and treatments was established to allow for concurrent measurement of soil mineral N transformations. Moist soil (300 g oven dry equivalent) was weighed into plastic bags and matching treatments applied. The bags were stored as per the gas jars allowing for normal gas exchange.

Gas flux measurement

Nitrous oxide production was measured 13 times over a 60 day period of incubation (1, 2, 3, 6, 8, 11, 14, 18, 22, 28, 35, 46 and 60 days following urine addition). By this time N_2O production from urine treatments had returned to the same level as the control. Measurements were more frequent early in the incubation to capture the initial flush following urine addition. Headspace N_2O concentrations were measured using a Trace Gas Analyser (TGA; Innova 1412i-2, Lumasense Technologies, Denmark). Jars were sealed with gas tight lids containing ports for attachment to the TGA and production was measured following 1 hour of accumulation. Prior to entering the TGA, samples were drawn through a soda lime filter to minimise interference by CO_2 and the TGA corrected for interference from water vapour and any remaining CO_2. Samples were drawn at T_0 and T_{60} minutes as preliminary work demonstrated that N_2O accumulation was linear over this period at this soil to headspace ratio. Hourly N_2O fluxes were calculated for each jar from the increase in headspace concentration. The hourly fluxes were integrated to estimate total emission and urea derived emission and % of applied N emitted as N_2O-N over the study period. % of applied N emitted as N_2O-N was calculated as follows:

$$\%\ of\ applied\ N\ emitted\ as\ N_2O = \frac{N_2O - N\ total\ (Urine) - N_2O - N\ total\ (Control)}{Urine\ N\ applied} \times 100$$

Carbon dioxide (CO_2) fluxes were measured following 16 hour headspace accumulation. Jars were sealed with gas tight lids containing a septum and CO_2 samples (12 ml) were collected and stored in over-pressurised glass vials (6 ml). CO_2 was analysed by infrared gas analysis (IRGA, LI-COR® LI7000). As with N_2O, fluxes were calculated from the increase in headspace CO_2 concentration over the 16 hours.

N transformations

Soil samples were analysed 7 times throughout the study period (1, 3, 7, 14, 22, 28, and 39 days following urine addition) for soil mineral N, pH and moisture. Soil pH

was measured in water using a 1:2.5 ratio, and soil moisture was determined by oven drying moist soil at 105ºC for 24 hours. For soil ammonium-N (NH_4^+-N) and nitrite (NO_2^--N) + nitrate-N (NO_3^--N), soil (3 g oven dry equivalent) was extracted with 2M potassium chloride solution (KCl; 30 ml). Concentrations of NH_4^+-N and NO_2^- + NO_3^--N in the KCl extracts were analysed colourmetrically using a scalar SAN^{++} segmented flow analyser (Skalar Analytical B. V., The Netherlands) (Blakemore *et al.*, 1987).

Microbial community quantification

On day 14, the soil from the bags of all treatments (4 reps only) were also subsampled for comparison of *amoA* gene abundance to determine if ammonia oxidising bacteria (AOB) numbers were reduced in kale urine treatments relative to the pasture urine treatments. Only AOB were enumerated as Di *et al.* (2009) demonstrated that AOB as opposed to ammonia oxidising archaea (AOA) are largely responsible for ammonia oxidation in pastoral soils and under high N loads. Total soil genomic DNA was extracted using a Qiagen DNeasy® Powerlyzer® Powersoil® (Germany) extraction kit according to the manufacturer's instructions. Soil samples were stored at -80 ºC between collection and DNA extraction. The PCR primer pair amoA1F/amoA2R was used to amplify regions of the bacterial *amoA* gene (Rotthauwe *et al.*, 1997). 16 µl reactions contained 1.5 µl DNA (diluted 1:10 to reduce potential PCR inhibition), 0.4 µl of each primer (10 µM concentration), 8 µl SYBR$^{®Premix}$ Ex Taq^{TM} (Takara, Japan), and 5.7 µl milli-Q water. Real time qPCR analysis was performed on a Rotor-Gene^{TM} 6000 (Corbett Life Science, Australia). Raw data analysis was carried out using Rotor-GeneTM 6000 series software version 2.1. Melting curve analysis was performed following each run to confirm PCR product specificity. Standard curves for qPCR analysis was developed by cloning *amoA* gene amplicons into pGEM® –T Easy vectors (Promega, USA) and transforming TOP10 E. coli (Thermo Fisher, USA). Selected clones were incubated overnight in Luria Broth liquid medium and the plasmid DNA was subsequently extracted using a PureLink^{TM} Quick Plasmid MiniPrep Kit (Thermo Fisher, USA). Plasmid DNA concentration was measured on a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA) and a standard curve was subsequently generated by dilution series.

Short term enzyme assays

In order to determine whether the different soils exhibited differences in their potential for nitrification and denitrification prior to treatment application, a short term nitrification enzyme assay and a denitrification enzyme assay were carried out.

The short term nitrification enzyme assay followed methodology from Schmidt and Belser (1994). Moist soil (20 g) was weighed into 250 ml conical flasks. 0.5 mM phosphate buffer solution (90 ml containing: 3.48 g 100 ml^{-1} 0.2M K_2HPO_4 and 2.72 g 100 ml^{-1} 0.2 M KH_2PO_4 and 0.25M $(NH_4)_2SO_4$ solution (0.2 ml) were added. Flasks were placed on an orbital shaker and 1M $KClO_3$ solution (1 ml) was added. After 5 minutes, a 5 ml aliquot was removed from the flask, placed into a test tube containing 1% merthiolate solution (0.05 ml) to stop the reaction, and was subsequently filtered through Whatman 42 filters. Four subsequent 5 ml aliquots were removed at 60, 120, 180 and 340 minutes and the process repeated. This was repeated 4 times for each soil. Nitrite (NO_2^--N) concentrations of the filtrates were analysed colourmetrically using a scalar SAN^{++} segmented flow analyser (Skalar Analytical B. V., The Netherlands).

The denitrification enzyme assay involved the anaerobic incubation of soil samples in the presence of acetylene (C_2H_2) to prevent the conversion of N_2O to N_2 during the denitrification process. The methodology was adapted from those of Smith and Tiedje (1979) and Tiedje (1994). Moist soil (85 g oven dry equivalent) was weighed into 1 litre preserving jars. KNO_3 solution (500 μg N g^{-1} soil) and glucose solution (105.98 μg C g^{-1} soil) were added to create a slurry. The jars were sealed and made anoxic by alternately flushing with nitrogen (N_2) gas and evacuating 4 times. Purified C_2H_2 was added to achieve 10% concentration in the jar headspace. The jars were placed on an orbital shaker and headspace samples (10 ml) were removed at time points T_0, T_{30}, T_{60} and T_{90} minutes and stored in pre-evacuated glass vials (6 ml) with a rubber septum. N_2O concentrations were analysed using a SRI 8610 automated gas chromatograph equipped with a ^{63}Ni-electron capture detector (310ºC), a HayesepD column (40◦C) and N_2 as carrier gas. This was repeated 4 times for each soil.

Potential rates of nitrification and denitrification were calculated by linear regression of accumulated NO_2^- and N_2O over time, respectively. Both NO_2^- and N_2O production were linear with time (r^2 >0.9 in all cases).

Statistical analysis

Daily N_2O flux data for different treatments was analysed using the mixed model smoothing programme FLEXI (Upsdell, 1994), which allows for comparison of multiple curves. One-way ANOVA was carried out on the total emissions, % of applied N emitted as N_2O-N, respiration, and microbial community data to determine the significance of the difference between treatments.

Results

Nitrous oxide flux and total emissions

All treatments that received urine showed a high N_2O flux immediately following treatment addition, and a second smaller peak occurred 8 days following treatment application. The peaks from urine applied to the crop soil were all higher than from the corresponding treatments of the pasture soil ($p < 0.05$). The Nil N treatments showed a small peak following treatment (water) addition in the crop soil only (Figure 1).

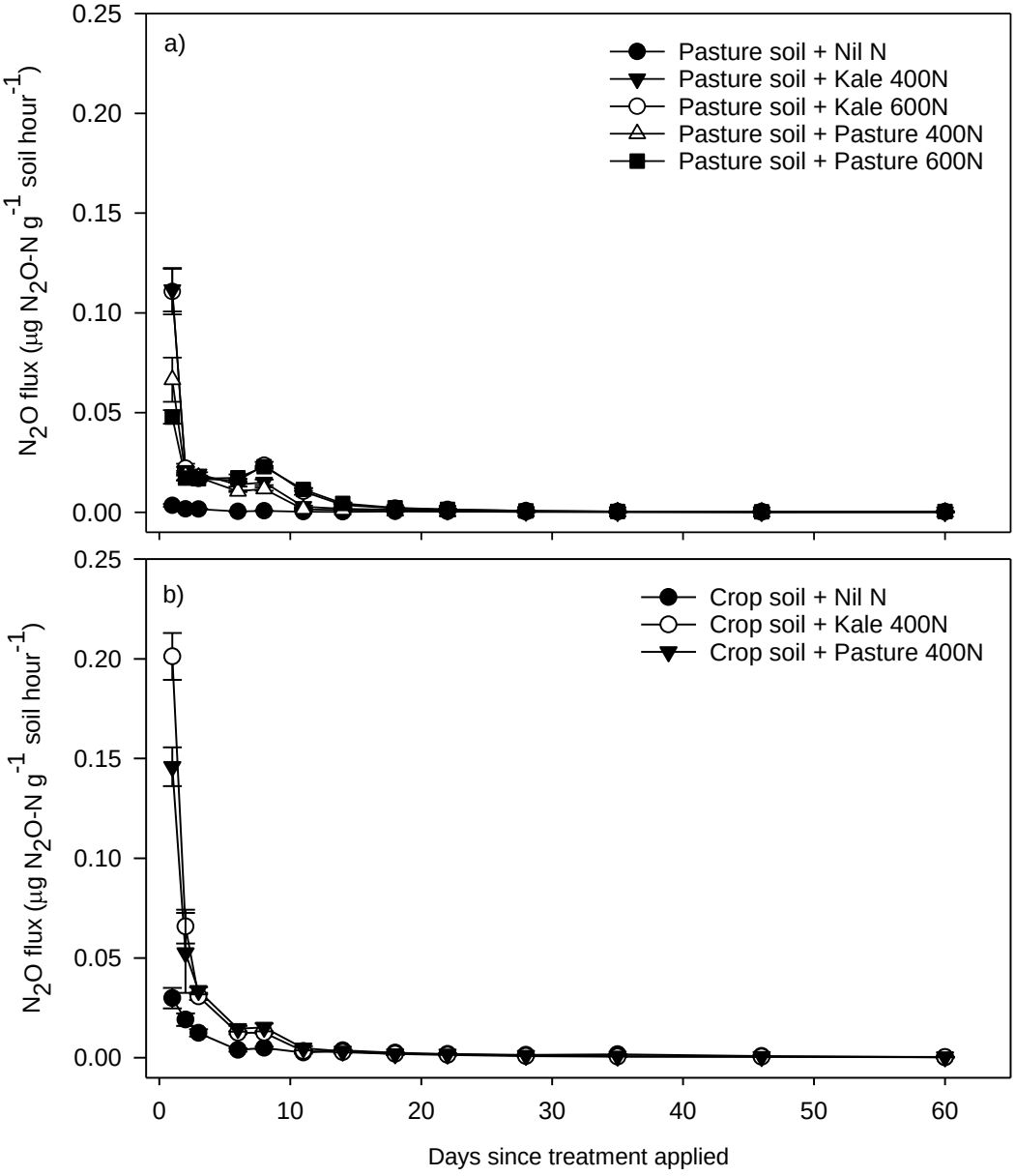

Figure 1: Daily nitrous oxide fluxes from incubated soils following addition of urine from kale and pasture fed cows: a) pasture soil; b) kale crop soil. Error bars represent the standard errors of the mean (n = 5).

Total N_2O emissions from the urine applied to kale cropped soils (range 3.65 – 9.8 µg N_2O-N g soil^{-1}) were significantly higher ($p < 0.05$) than those applied to pasture soils (range 0.56 – 7.59 µg N_2O-N g soil^{-1}) (Table 2). There was no difference between emissions from the different urine types when applied to crop soil. Total N_2O emissions were significantly higher ($p < 0.05$) from the higher N rate in both urine types. Application of kale urine resulted in significantly higher total emissions than pasture urine at the same N concentration when applied to pasture soil

(Table 2). There was a general trend for % of applied N emitted as N_2O-N to be higher following application of kale urine relative to application of pasture urine. However, this difference was only statistically significant ($P < 0.05$) when the urines were applied to the pasture soil and at the rate of 400 μg N g^{-1} soil.

Table 2: Total N_2O emissions and percent of applied N emitted as N_2O-N from incubated (60 days) pasture and kale crop soils following application of kale and pasture derived urine. Least significant differences (LSDs) ($p < 0.05$) are presented. Different letters in the same column denote a significant difference between treatments.

Treatment	***Total N_2O emissions (μg N_2O-N g^{-1} soil)***	***% of applied N emitted as N_2O***	***Change relative to pasture urine****
Pasture soil + Nil N	0.56^{a}		
Pasture soil + Pasture 400N	4.20^{b}	0.91^{a}	
Pasture soil + Pasture 600N	6.25^{c}	0.95^{ab}	
Pasture soil + Kale 400N	6.12^{c}	1.39^{cd}	+53%
Pasture soil + Kale 600N	7.59^{d}	1.17^{bc}	+23%
Crop soil + Nil N	3.65^{b}		
Crop soil + Pasture 400N	8.83^{e}	1.29^{cd}	
Crop soil + Kale 400N	9.8^{e}	1.54^{d}	+19%
LSD ($p < 0.05$)	1.10	0.258	

*Calculated as the percentage difference between kale urine % of applied N emitted as N_2O-N and pasture urine % of applied Urea-N emitted as N_2O;

$$\left(1 - \frac{kale\ urine\ 600N}{pasture\ urine\ 600N}\right) \times 100$$

Soil respiration

Soil respiration increased sharply immediately following addition of urine to both soil types. The Nil N treatments for both pasture and cropped soils had a smaller peak in respiration 3 days after treatments were applied. In the pasture soil, soil respiration in the urine treatments reduced to the level of the Nil N treatment by 7 days following treatment addition and remained at that level for the remainder of

the study. In the kale cropped soil, however, soil respiration in the urine treatments reduced to a level significantly lower than that of the Nil N treatment ($p < 0.001$) and remained lower for the remainder of the study. Following the initial peak, soil respiration was significantly higher ($p < 0.001$) in the cropped soil than in the pasture soil (Figure 2).

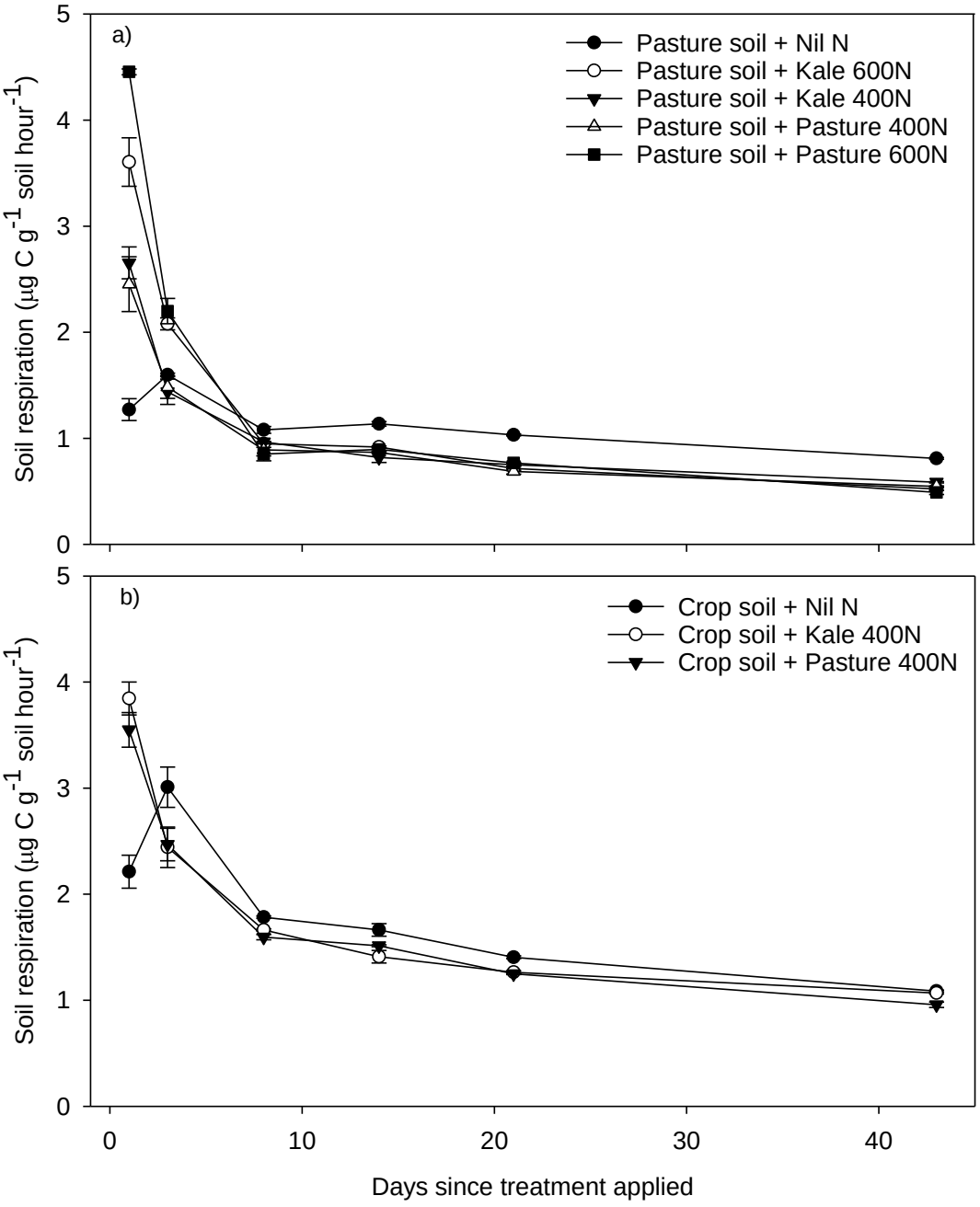

Figure 2: Soil respiration rates (CO_2-C flux) from incubated soils following application of kale and pasture fed urine: a) pasture soil, and b) kale crop soil. Error bars represent the standard error of the mean (n = 5).

Mineral N transformations

Soil NH_4^+- N concentrations peaked immediately following urine addition in both the pasture and crop soils. The 600N peaks were significantly higher (p <0.001) than the 400N peaks, which were significantly higher than those from the Nil N rate for both urine types. There was no difference in peak heights between the pasture and crop soils or between urine types. Soil NH_4^+- N concentrations had reduced to background levels by 14 days following addition of urine. There was no change in soil NH_4^+- N concentrations in the Nil N treatments (Figure 3). Soil NO_3^--N concentrations began to increase within 3 days following addition of urine in both soils types. In the pasture soil, NO_3^--N concentrations peaked at approximately 30 days following treatment addition. In the cropped soil, however, NO_3^--N concentrations were still increasing at the end of the study period and had reached around 600 µg N g^{-1} soil although only 400 µg N g^{-1} soil equivalent was applied in urine. The 600N peaks were significantly higher (p <0.001) than those from the 400N rate for both urine types, however, there was no difference between the urine types. In the Nil N treatments, soil NO_3^--N concentrations continually increased over the study period under both soil types, reaching 139 and 293 µg N g^{-1} soil for the pasture and crop soils, respectively (Figure 3).

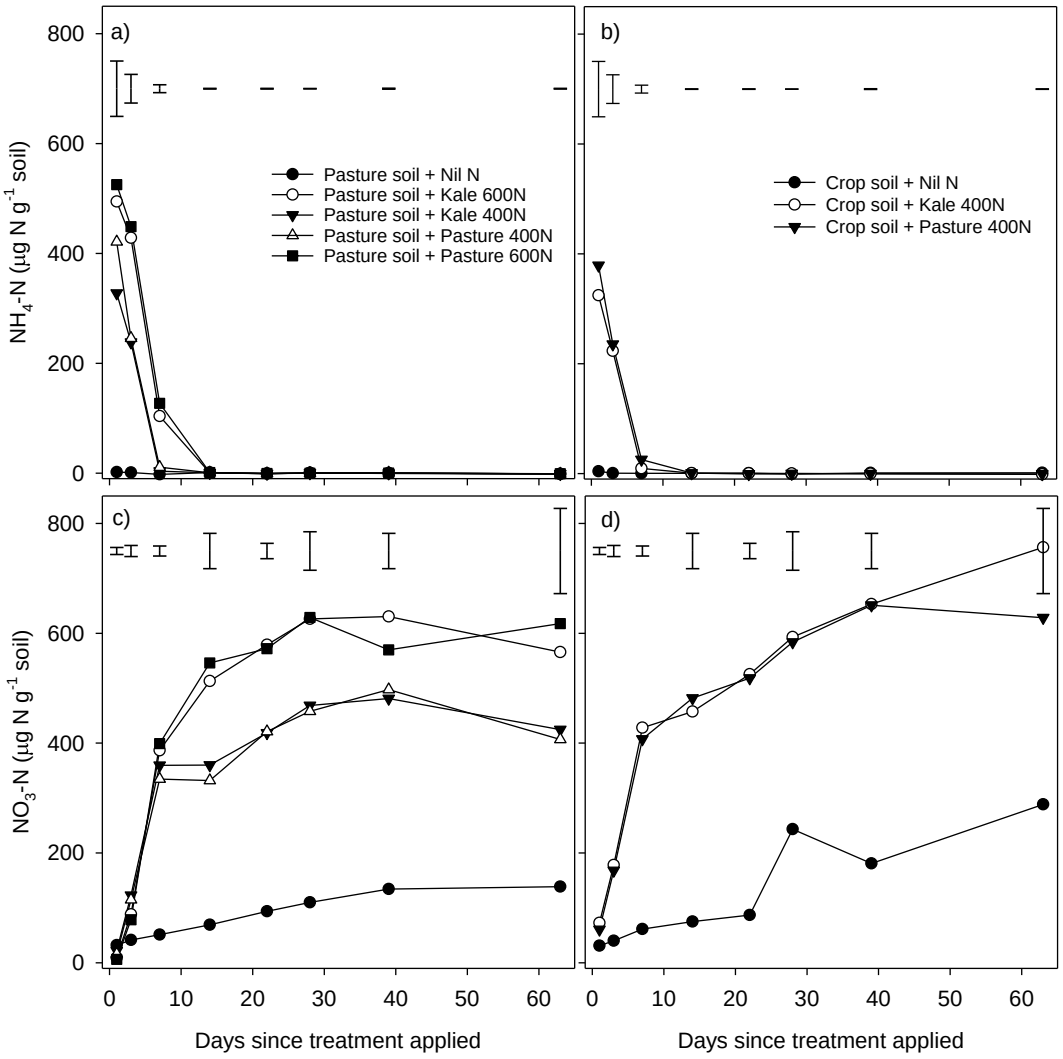

Figure 3: Soil mineral N concentrations in incubated soils following application of kale and pasture derived cow urine: a) pasture soil treatments, soil ammonium N concentrations; b) kale crop soil treatments, soil ammonium-N concentrations; c) pasture soil treatments, soil nitrate-N concentrations; and d) kale crop soil treatments, soil ammonium-N concentrations. Error bars represent the least significant differences (n = 5).

Microbial community

AmoA gene copy numbers increased following the addition of both urine types and in both the pasture and crop soils (Figure 4). There was a trend for higher copy numbers in the higher N treatments, however, this was not statistically significant. There was no difference in copy numbers between urine or soil types.

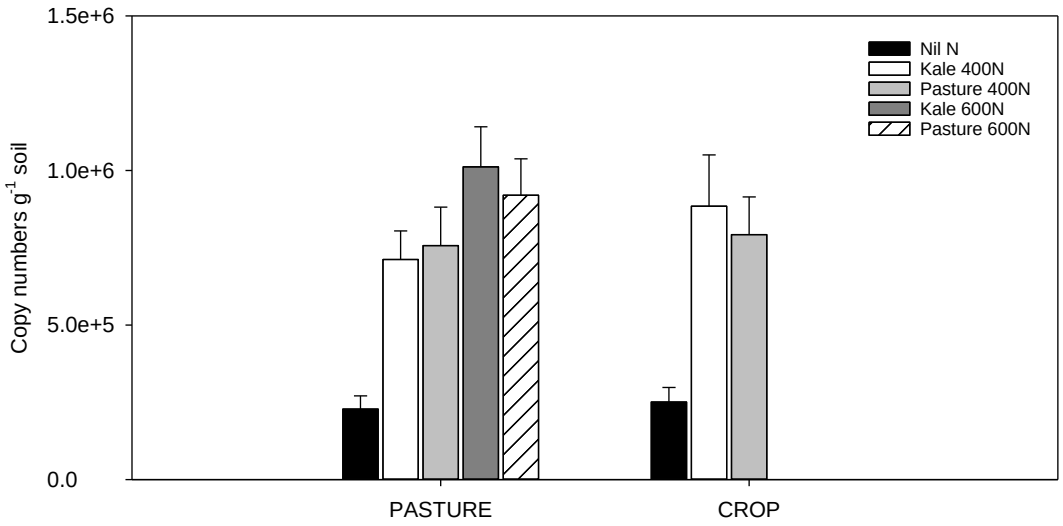

Figure 4: Ammonia oxidising bacteria *amoA* gene copy numbers as affected by different urine types (pasture and kale fed at 2 rates) and soil type (pasture and crop) on day 14 of the incubation. Error bars represent the standard error of the mean (n = 4).

Enzyme Assays

There was no difference in the nitrification enzyme activity between the pasture and kale crop soils prior to treatment application, with nitrification rates of 7.78 and 8.69 ng NO_2^- g^{-1} soil $hour^{-1}$, respectively (Table 3). There was also no difference in denitrification enzyme activity between the soils prior to treatment application. Denitrification enzyme activities were 1.11 and 1.76 µg N_2O g^{-1} soil $hour^{-1}$ for the pasture and kale crop soils, respectively (Table 3).

Table 3: Average nitrification and denitrification enzyme activity (± SEM) of the pasture and crop soils prior to treatment application. P values for the differences between soils for the two assays are presented (n = 4).

	Nitrification enzyme activity	***Denitrification enzyme activity***
Soil type	*(ng NO_2^- g^{-1} soil $hour^{-1}$)*	*(µg N_2O g^{-1} soil $hour^{-1}$)*
Pasture Soil	7.78 ±2.17	1.11 ±0.13
Crop Soil	8.69 ±7.33	1.76 ±0.17
p value	0.915	0.083

Discussion

Effect of urine type

No difference was observed in peak N_2O fluxes between the two urine types and total N_2O emissions were higher from the kale derived urine than the pasture urine. This was in contrast to our hypothesis that N_2O production would be lower from kale derived urine because of BNI in brassicas. Percent of applied N emitted as N_2O-N was also higher from the kale derived urine than the pasture urine. This is similar to that observed by Hoogendoorn *et al.* (2016) who measured higher emission factors (EF_3) from brassica (forage rape) urine than from pasture urine. Percent of applied N emitted as N_2O-N is used in the current study as a proxy for an EF_3. Although % of applied N emitted as N_2O-N and EF_3 are calculated the same way, an EF_3 is generally based on data collected from the field, and over a longer period of time than was measured in this incubation study. EF_3s are reported to be unresponsive to urine-N concentration (de Klein *et al.*, 2014), which was supported by our data as there was no difference in the % of applied N emitted as N_2O-N between the different N rates of each urine type.

There was also no evidence to suggest that the kale derived urine reduced nitrification relative to pasture derived urine as was hypothesised. There was no difference observed between the urine types in the soil mineral N transformations or the microbial community data. If nitrification (the conversion of NH_4^+-N to NO_3^--N) was inhibited, we would have expected to see a reduction in soil NO_3^--N in the brassica urine treatments, as well as fewer copies of the *amoA* gene relative to the pasture urine treatments. Balvert *et al.* (2017) found different GLS hydrolysis products had different effects on nitrification; some inhibited nitrification while others did not. It is possible that kale does not contain sufficient concentration of the parent GLS types that inhibit nitrification and so the hydrolysis products are not effective in the urine. For example, forage rape contains higher concentrations (following autolysis) than kale of phenylethyl isothiocyanate (PEITC) (Cole, 1976), a hydrolysis product that Balvert *et al.* (2017) found to effectively inhibit nitrification. This, however, does not explain the difference in results between the Hoogendoorn *et al.* (2016) and the Luo *et al.* (2015b) trials as they both used urine from animals fed on forage rape.

Another possibility is that the amount of kale fed to the cows (approx. 82% of diet) was not a high enough percentage of the diet to achieve an effective concentration of GLS hydrolysis products in the urine. Urinary concentration of GLS hydrolysis products was not measured in this study, however, if the lack of concentration of GLS hydrolysis products was the reason there was no observed reduction in nitrification or N_2O production, using brassica crops to reduce N_2O emissions would not be feasible in farm systems.

The sharp and brief increase in soil respiration immediately following urine application was expected and was likely due to the release of N and C following the hydrolysis of urea (Ambus *et al.*, 2007), as well as the increase in moisture content that stimulated microbial activity (Borken *et al.*, 2003). This also likely contributed to the corresponding initial peak in N_2O emissions as the N and C substrates would initially drive N_2O production *via* nitrification, and the increase in moisture content would subsequently drive production *via* denitrification in anaerobic microsites.

Effect of soil type

Peak and total N_2O emissions, from all urine treatments, were both higher from the kale crop soil than from the pasture soil under all urine treatments. This difference was potentially due to the lower pH and higher soil fertility status of the crop soil. N_2O production is known to be influenced by pH with emissions decreasing with increasing pH (Saggar *et al.*, 2013; Samad *et al.*, 2016). The greater concentrations of total N and C under the crop soil would lead to more available nutrients and thus a greater abundance and activity of soil microbes. These factors combined would be expected to increase N_2O emissions. Soil Olsen P was also higher under the crop soil. Soil P concentration is not in itself expected to directly alter N_2O emissions, however, Olsen P concentrations may be used as an indicator of soil fertility status and Olsen P has been shown to be positively correlated with N_2O emissions (Luo *et al.*, 2013a). The higher soil respiration from the kale crop soil also indicated higher microbial activity in this soil.

However, despite higher N_2O emissions from cropped soils, there were no significant differences in the nitrification and denitrification activity measurements. The rate of nitrification in soil has been shown to differ significantly depending on

the plant species the soil is supporting (Lata *et al.*, 2004; Osanai *et al.*, 2012; Subbarao *et al.*, 2015). Specifically, Rumberger and Marschner (2003) showed that some brassicas (in this case canola) released PEITC into the rhizosphere as root growth was likely accompanied by cell destruction. Given that PEITC has been demonstrated to be a strong inhibitor of nitrification, no observed difference between nitrification activities in this study was somewhat surprising. It is possible that the higher soil fertility of the crop soil, described above, resulted in higher nitrification rates which masked any inhibitory effect.

Conclusion

Overall, this study found no evidence for a reduction in nitrification or N_2O emissions following the addition of kale urine to soil compared to that following the addition of pasture urine. Contrary to our hypothesis, addition of kale urine to soil tended to produce higher total N_2O emissions than the addition of pasture urine. Mineral N dynamics, microbial enzyme activity and community data indicated this was not a result of a difference in nitrification or denitrification rate. Therefore, the BNI capacity of any GLS hydrolysis products contained within the kale urine was not sufficient to produce a reduction in N_2O emissions. N_2O emissions tended to be higher from the cropped soil compared to the ryegrass/clover pasture soil which was likely a result of the higher soil fertility of the crop soil. Further research is required to assess whether soils on which brassica crops are grown contain sufficient GLS hydrolysis products that might reduce nitrification rates. Additionally, further exploration of whether these products are found in urine derived from a wider range of brassicas also deserves attention.

Acknowledgments

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References

Ambus, P., Petersen, S. O., & Soussana, J. F. (2007). Short-term carbon and nitrogen cycling in urine patches assessed by combined carbon-13 and nitrogen-15 labelling. *Agriculture, Ecosystems & Environment, 121*, 84-92.

Balvert, S. F., Luo, J., & Schipper, L. A. (2017). Do glucosinolate hydrolysis products reduce nitrous oxide emissions from urine affected soil? *Science of the Total Environment, 603*(Supplement C), 370-380.

Bending, G. D., & Lincoln, S. D. (2000). Inhibition of soil nitrifying bacteria communities and their activities by glucosinolate hydrolysis products. *Soil Biology and Biochemistry, 32*, 1261-1269.

Blakemore, L. C., Searle, P. L., & Daley, B. K. (1987). *Methods for chemical analysis of soils.* New Zealand Soil Bureau Scientific Report No. 80, New Zealand Soil Bureau, Wellington, New Zealand.

Blažević, I., Montaut, S., Burčul, F., & Rollin, P. (2017). Glucosinolates: Novel sources and biological potential. In J. Merillon & K. G. Ramawat (Eds.), *Glucosinolates* (pp. 15-73). Switzerland: Springer International Publishing.

Borken, W., Davidson, E. A., Savage, K., Gaudinski, J., & Trumbore, S. E. (2003). Drying and Wetting Effects on Carbon Dioxide Release from Organic Horizons. *Soil Science Society of America Journal, 67*, 1888-1896.

Brown, P. D., & Morra, M. J. (2009). Brassicaceae tissues as inhibbitors of nitrification in soil. *Journal of Agricultural and Food Chemistry, 57*, 7706-7711.

Butterbach-Bahl, K., Baggs, E. M., Dannenmann, M., Kiese, R., & Zechmeister-Boltenstern, S. (2013). Nitrous oxide emissions from soils: how well do we understand the processes and their controls? *Philosophical Transactions of the Royal Society B: Biological Sciences, 368*(1621), 20130122.

Cole, R. A. (1976). Isothiocyanates, nitriles and thiocyanates as products of autolysis of glucosinolates in Cruciferae. *Phytochemistry, 15*, 759-762.

Collett, M. G., Stegelmeier, B. L., & Tapper, B. A. (2014). Could nitrile derivitaves of turnip (*Brassica rapa*) glucosinolates be hepato- or cholangiotoxic in cattle? *Journal of Agricultural and Food Chemistry, 62*, 7370-7375.

de Klein, C. A. M., Luo, J., Woodward, K. B., Styles, T., Wise, B., Lindsey, S., & Cox, N. (2014). The effect of nitrogen concentration in synthetic cattle urine on nitrous oxide emissions. *Agriculture, Ecosystems & Environment, 188*, 85-92.

Di, H. J., & Cameron, K. C. (2011). How does the application of different nitrification inhibitors affect nitrous oxide emissions and nitrate leaching from cow urine in grazed pastures? *Soil Use and Management, 28*, 54-61.

Di, H. J., Cameron, K. C., Shen, J. P., Winefield, C. S., O/'Callaghan, M., Bowatte, S., & He, J. Z. (2009). Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature Geosci, 2*, 621-624.

Duncan, A. J., & Milne, J. A. (2007). Effects of oral administration of brassica secondary metabolites, allyl cyanide, allyl isothiocyanate and dimethyl disulphide, on the voluntary food intake and metabolism of sheep. *British Journal of Nutrition, 70*, 631-645.

Estell, R. E. (2010). Coping with shrub secondary metabolites by ruminants. *Small Ruminant Research, 94*, 1-9.

Firestone, M. K., & Davidson, E. A. (1989). Microbial basis of NO and N_2O production and consumption. In M. O. Andreae & D. S. Schimel (Eds.), *Exchange of trace gases between terrestrial ecosystems and the atmosphere* (pp. 7-21). New York, United States of America: John Wiley and Sons Ltd.

Fowler, D., Coyle, M., Skiba, U., Sutton, M. A., Cape, J. N., Reis, S., Sheppard, L. J., Jenkins, A., Grizzetti, B., Galloway, J. N., Vitousek, P., Leach, A., Bouwman, A. F., Butterbach-Bahl, K., Dentener, F., Stevenson, D., Amann, M., & Voss, M. (2013). The global nitrogen cycle in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences, 368*, 20130164.

Hewitt, A. E. (1998). *New Zealand Soil Classification - Landcare Research Science Series No. 1.* Lincoln, Canterbury, New Zealand: Manaaki Whenua Press.

Hoogendoorn, C. J., Luo, J., Lloyd-West, C. M., Devantier, B. P., Lindsey, S. B., Sun, S., Pacheco, D., Li, Y., Theobald, P. W., & Judge, A. (2016). Nitrous oxide emission factors for urine from sheep and cattle fed forage rape (Brassica napus L.) or perennial ryegrass/white clover pasture (Lolium perenne L./Trifolium repens). *Agriculture, Ecosystems & Environment, 227*, 11-23.

Hu, H.-W., Chen, D., & He, J.-Z. (2015). Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. *FEMS Microbiology Reviews, 39*, 729-749.

Kirkegaard, J. A., Howe, G. N., & Mele, P. M. (1999). Enhanced accumulation of mineral-N following Canola. *Australian Journal of Experimental Agriculture, 39*, 587-593.

Lata, J. C., Degrange, V., Raynaud, X., Maron, P. A., Lensi, R., & Abbadie, L. (2004). Grass populations control nitrification in savanna soils. *Functional Ecology, 18*, 605-611.

Luo, J., Hoogendoorn, C. J., Van der Weerden, T. J., Saggar, S., De Klein, C., Giltrap, D., Rollo, M., & Rys, G. (2013a). Nitrous oxide emissions from grazed hill land in New Zealand. *Agriculture, Ecosystems & Environment, 181*, 58-68.

Luo, J., Ledgard, S., Wise, B., Welten, B., Lindsey, S., Judge, A., & Sprosen, M. (2015a). Effect of dicyandiamide (DCD) delivery method, application rate, and season on pasture urine patch nitrous oxide emissions. *Biology and Fertility of Soils, 51*, 453-464.

Luo, J., Ledgard, S. F., & Lindsey, S. B. (2013b). Nitrous oxide and greenhouse gas emissions from grazed pastures as affected by use of nitrification inhibitor and restricted grazing regime. *Science of the Total Environment, 465*, 107-114.

Luo, J., Sun, X. Z., Pacheco, D., Ledgard, S., Lindsey, S., Hoogendoorn, C. J., Wise, B., & Watkins, N. (2015b). Nitrous oxide emission factors for urine and dung from sheep fed either fresh forage rape (*Brassica napus* L.) or fresh perennial rygrass (*Lolium perenne* L.). *Animal, 9*(3), 534-543.

Monaghan, R. M., Smith, L. C., & de Klein, C. A. M. (2013). The effectiveness of the nitrification inhibitor dicyandiamide (DCD) in reducing nitrate leaching and nitrous oxide emissions from a grazed winter forage crop in southern New Zealand. *Agriculture, Ecosystems & Environment, 175*, 29-38.

Myhre, G., Shindell, D., Breon, F.-M., Collins, W., Fuglestvedt, J., Huang, J., Koch, D., Lamarque, J.-F., Lee, D., Mendoza, B., Nakajima, T., Robock, A., Stephens, G., Tekemura, T., & Zhang, H. (2013). Anthropogenic and natural radiative forcing. In T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex & P. M. Midgley (Eds.), *Climate Change 2013: The physical science basis. Contribution of Working Group I to the fifth assessment report of the Intergovernmental Panel on Climate Change.* (pp. 659-740). Cambridge: Cambridge University Press.

O'Sullivan, C. A., Duncan, E. G., Whisson, K., Treble, K., Roper, M. M., & Peoples, M. B. (2016) Changes in N cycling in the rhizosphere of canola lead to decreased N requirements in a following wheat crop. In *7th International Nitrogen Initiative Conference: Solutions to improve nitrogen use efficiency for the world*. Melbourne, Australia.

Osanai, Y., Flittner, A., Janes, J. K., Theobald, P., Pendall, E., Newton, P. C. D., & Hovenden, M. J. (2012). Decomposition and nitrogen transformation rates in a temperate grassland vary among co-occurring plant species. *Plant and Soil, 350*, 365-378.

Ravishankara, A. R., Daniel, J. S., & Portmann, R. W. (2009). Nitrous oxide (N_2O): The dominant ozone-depleting substance emitted in the 21st century. *Science, 326*, 123-125.

Reardon, C. L., Strauss, S. L., & Mazzola, M. (2013). Changes in available nitrogen and nematode abundance in response to *Brassica* seed meal amendment of orchard soil. *Soil Biology and Biochemistry, 57*, 22-29.

Rotthauwe, J. H., Witzel, K. P., & Liesack, W. (1997). The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology, 63*, 4704-12.

Rumberger, A., & Marschner, P. (2003). 2-Phenylethylisothiocyanate concentration and microbial community composition in the rhizosphere of canola. *Soil Biology and Biochemistry, 35*, 445-452.

Ryan, M. H., Kirkegaard, J. A., & Angus, J. F. (2006). *Brassica* crops stimulate soil mineral N accumulation. *Australian Journal of Soil Research, 44*, 367-377.

Saggar, S., Jha, N., Deslippe, J., Bolan, N. S., Luo, J., Giltrap, D. L., Kim, D. G., Zaman, M., & Tillman, R. W. (2013). Denitrification and N_2O:N_2 production in temperate grasslands: Processes, measurements, modelling and mitigating negative impacts. *Science of the Total Environment, 465*(Supplement C), 173-195.

Samad, M. S., Bakken, L. R., Nadeem, S., Clough, T. J., de Klein, C. A. M., Richards, K. G., Lanigan, G. J., & Morales, S. E. (2016). High-Resolution Denitrification Kinetics in Pasture Soils Link N_2O Emissions to pH, and Denitrification to C Mineralization. *PLOS ONE, 11*, e0151713.

Schmidt, E. L., & Belser, L. W. (1994). Autotrophic Nitrifying Bacteria. In S. H. Mickelson (Ed.), *Methods of Soil Analysis: Part 2 Micriobiological and Biochemical Properties* (pp. 159-178). USA: Soil Science Society of America.

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

Smith, L. C., de Klein, C. A. M., Monaghan, R. M., & Catto, W. D. (2008). The effectiveness of dicyandiamide in reducing nitrous oxide emissions from a cattle-grazed, winter forage crop in Southland, New Zealand. *Australian Journal of Experimental Agriculture, 48*, 160-164.

Smith, M. S., & Tiedje, J. M. (1979). Phases of denitrification following oxygen depletion in soil. *Soil Biology and Biochemistry, 11*, 261-267.

Subbarao, G. V., Yoshihashi, T., Worthington, M., Nakahara, K., Ando, Y., Sahrawat, K. L., Rao, I. M., Lata, J. C., Kishii, M., & Braun, H. J. (2015). Suppression of soil nitrification by plants. *Plant Science, 233*, 155-164.

Tiedje, J. M. (1994). Denitrifiers. In S. H. Mickelson (Ed.), *Methods of Soil Analysis: Part 2 Microbiological and Biochemical Properties* (pp. 245-268). USA: Soil Science Society of America.

Upsdell, M. P. (1994). Bayesian smoothers as an extension of nonlinear regression. *The New Zealand Statistician, 29*, 66-81.

van der Weerden, T. J., Styles, T. M., Rutherford, A. J., de Klein, C. A. M., & Dynes, R. (2017). Nitrous oxide emissions from cattle urine deposited onto soil supporting a winter forage kale crop. *New Zealand Journal of Agricultural Research, 60*, 119-130.

Wrage, N., Groenigen, J. W. v., Oenema, O., & Baggs, E. M. (2005). A novel dual-isotope labelling method for distinguishing between soil sources of N_2O. *Rapid Communications in Mass Spectrometry, 19*, 3298-3306.

Chapter 6

Summary and Conclusions

6.1 Introduction

Nitrogen (N) is extremely important in agricultural systems to enhance production of food required for the rapidly growing world population. However, due to its mobility, a considerable amount of the N applied to agricultural systems is lost to downstream ecosystems such as watersheds or the atmosphere (Galloway *et al.*, 2004; Robertson & Vitousek, 2009). Losses to the atmosphere include emissions of the greenhouse gas nitrous oxide (N_2O). Nitrous oxide is of concern as it has a global warming potential about 298 times that of carbon dioxide (Myhre *et al.*, 2013) and significantly depletes stratospheric ozone (Ravishankara *et al.*, 2009). Increasing atmospheric N_2O concentrations threatens the sustainability of agricultural systems so there is a need to mitigate agriculturally derived N_2O.

Inhibition of nitrification has been identified as an effective tool for mitigating N_2O emissions (IPCC, 2014), and the use of synthetic inhibitors for reducing emissions has been widely researched (e.g. Barneze *et al.*, 2015; Di & Cameron, 2011; Li *et al.*, 2014). However, the use of synthetic inhibitors in New Zealand agriculture was temporarily halted in 2013, waiting for the establishment of standards by the Codex Committee for Food. Consequently, alternative 'biological' inhibitors have been investigated. Biological nitrification inhibitors (BNIs) are secondary metabolites that are produced in, and are released to the soil by plants, and inhibit nitrification (Subbarao *et al.*, 2007).

Brassica forage crops are commonly used in New Zealand agricultural systems in order to meet feed deficits during periods of low pasture growth. Brassica crops also contain glucosinolates (GLS), whose hydrolysis products have been shown to inhibit soil nitrification (Bending & Lincoln, 2000) and so have the potential to be utilised as BNIs and reduce N_2O emissions from agriculture. Therefore, research presented in this thesis focused on developing understanding of the effectiveness of

GLS hydrolysis products as nitrification inhibitors and their potential for reducing N_2O emissions.

This chapter describes the main findings of each objective, as well as the practical considerations and limitations of using brassica forage crops for reducing N_2O emissions, and finishes with recommendations for future research.

6.2 Research summary and implications

6.2.1 Objective 1

The first objective of this thesis was:

To examine whether secondary metabolites from brassica forage crops (i.e. glucosinolate and its hydrolysis products) have an inhibitory effect on soil nitrification and N_2O emissions when applied to soil with high N inputs.

This objective was addressed through both a laboratory incubation and a field plot study. The laboratory incubation study showed that some GLS hydrolysis products (phenylethyl isothiocyanate and 4-pen-1-yl isothiocyanate) reduced N_2O emissions when applied to soil amended with high concentrations of urea-N. Evidence from the soil mineral N data and the microbial community data showed that there was some inhibition of nitrification by these products which was the likely cause of the reduction in N_2O emissions. The inhibition of nitrification, however, was not particularly strong and so where it occurred the impact appeared to be short lived. There was no measured difference in the impact by the application of different rates, suggesting that stronger inhibition might not be able to be achieved by applying a higher concentration of GLS hydrolysis product. Therefore, multiple applications of GLS hydrolysis products may be required to achieve significant inhibition of nitrification and meaningful reductions in N_2O production.

The mechanisms behind the inhibition of nitrification were not determined in this study. The results, however, showed that GLS hydrolysis products with different side chains (designated 'R') inhibited nitrification to varying degrees, e.g. phenylethyl isothiocyanate reduced N_2O emissions by about 50%, whereas 4-

pentene nitrile increased N_2O emissions. This suggests that it was not the functional group (i.e. the isothiocyanate or nitrile group) but rather the R group of the parent GLS that was partially responsible for the inhibition of nitrification. Only 3 hydrolysis products were examined in this study, and given the large number of GLS present in brassica plants there are possibly multiple other hydrolysis products that also inhibit nitrification. Practically, however, investigating all GLS hydrolysis products in this manner is not easily achieved. Additionally, it is possible that some GLS hydrolysis products could act synergistically when combined, as they may inhibit different enzymatic pathways.

In contrast to the lab study, there was no inhibition of nitrification, or reduction of N_2O emissions measured in the field study. There were many environmental and experimental factors that mean field efficacy may have been difficult to demonstrate. In particular, GLS hydrolysis products as pure compounds are extremely volatile and may have been partially lost following application. Therefore, field application, and concentrations achieved, are likely highly dependent on environmental conditions. However, dicyandiamide (DCD), which was used as benchmark inhibitor, also did not inhibit N_2O emissions in this field study suggesting other environmental factors reduced the efficacy of the nitrification inhibitors more generally.

The volatility of GLS hydrolysis products does, however, highlight a practical consideration for the potential adoption and application of GLS hydrolysis products for reducing N_2O emissions from agricultural systems ‘on farm’. Significant thought would need to be given into how applying GLS hydrolysis compounds to urine deposited onto soil might be achieved. Also, the cost and ability to obtain GLS hydrolysis products are an additional limitation as they are expensive to synthesise and there is currently no large scale, commercial production of these compounds. Overall, this study tested and supported the concept that brassica derived GLS hydrolysis products could act as potential BNIs and are worth investigating further.

6.2.2 Objective 2

The second objective of the thesis was:

To determine the effects of incorporating brassica crop tissues into soil on subsequent nitrification and nitrous oxide emissions under high N loadings.

This laboratory incubation study showed that incorporation of brassica tissues reduced urea-derived N_2O emissions, relative to the ryegrass treatment, when incorporated into soil amended with high concentrations of urea-N. Evidence from the soil mineral N data and the microbial community data, however, did not provide any evidence that inhibition of nitrification was the cause of the reduction in urea-derived N_2O emissions. No difference was observed in soil mineral N transformations or ammonia oxidising bacteria *amo-A* gene copy abundance between brassica and ryegrass treatments. While the mechanism for reduced N_2O emissions from urea was not elucidated in this study, based on the different patterns of N_2O production, it was hypothesised that this reduction was related to the relative availability of carbon (C) in the different tissues. Brassica tissues contain a large quantity of soluble sugars, which rapidly degrade and become available for microorganisms. Ryegrass tissues, on the other hand, contain more lignified fibre which would degrade more slowly resulting in a delayed, and possibly sustained, supply of labile C to the microbial pool.

Although incorporation of brassica tissues decreased N_2O released from urea, this study also showed that these tissues actually supported increased total N_2O emissions. This increase was presumably due to the labile C and N delivered by the decomposition of the plant tissues providing substrates for increased nitrification and denitrification. This increase would therefore need to be taken into consideration when determining the feasibility of tissue incorporation as a tool for mitigation of N_2O emissions.

While this study suggested there may be scope to utilise brassicas as a N_2O management tool, the contribution of tissue trampled into soil or not utilised by grazing animals has not been quantified. It is therefore not known whether this source of brassica tissue would be sufficient to achieve the reductions in urea-derived emissions observed in this study. Additionally, as the incorporation of

tissues resulted in an increase in total N_2O emissions, improving crop utilisation and minimising wastage through trampling, is potentially a more effective means of reducing N_2O emissions from soils following grazing of brassica crops. However, the level of reduction measured was significant, reducing the % of applied urea-N emitted as N_2O by up to 62%. This reduction may be sufficient to balance the inevitably high urea derived N_2O emissions resulting from the very high stocking densities which occur during grazing of forage crops. If stocking density and crop utilisation is similar between different forage crops, this could imply that brassica crops may be a preferred option for minimising N_2O emissions from the intensive grazing of forage crops.

6.2.3 Objective 3

The third objective of this thesis was:

To determine whether urine from animals fed on a predominantly brassica diet applied to soil resulted in lower N_2O emissions than urine from animals fed on a predominantly pasture diet.

This laboratory incubation study found no evidence for a reduction in nitrification or N_2O emissions following the addition of urine from a predominantly brassica fed (in this case kale) diet, compared to that following addition of urine from a predominantly pasture (ryegrass/clover) fed diet. In fact, N_2O emissions following addition of kale urine tended to be higher than following pasture urine, which was in contrast to our hypothesis. The impetus for this work was from two studies that had previously investigated this same question, but produced opposing results (Hoogendoorn *et al.*, 2016; Luo *et al.*, 2015). Both of these previous studies had been carried out in the field so this study was conducted in the laboratory under controlled conditions to reduce environmental variables that may influence results. Results from this current study were in line with that of Hoogendoorn *et al.* (2016) who also reported higher N_2O emissions from the brassica derived urine.

According to the mineral N data, the observed difference in N_2O emissions was not a result of a difference in nitrification rate. The *amoA* gene abundance data also

showed ammonia oxidising bacteria were not inhibited by either urine type. This was despite evidence that GLS hydrolysis products could be excreted in urine and that they have been reported to reduce soil nitrification as corroborated by objective 1. These results suggest that any BNI capacity of the GLS hydrolysis products, either does not carry through to urine, or was not in concentrations sufficiently high enough to produce a difference in N_2O emissions following application of that urine to soil. Additionally, other urinary constituents might also differ as a result of the two diets, and influence N_2O production.

If, however, insufficient concentration of GLS hydrolysis products in the kale derived urine was the reason for no observed reduction in nitrification rate or N_2O emissions, this mitigation method would likely not be viable on farm. In this study, kale constituted over 80% of the feed provided to the subject cows. It would not be feasible in commercial farm systems in NZ to provide more than this amount of forage brassica in an animal's diet.

A smaller, secondary objective of this study, investigated whether soils collected from beneath a growing brassica crop exhibited any BNI activity that reduced N_2O emissions following addition of urine. As such, soils were collected from beneath both a ryegrass/clover pasture, and an adjacent kale crop. Nitrification and denitrification enzyme activities measured prior to urine application showed no difference between the soils, however, N_2O emissions were higher from the kale cropped soil than the pasture soil.

The higher N_2O emissions were likely a result of the higher fertility status of this kale cropped soil. Ideally, the fertility of the two soils would have been the same for this incubation, however, the decision was made not to add nutrients to the pasture soil to avoid changing, or priming of, the native microbial community. As such, the crop soil had greater concentrations of total N, total C, and Olsen P, all of which would increase the nutrient status of the soil and may increase N_2O production. Additionally, the kale cropped soil had lower pH compared to that of the pasture soil which would also result in higher N_2O emissions from the cropped soil. However, despite this discrepancy in soil fertility status of the two soils, no difference in soil nitrification rate was observed. It is possible that GLS hydrolysis

products present in the kale cropped soil did in fact reduce nitrification, but this was masked by the higher fertility.

Due to the nature of this study, where only a single paired site was examined and the difference in soil fertility, it was difficult to draw any conclusions as to whether N cycling in soil growing brassica crops was decreased as a result of the presence of GLS hydrolysis products. Multiple paired sites and equivalent soil fertility would be required for further assessment.

6.3 Final conclusions

The overarching hypothesis of this thesis was:

Naturally occurring glucosinolate related compounds from brassica crops act as biological nitrification inhibitors and can reduce nitrous oxide emissions from a urine patch.

Overall, the hypothesis was partially supported in that some GLS hydrolysis products inhibited soil nitrification and reduced N_2O emissions following addition to soil (Chapter 3). However, this observation was not consistent across all forms of potential GLS hydrolysis product delivery to soil (Chapters 4 and 5). The BNI activity displayed by GLS hydrolysis products was very weak and short lived. Consequently, this BNI activity was not observed when GLS hydrolysis products were applied by tissue incorporation, or by application of urine derived from cows fed brassicas.

Additionally, this hypothesis was partially supported in that N_2O emissions were reduced in two of the studies conducted (Chapters 3 and 4). However, this could not always be attributed to inhibition of nitrification (Chapter 4).

The main conclusions of this thesis were:

1. Some GLS hydrolysis products showed BNI capacity and reduced N_2O emissions when applied to soil with a high concentration of urea-N. However, this inhibition was weak and short-lived.

2. Brassica plant tissues reduced urea derived N_2O emissions relative to ryegrass tissues when incorporated into soil with a high concentration of urea-N, however, the reduction in N_2O emissions could not be attributed to inhibition of nitrification by GLS hydrolysis products.

3. Incorporation of plant tissues into soil increased total N_2O emissions likely due to the provision of N and C substrates from the added plants.

4. The BNI capacity of GLS hydrolysis products was not carried through to the urine of animals grazing brassica crops in sufficient quantity to reduce N_2O emissions when urine was applied to soil.

Research that has been conducted, on brassicas and GLS, to date is summarised in Table 6.1. This, along with the literature review, highlight that very little research has been carried out on the impact that brassicas, or their secondary metabolites, have on N_2O emissions. Additionally, most of the work investigating the influence of brassicas and GLS on N cycling has been in low N environments which are different to what would be expected in a grazing system.

Overall, my data and that from literature provides strong evidence that BNI activity exists in brassicas. BNI activity has been observed in both high and low N environments, and where inhibition of nitrification was recorded, it was also generally supported by evidence of the specific inhibition of nitrifying bacteria. However, the degree of inhibition appears to be low and there is little data on the mechanism by which this inhibition might occur.

Table 6.1: Summary of research involving brassica crops or GLS hydrolysis products as they apply to each of the thesis objectives.

PURE COMPOUNDS ADDED TO SOIL – *Objective 1*

Author	*Field/ Lab*	*N rate ($\mu g\ N\ g^{-1}$ soil or kg N ha^{-1})*	*N form*	*Process inhibited*	*Nitrous oxide reduced?*
Balvert *et al.* (2017) (Chapter 3)	Lab	600	Urea	Nitrification	Yes
Balvert *et al.* (2017) (Chapter 3)	Field	600	Artificial urine	None	No
Bending and Lincoln (2000)	Lab	80	NH_4SO_4	Nitrification	Not measured
Welsh *et al.* (1998)	Field	144	NH_4SO_4	Undefined N cycling	Not measured

PLANT TISSUES INCORPORATED INTO SOIL – *Objective 2*

Author	*Field/ Lab*	*N rate* ($\mu g\ N\ g^{-1}$ soil or kg N ha^{-1})*	*N form*	*Process inhibited*	*Nitrous oxide reduced?*
Balvert *et al.* (Chapter 4)	Lab	600	Urea	Undefined N cycling	Yes
Brown and Morra (2009)	Lab	40	Urea	Nitrification	Not measured
Reardon *et al.* (2013)	Lab	0		Nitrification	Not measured
Ryan *et al.* (2006)	Lab	0		Undefined N cycling	Not measured
Snyder *et al.* (2010)	Lab	0		Nitrification	Not measured
Velthof *et al.* (2002)	Lab	50	NO_3^-	None	No

URINE ADDED TO SOIL – *Objective 3*

Author	*Field/ Lab*	*N rate ($\mu g\ N\ g^{-1}$ soil or kg N ha^{-1})*	*N form*	*Process inhibited*	*Nitrous oxide reduced?*
Balvert *et al.* (Chapter 5)	Lab	600	Urine	None	No
Luo *et al.* (2015)	Field	155 & 441	Urine	Undefined N cycling	Yes
Hoogendoorn *et al.* (2016)	Field	150 & 300	Urine	None	No

ROOT EXUDATES IN SOIL – *Objective 3b*

Author	*Field/ Lab*	*N rate ($\mu g\ N\ g^{-1}$ soil or kg N ha^{-1})*	*N form*	*Process inhibited*	*Nitrous oxide reduced?*
Balvert *et al.* (Chapter 5)	Lab	600	Urine	None	No
Kirkegaard *et al.* (1999)	Field	20	?	Nitrification	Not measured
O'Sullivan *et al.* (2016)	Field	?	NH_4NO_3	Nitrification	Not measured
Ryan *et al.* (2006)	Field	25	?	Undefined N cycling	Not measured

*Additional to plant N

Evidence for the ability of brassicas or GLS hydrolysis products to reduce N_2O emissions as a result of BNI activity is less certain. Objective 1 of this thesis is the only study that has examined this question. Although the results were positive, this needs to be repeated, and examined under different soil types and moisture regimes before any final conclusions can be made. Objective 2 also showed a reduction in N_2O emissions following incorporation of brassica tissues, however, this could not be attributed to BNI activity.

There has been little evidence that the BNI capacity of brassicas is carried through to the urine derived from animals fed a brassica diet.

6.4 Future research

If brassica crops or GLS are to be recommended as a N_2O mitigation strategy in grazed agricultural systems, there are some key areas that require further research. Areas that have been highlighted as a result of this thesis are discussed below:

Plant extracts

As discussed in the first objective (section 6.2.1), there are many GLS contained within each brassica plant and the degree of impact each hydrolysis product had on nitrification appeared to be related to the parent GLS. Also, there is a potential for synergistic effects of combining GLS hydrolysis products. Testing all potential GLS hydrolysis products and combinations would require a significant investment of time, thus, it is potentially more worthwhile to focus any further research on whole plant extracts to establish which combinations are likely to naturally occur, and which particular brassica plant shows the most promise for further investigation.

Quantification of crop utilisation and crop wastage following grazing

Incorporation of crop tissues was shown to reduce urea derived N_2O emissions, however, as outlined in objective 2 (section 6.2.2), the quantity of crop tissues left behind following grazing by animals is unknown. Additionally, grazing of certain brassicas would result in remaining stubble and dying roots which would also

release GLS hydrolysis products. Therefore, research on both the amount of un-utilised or trampled crop remaining, and the contribution of that to N_2O emissions is required.

Root exudation of GLS hydrolysis products and crop-soil interactions

The comparison of pasture and cropped soils examined in Chapter 5 was based on the research outlined in Table 6.1 where growing brassica crops have inhibited either nitrification or another undefined pathway of the N cycle. However, this thesis only examined a single paired site. Therefore, an in-depth comparison of multiple paired brassica/pasture sites is required to understand whether the BNI capacity of growing brassicas observed in the previous studies can also be found in forage brassicas growing in NZ, and the impact that may have on N_2O production. Pasture and cropping soils examined should be of similar fertility status for a true comparison as outlined in section 6.2.3., although this could be difficult on a commercial farm, as crops require additional nutrients to achieve the required dry matter and soils are generally fertilised at sowing.

GLS hydrolysis products in urine and other urinary constituents

There has been no detailed comparison of urines derived from animals fed on pasture and forage brassicas. As discussed in objective 3 (section 6.2.3), along with the presence of GLS hydrolysis products in brassica derived urine, other urinary constituents might also differ as a result of the two diets. Further research is required to elucidate the difference in urinary constituents between urine types and any influence they may have on N_2O production.

Potential fungicidal properties of GLS hydrolysis products

Some brassica crops and GLS hydrolysis products are used as fumigants to control soil fungal pests in cropping systems. Nitrous oxide production by fungi was not investigated in this study, however, there is the potential that GLS hydrolysis products may inhibit both fungal and bacterial production of N_2O. Although fungal N_2O production is considerably lower than that of prokaryotes, fungi may

contribute significantly to N_2O production due to the high biomass of fungi in soils (Mothapo *et al.*, 2013). Further research is required to understand the fungicidal properties of GLS hydrolysis products and the effect this has on fungal N_2O production.

Agricultural systems modelling

The use of agricultural systems models (e.g. Overseer and APSIM) would be required in order to determine whether forage brassicas and GLS hydrolysis products may be utilised on farm to achieve a meaningful reduction in N_2O emissions. This would be particularly useful for assessing a wide variety of farm systems, climatic conditions and soil types. Models (e.g. FARMAX at farm level, or abatement curves at regional level) may also provide insight into the economic viability of the use of forage brassicas as a N_2O mitigation tool, particularly once agriculture is included in the New Zealand greenhouse gas emissions trading scheme. This would inform as to the value of continuing to investigate brassicas as a potential N_2O mitigation tool.

6.5 References

Balvert, S. F., Luo, J., & Schipper, L. A. (2017). Do glucosinolate hydrolysis products reduce nitrous oxide emissions from urine affected soil? *Science of the Total Environment, 603*(Supplement C), 370-380.

Barneze, A. S., Minet, E. P., Cerri, C. C., & Misselbrook, T. (2015). The effect of nitrification inhibitors on nitrous oxide emissions from cattle urine depositions to grassland under summer conditions in the UK. *Chemosphere, 119*, 122-129.

Bending, G. D., & Lincoln, S. D. (2000). Inhibition of soil nitrifying bacteria communities and their activities by glucosinolate hydrolysis products. *Soil Biology and Biochemistry, 32*, 1261-1269.

Brown, P. D., & Morra, M. J. (2009). Brassicaceae tissues as inhibbitors of nitrification in soil. *Journal of Agricultural and Food Chemistry, 57*, 7706-7711.

Di, H. J., & Cameron, K. C. (2011). How does the application of different nitrification inhibitors affect nitrous oxide emissions and nitrate leaching from cow urine in grazed pastures? *Soil Use and Management, 28*, 54-61.

Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., Asner, G. P., Cleveland, C. C., Green, P. A., Holland, E. A., Karl, D. M., Michaels, A. F., Porter, J. H., Townsend, A. R., & Vöosmarty, C. J. (2004). Nitrogen cycles: past, present, and future. *Biogeochemistry, 70*, 153-226.

Hoogendoorn, C. J., Luo, J., Lloyd-West, C. M., Devantier, B. P., Lindsey, S. B., Sun, S., Pacheco, D., Li, Y., Theobald, P. W., & Judge, A. (2016). Nitrous oxide emission factors for urine from sheep and cattle fed forage rape (Brassica napus L.) or perennial ryegrass/white clover pasture (Lolium perenne L./Trifolium repens). *Agriculture, Ecosystems & Environment, 227*, 11-23.

IPCC. (2014). *Climate change 2014: Mitigation of climate change. Contribution of working group III to the fifth assessment report of the intergovernmental panel on climate change.*, Cambridge, United Kingdom and New York, United States of America. 1454p.

Kirkegaard, J. A., Howe, G. N., & Mele, P. M. (1999). Enhanced accumulation of mineral-N following Canola. *Australian Journal of Experimental Agriculture, 39*, 587-593.

Li, J., Shi, Y., Luo, J., Zaman, M., Houlbrooke, D., Ding, W., Ledgard, S., & Ghani, A. (2014). Use of nitrogen process inhibitors for reducing gaseous nitrogen losses from land-applied farm effluents. *Biology and Fertility of Soils, 50*, 133-145.

Luo, J., Sun, X. Z., Pacheco, D., Ledgard, S., Lindsey, S., Hoogendoorn, C. J., Wise, B., & Watkins, N. (2015). Nitrous oxide emission factors for urine and dung from sheep fed either fresh forage rape (*Brassica napus* L.) or fresh perennial rygrass (*Lolium perenne* L.). *Animal, 9*, 534-543.

Mothapo, N. V., Chen, H., Cubeta, M. A., & Shi, W. (2013). Nitrous oxide producing activity of diverse fungi from distinct agroecosystems. *Soil Biology and Biochemistry, 66*, 94-101.

Myhre, G., Shindell, D., Breon, F.-M., Collins, W., Fuglestvedt, J., Huang, J., Koch, D., Lamarque, J.-F., Lee, D., Mendoza, B., Nakajima, T., Robock, A., Stephens, G., Tekemura, T., & Zhang, H. (2013). Anthropogenic and natural radiative forcing. In T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex & P. M. Midgley (Eds.), *Climate Change 2013: The physical science basis. Contribution of Working Group I to the fifth assessment report of the Intergovernmental Panel on Climate Change.* (pp. 659-740). Cambridge: Cambridge University Press.

O'Sullivan, C. A., Duncan, E. G., Whisson, K., Treble, K., Roper, M. M., & Peoples, M. B. (2016) Changes in N cycling in the rhizosphere of canola lead to decreased N reaquirements in a following wheat crop. In *7th International Nitrogen Initiative Conference: Solutions to improve nitrogen use efficiency for the world*. Melbourne, Australia.

Ravishankara, A. R., Daniel, J. S., & Portmann, R. W. (2009). Nitrous oxide (N_2O): The dominant ozone-depleting substance emitted in the 21st century. *Science, 326*, 123-125.

Reardon, C. L., Strauss, S. L., & Mazzola, M. (2013). Changes in available nitrogen and nematode abundance in response to *Brassica* seed meal amendment of orchard soil. *Soil Biology and Biochemistry, 57*, 22-29.

Robertson, G. P., & Vitousek, P. M. (2009). Nitrogen in agriculture: Balancing the cost of an essential resource. *Annual Review of Environment and Resources, 34*, 97-125.

Ryan, M. H., Kirkegaard, J. A., & Angus, J. F. (2006). *Brassica* crops stimulate soil mineral N accumulation. *Australian Journal of Soil Research, 44*, 367-377.

Snyder, A. J., Johnson-Maynard, J. L., & Morra, M. J. (2010). Nitrogen mineralization in soil incubated with 15N-labeled Brassicaceae seed meals. *Applied Soil Ecology, 46*, 73-80.

Subbarao, G. V., Rondon, M., Ito, O., Ishikawa, T., Rao, I. M., Nakahara, K., Lascano, C., & Berry, W. (2007). Biological nitrification inhibition - is it a widespread phenomenon? *Plant Soil, 294*, 5-18.

Velthof, G., Kuikman, P., & Oenema, O. (2002). Nitrous oxide emission from soils amended with crop residues. *Nutrient Cycling in Agroecosystems, 62*, 249-261.

Welsh, C. E., Guertal, E. A., & Wood, C. W. (1998). Effects of soil fumigation and N source on soil inorganic N and tomato growth. *Nutrient Cycling in Agroecosystems, 52*, 37-44.

Appendix A

Dietary Manipulation as a Tool for Mitigating Nitrous Oxide Emissions

This document was published in the following edited book and is presented here for reference:

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DIETARY MANIPULATION AS A TOOL FOR MITIGATING NITROUS OXIDE EMISSIONS

Sheree Balvert [*] ***and Jiafa Luo***
AgResearch, Hamilton, New Zealand

1. INTRODUCTION

Currently, for every 100 units of nitrogen (N) used in agriculture, only about 15 are consumed as crop, dairy or meat products (Steinfeld et al., 2006; Robertson and Vitousek 2009). This points to very low N use efficiency in most agricultural systems. In the dairy industry for example, despite improved genetic potential of cows with high nutrient utilisation, the increased external input of feed concentrates and use of fertiliser N have decreased N use efficiency in these systems (Huhtanen et al., 2008). Increasing N use efficiency is a key strategy by which the increasing food demand might be met without a corresponding increase in N losses and nitrous oxide (N_2O) emissions.

This chapter focuses on the use of dietary intervention or manipulation by incorporating diverse forages or crops into animal based agriculture as an approach for increasing N use efficiency. Dietary intervention is primarily aimed at either reducing the concentration and amount of N in animal excreta, or changing the partitioning of N in urine and dung, while preferably having no detrimental effect on productivity. This may include, but is not limited to, using low N feed supplements to reduce the amount of N consumed and hence lower the amount of surplus N being excreted, or specific dietary supplementation to encourage the partitioning of N toward dung rather than urine. Additionally, forages may be selected for their potential to inhibit soil N transformations by either the root exudation or urine-excretion of plant secondary metabolites. Dietary intervention may not be feasible in many low input, pasture based agricultural systems, however, in more intensive systems such as the New Zealand dairy industry, particularly with the increasing use of animal feed pads and housing practices, supplements are commonly imported and so dietary intervention is more practical.

2. URINE PATCHES AND NITROUS OXIDE

In order to understand the initiative to reduce excreta N, and urinary N in particular, it is necessary to understand the N dynamics of the urine patch in grazing systems and how they relate to N_2O emissions. Grazing ruminants are poor utilisers of dietary N, with only 10–30% of the N consumed being retained in body tissues and in products. The remainder is excreted in urine or dung (e.g., Selbie et al., 2015). This is generally because plants require more N to grow than ruminants require for protein synthesis (Haynes and Williams 1993) and so excess N is consumed by the ruminant. There have been several studies that have demonstrated a strong linear relationship between N intake and N excretion (e.g., Castillo et al., 2000; Kebreab et al., 2001; Mulligan et al. 2004; Huhtanen et al., 2008) and in New Zealand about 80% of the total N_2O emissions come from urine and dung deposition.

Urine deposited onto grazed pastures is considered a hotspot for N_2O production as urine provides high localised concentrations of available N and carbon in soils. In New Zealand, the

[*]Corresponding author: Sheree Balvert, AgResearch, 10 Bisley Road, Private Bag 3123, Hamilton 3214, New Zealand. Email: sheree.balvert@agresearch.co.nz.

average urine N loading rate is about 600 kg N ha^{-1} for dairy cattle, and about 1000 kg N ha^{-1} for sheep (Selbie et al., 2015). Additionally, other abiotic conditions found in the urine patch, in particular soil water filled pore space, encourage anaerobic denitrification which is the main N_2O forming pathway.

3. REDUCING DIETARY N

Due to the strong relationship between N intake and N excretion, one of the simpler ways to reduce N excreted (and hence N available as a substrate for N_2O production) is to reduce the N consumed in the diet through the use of diverse feeds. As N in pasture is generally in excess of animal requirements, reducing N intake should presumably not reduce production. Care however must be taken to ensure dietary N/protein is not reduced to a point where it is no longer sufficient to meet the animal's metabolic requirements.

Many studies have shown that reducing the N content in the diet through low N supplements reduces total and urinary N excreted in ruminants (e.g., Edwards et al., 2014; Huhtanen et al., 2008; Kebreab et al., 2001; Misselbrook et al., 2005; Mulligan et al. 2004 (and references therein); Nielsen et al., 2003; Steinshamn et al., 2006). In a New Zealand example, Luo et al., (2008) in a study on a pasture based dairy system, showed that using low N feed supplements such as maize to reduce the amount of N consumed, significantly reduced the amount of surplus N being excreted and showed an increase in milk production per unit of N and thus overall N use efficiency. Additionally, Burke et al., (2008) carried out a study on an Irish dairy system, which showed a reduction in both urinary and total N excretion, and no effect on production, when supplementing the diet with low N citrus pulp.

4. N PARTITIONING – PROTEIN VS EXCRETA

The low level of utilisation of dietary N is largely a result of insufficient carbohydrate-derived energy for conversion of the feed N to microbial N in the rumen. The surplus N is then lost through the rumen wall as ammonia and subsequently excreted in urine following hepatic conversion to urea N (Miller et al., 2001). It therefore suggests that increasing dietary soluble carbohydrate content may increase utilisation of feed N in the rumen and thus reduce N excretion.

Miller et al., (2001) and Moorby et al., (2006) showed that the addition of 'high sugar grasses', or grass varieties with a high content of water soluble carbohydrate, to the diet increased milk production in dairy cows through more efficient utilisation of the feed N in the rumen. They also measured a reduction in total excreted N and urinary N. Additionally, Lee et al., (2002) found reduced ammonia-N concentrations in the rumen of steers fed high sugar grasses compared with steers offered the control perennial ryegrass variety, which translated through to reduced urine N excretion. These results are supported by others (e.g., Castillo et al., 2000; Broderick 2003; Steinshamn et al., 2006) who have reported improved N utilisation and reduced excretal N following supplementation of high energy feeds to cattle.

5. N PARTITIONING – DUNG VS URINE

Although the relationship between N intake and N excreted is linear for both dung and urine, the line is much steeper for urine than for dung (e.g., Dijkstra et al., 2013), indicating that urine is the main removal pathway for excess dietary N. N_2O emissions from dung deposits are considerably lower than from urine patches as the N transformation processes are much slower in dung, and it has been found that the emission factor (percentage of applied N emitted as N_2O-N) for dung is significantly lower than that of urinary N (van der Weerden et al, 2011). It is therefore likely that encouraging excess dietary N to be excreted in dung rather than urine would result in an overall reduction of N_2O emissions.

Supplementation of diet with condensed tannins, for example, has been shown to result in a greater partitioning of excreta N in dung rather than urine. Condensed tannins bind with proteins in the rumen, slowing protein degradation and leading to decreased ruminal NH_3 concentration and subsequently decreased urinary N excretion, without affecting production (Carulla et al., 2005; Misselbrook et al., 2005).

6. PLANT SECONDARY METABOLITES

There is extensive evidence to show that plant tissues contain a wide variety of chemical compounds which are not involved in primary metabolism. These compounds, termed 'secondary metabolites', vary according to family and species and play a variety of roles in protecting plants from a range of stresses (e.g., predation and competition, Bennett and Wallsgrove 1994; Erickson et al., 2000) including through suppression or inhibition of soil N transformation processes. Inhibitors of soil N processes, such as urease and nitrification inhibitors, have the potential to reduce N_2O emissions from animal excreta deposited to land (Dell et al., 2014; Li et al., 2014). Urease inhibitors retard the hydrolysis of urea to NH_4^+ whereas nitrification inhibitors retard the conversion of NH_4^+ to NO_3^-. These both prolong the length of time taken for applied N (e.g., excreta or fertiliser N) to be converted to NO_3^- thereby allowing for more uptake by plants and improving N use efficiency (Amberger 1989). This results in less available NO_3^- for both direct N_2O emissions and indirect emissions following leaching.

The suppression of nitrification has been observed to occur naturally in some ecosystems where certain plant species release organic molecules from their roots that suppress the function and growth of nitrifying bacteria (Subbarao et al., 2007). This process is termed biological nitrification inhibition (BNI). It has been hypothesised that BNI has evolved as part of some plants' adaptation mechanisms to conserve and use N efficiently in systems that are naturally limited in mineral N (Al-Ansari & Abdulkareem 2014; Subbarao et al., 2007) and appears to be stimulated by high NH_4^+ concentration in the soil (Subbarao et al., 2015). BNI has the potential to improve agronomic nutrient use efficiency and, as a result, reduce both leached and gaseous N loss from agricultural systems. A field study carried out over two years in China, showed higher vegetable yield and nutrient use efficiency for crops co-planted with known biological nitrification inhibiting plants, as well as a reduction in N_2O emissions (Zhang et al., 2015). Several BNIs have been isolated from root exudates and plant tissues and BNIs that belong to many different chemical classes have been identified (Gopalakrishnan et al., 2007; Subbarao et al., 2013).

Additionally, residues of plant tissues are also known to release allelo-chemicals that inhibit nitrification in soil (Bremner and McCarty 1993). As these chemicals are released during destruction or decomposition of the plant, it is thought they are a defence mechanism against herbivores, pests or pathogens, but also inhibit nitrification (Bending and Lincoln 2000; Bennett and Wallsgrove 1994). Some examples of plants where tissue residues or extracts of tissues inhibit nitrification include neem, karanj, plantain, sorghum and brassica species (Luo unpublished data; Prasad and Power 1995; Zhang et al., 2015).

Incorporating plant tissues into soil may inhibit nitrification in cropping systems, however, this approach is less suitable for grazing systems where pastures are generally only renewed every 5-10 years so there is little opportunity to incorporate plant material. Also, N substrate supplied in the excreta of grazing animals is the main driver of nitrification in these systems. Therefore, rather than incorporating plant residues, it may be possible to deliver BNI compounds to the soil through the grazing animal. Grazers eliminate unprocessed plant material, and presumably secondary metabolites, in excreta (dung and urine). One advantage of this approach is that the inhibitor is delivered with the main N source for N_2O production. For example, Ledgard et al., (2008) and Welten et al., (2013) showed that urine patches could be individually targeted with a synthetic nitrification inhibitor (in this instance dicyandiamide) by orally administering the inhibitor to grazing animals that was subsequently excreted in the urine. It is possible that this method could also be used for administering plant derived inhibitors to urine patches. Luo et al., (2015) recently reported reduced N_2O emissions from urine patches derived from animals fed a brassica crop compared to urine from animals fed on perennial pasture. The N content of urine from brassica fed animals is lower than that of ryegrass fed animals and so lower N2O emissions would be expected, however, they also reported reduced emission factors which accounts for the differences in N loading. One possible explanation for this reduction is that brassica-derived compounds may be delivered to the soil in the urine and nitrification may subsequently be inhibited in the urine patch. There is an array of potential BNIs that could possibly be utilised for reducing N_2O production in agricultural systems. In New Zealand systems, fodder crops that are known to have BNI potential include: leafy turnips, bulb turnips, swedes, kale, forage rape and plantain (de Ruiter et al., 2009). However, there is little research that has examined the potential for these feeds to reduce nitrification and N_2O emissions in practice.

CONCLUSION

This chapter outlines the potential for using dietary manipulation as a tool for mitigating N_2O emissions, and indeed improving N use efficiency in general, from intensive animal agricultural systems.

Most of the approaches are aimed at reducing N excreted in the urine patch and associated N_2O formed under patch conditions. A number of studies have already been carried out in this area and much is known about which feed characteristics can be used to achieve this. It is however, important to ensure that animal metabolic requirements are always met.

Other approaches focus on inhibiting soil N transformation processes through the use of plant secondary metabolites. Although there are studies that confirm the presence and potential of such compounds, their effectiveness via a feed manipulation route requires further testing.

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REFERENCES

Al-Ansari A. M. S. and Abdulkareem M. A. (2014) Some plant extracts retarde nitrification in soil. *Acta Agriculturae Slovenica* 103: 5-13.

Amberger A. (1989) Research on dicyandiamide as a nitrification inhibitor and future outlook. *Communications in Soil Science and Plant Analysis* 20: 1933-1955.

Bending G. D. and Lincoln S. D. (2000) Inhibition of soil nitrifying bacteria communities and their activities by glucosinolate hydrolysis products. *Soil Biology and Biochemistry* 32: 1261-1269.

Bennett R. N. and Wallsgrove R. M. (1994) Tansley Review No. 72. Secondary metabolites in plant defence mechanisms. *New Phytologist* 127: 617-633.

Bremner J. M. and McCarty G. W. (1993) Inhibition of nitrification in soil by allelochemicals derived from plants and plant residues. *Soil Biochemistry* 8: 181-218).

Broderick G. A. (2003) Effects of Varying Dietary Protein and Energy Levels on the Production of Lactating Dairy Cows1. *Journal of Dairy Science* 86: 1370-1381.

Burke F., O'Donovan M. A., Murphy J. J., O'Mara F. P. and Mulligan F. J. (2008) Effect of pasture allowance and supplementation with maize silage and concentrates differing in crude protein concentration on milk production and nitrogen excretion by dairy cows. *Livestock Science* 114: 325-335.

Carulla J. E., Kreuzer M., Machmüller A. and Hess, H. D. (2005) Supplementation of Acacia mearnsii tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. *Australian Journal of Agricultural Research* 56: 961-970.

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 to environmental pollution. J*ournal of Animal and Feed Sciences* 9: 1-32.

de Ruiter J., Wilson D., Maley S., Fletcher A., Fraser T., Scott W., Berryman S., Dumbleton A. and Nichol,W. (2009) *Management practices for forage brassicas*. New Zealand. pp 70.

Dell C. J., Han K., Bryant R. B. and Schmidt J. P. (2014) Nitrous oxide emissions with enhanced efficiency nitrogen fertilizers in a rainfed system. *Agronomy Journal* 106: 723-731.

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(s2): 292-302.

Edwards G. C., De Ruiter J. M., Dalley D. E., Pinxterhuis J. B., Cameron K. C., Bryant R. H., Di H. J., Malcolm B. J. and Chapman D.F. (2014) Urinary nitrogen concentration of cows grazing fodder beet, kale and kale-oat forage systems in winter. *Australian Dairy Science Symposium:* 144-147.

Erickson A. J., Ramsewak R. S., Smucke, A. J. and Nair M. G. (2000) Nitrification Inhibitors from the roots of Leucaena leucocephala. *Journal of Agricultural and Food Chemistry* 48: 6174-6177.

Gopalakrishnan S., Subbarao G. V., Nakahara K., Yoshihashi T., Ito O., Maeda I., Ono H. and Yoshida M. (2007) Nitrification inhibitors from the root tissues of Brachiaria humidicola, a tropical grass. *Journal of Agricultural and Food Chemistry 55*: 1385-1388.

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

Huhtanen P., Nousiainen J. I., Rinne M., Kytölä K. and Khalili H. (2008) Utilization and Partition of Dietary Nitrogen in Dairy Cows Fed Grass Silage-Based Diets. *Journal of Dairy Science* 91: 3589-3599.

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.

Ledgard S. F., Menneer J. C., Dexter M. M., Kear M. J., Lindsey S., Peters J. S. and Pacheco D. (2008) A novel concept to reduce nitrogen losses from grazed pastures by administering soil nitrogen process inhibitors to ruminant animals: A study with sheep. *Agriculture, Ecosystems & Environment* 125: 148-158.

Lee M. R. F., Harris L. J., Moorby J. M., Humphreys M. O., Theodorou M. K., MacRae J. C. and Scollan N. D. (2002) Rumen metabolism and nitrogen flow to the small intestine in steers offered Lolium perenne containing different levels of water-soluble carbohydrate. *Animal Science* 74: 587-596.

Li J., Shi Y., Luo J., Zaman M., Houlbrooke D., Ding W., Ledgard S. and Ghani A. (2014) Use of nitrogen process inhibitors for reducing gaseous nitrogen losses from land-applied farm effluents. *Biology and Fertility of Soils* 50: 133-145.

Luo J., Ledgard S. F., de Klein C. A. M., Lindsey S.B. and Kear M. (2008) Effects of dairy farming intensification on nitrous oxide emissions. *Plant and Soil* 309): 227-237.

Luo J., Sun X. Z., Pacheco D., Ledgard S., Lindsey S., Hoogendoorn C.J., Wise B. and Watkins N. (2015). Nitrous oxide emission factors for urine and dung from sheep fed either fresh forage rape (Brassica napus L.) or fresh perennial rygrass (Lolium perenne L.). *Animal* 9: 534-543.

Miller L. A., Moorby J. M., Davies D. R., Humphreys M. O., Scollan N. D., MacRae J. C. and Theodorou M. K. (2001) Increased concentration of water-soluble carbohydrate in perennial ryegrass (Lolium perenne L.): milk production from late-lactation dairy cows. *Grass and Forage Science* 56: 383-394.

Misselbrook T. H., Powell J. M., Broderick G. A. and Grabber J. H. (2005) Dietary Manipulation in Dairy Cattle: Laboratory Experiments to Assess the Influence on Ammonia Emissions. *Journal of Dairy Science* 88: 1765-1777.

Moorby J. M., Evans R. T., Scollan N. D., MacRae J. C. and Theodorou M. K. (2006) Increased concentration of water-soluble carbohydrate in perennial ryegrass (Lolium perenne L.). Evaluation in dairy cows in early lactation. *Grass and Forage Science* 61: 52-59.

Mulligan F. J., Dillon P., Callan J. J., Rath M. and O'Mara F. P. (2004) Supplementary Concentrate Type Affects Nitrogen Excretion of Grazing Dairy Cows. *Journal of Dairy Science* 87: 3451-3460.

Nielsen N. M., Kristensen T., Nørgaard P. and Hansen H. (2003) The effect of low protein supplementation to dairy cows grazing clover grass during half of the day. *Livestock Production Science* 81: 293-306.

Prasad R. and Power J. F. (1995) Nitrification inhibitors for agriculture, health, and the environment. *Advances in Agronomy* 54: 233-281.

Robertson G. P. and Vitousek P. M. (2009) Nitrogen in agriculture: Balancing the cost of an essential resource. *Annual Review of Environment and Resources* 34: 97-125.

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.

Steinfeld H., Gerber P., Wassenaar T., Castel V., Rosales M. and de Haan C. (2006) Livestock's long shadow: Environmental issues and options. Renewable Resources Journal 24: 15-17.

Steinshamn H., Höglind M., Garmo T. H., Thuen E. and Brenøe U. T. (2006) Feed nitrogen conversion in lactating dairy cows on pasture as affected by concentrate supplementation. *Animal Feed Science and Technology* 131: 25-41.

Subbarao G. V., Rao I. M., Nakahara K., Ando Y., Sahrawat K. L., Tesfamariam T., Lata J. C., Boudsocq S., Miles J. W., Ishitani M. and Peters M. (2013) Nitrogen management in grasslands and forage-based production systems - Role of biological nitrification inhibition (BNI). *Tropical Grasslands - Forajes Tropicales* 1: 168-174.

Subbarao G. V., Rondon M., Ito O., Ishikawa T., Rao I. M., Nakahara K., Lascano C. and Berry W. (2007) Biological nitrification inhibition - is it a widespread phenomenon? *Plant Soil* 294: 5-18.

Subbarao G. V., Yoshihashi T., Worthington M., Nakahara K., Ando Y., Sahrawat, K. L., Rao I. M., Lata J. C., Kishii M. and Braun H.J. (2015) Suppression of soil nitrification by plants. *Plant Science* 233: 155-164.

van der Weerden T. J., Luo J., de Klein, C. A. M., Hoogendoorn C. J., Littlejohn R. P. and Rys G. J. (2011) Disaggregating nitrous oxide emission factors for ruminant urine and dung deposited onto pastoral soils. *Agriculture, Ecosystems and Environment* 141: 426-436.

Welten B. G., Ledgard S. F., Schipper L. A. and Judge A. A. (2013) Effect of amending cattle urine with dicyandiamide on soil nitrogen dynamics and leaching of urinary-nitrogen. *Agriculture, Ecosystems and Environment* 167: 12-22.

Zhang M., Fan C. H., Li Q. L., Li B., Zhu Y. Y. and Xiong Z. Q. (2015) A 2-yr field assessment of the effects of chemical and biological nitrification inhibitors on nitrous oxide emissions and nitrogen use efficiency in an intensively managed vegetable cropping system. *Agriculture, Ecosystems and Environment* 201: 43-50.

Appendix B

Co-Authorship Forms for Research Chapters

THE UNIVERSITY OF WAIKATO
Te Whare Wānanga o Waikato

Co-Authorship Form

Postgraduate Studies Office
Student and Academic Services Division
Wahanga Ratonga Matauranga Akonga
The University of Waikato
Private Bag 3105
Hamilton 3240, New Zealand
Phone +64 7 838 4439
Website: http://www.waikato.ac.nz/sasd/postgraduate/

This form is to accompany the submission of any PhD that contains research reported in published or unpublished co-authored work. **Please include one copy of this form for each co-authored work.** Completed forms should be included in your appendices for all the copies of your thesis submitted for examination and library deposit (including digital deposit).

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 3 has been published in the journal *Science of the Total Environment*. The title of this paper is "Do glucosinolate hydrolysis products reduce nitrous oxide emissions from urine affected soil?"

Nature of contribution by PhD candidate	Collaborated on development of research objectives and protocol. Developed experimental design and set-up trials. Carried out sample collection and the majority of sample analyses. Conducted data analysis and writing of manuscript.
Extent of contribution by PhD candidate (%)	90

CO-AUTHORS

Name	Nature of Contribution
Jiafa Luo	Collaborated on development of research objectives and protocol. Assisted in data interpretation and provided comments on manuscript.
Louis Schipper	Collaborated on development of research objectives and protocol. Assisted in data interpretation and provided comments on manuscript.

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

Name	Signature	Date
Jiafa Luo		7/5/18
Louis Schipper	L A Schipper	2/5/18

July 2015

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Postgraduate Studies Office
Student and Academic Services Division
Wahanga Ratonga Matauranga Akonga
The University of Waikato
Private Bag 3105
Hamilton 3240, New Zealand
Phone +64 7 838 4439
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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 4 has been submitted for publication in the journal *Environmental Quality*. The title of the paper is "Can incorporating brassica tissues into soil reduce nitrification rates and nitrous oxide emissions?"

Nature of contribution by PhD candidate	Collaborated on development of research objectives and protocol. Developed experimental design and set-up trials. Carried out sample collection and the majority of sample analyses. Conducted data analysis and writing of manuscript.
Extent of contribution by PhD candidate (%)	90

CO-AUTHORS

Name	Nature of Contribution
Jiafa Luo	Collaborated on development of research objectives and protocol. Assisted in data interpretation and provided comments on manuscript.
Louis Schipper	Collaborated on development of research objectives and protocol. Assisted in data interpretation and provided comments on manuscript.

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

Name	Signature	Date
Jiafa Luo		7/5/18
Louis Schipper	L. A. Schipper	2/5/18

July 2015

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Te Whare Wānanga o Waikato

Co-Authorship Form

Postgraduate Studies Office
Student and Academic Services Division
Wahanga Ratonga Mataurange Akonga
The University of Waikato
Private Bag 3105
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Chapter 5 is being considered for submission to a peer reviewed journal. The title of this chapter is "Does urine from brassica fed cows added to soil have lower nitrous oxide emissions than addition of urine from cows fed pasture?"

Nature of contribution by PhD candidate	Collaborated on development of research objectives and protocol. Developed experimental design and set-up trials. Carried out sample collection and the majority of sample analyses. Conducted data analysis and writing of manuscript.
Extent of contribution by PhD candidate (%)	90

CO-AUTHORS

Name	Nature of Contribution
Jiafa Luo	Collaborated on development of research objectives and protocol. Assisted in data interpretation and provided comments on manuscript.
Louis Schipper	Collaborated on development of research objectives and protocol. Assisted in data interpretation and provided comments on manuscript.

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

Name	Signature	Date
Jiafa Luo		8/05/18
Louis Schipper	L A Schipper	8/5/18

July 2015