Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

MANIPULATING SOIL BIOAVAILABLE COPPER AS AN INNOVATIVE NITRATE LEACHING MITIGATING STRATEGY IN NEW ZEALAND PASTORAL SOILS

A thesis presented in partial fulfilment of the
requirements for the degree of

Doctor of Philosophy (PhD)
in
Soil Science

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

School of Agriculture and Environment,
College of Sciences, Massey University
Palmerston North, New Zealand

Dumsane Themba Matse

2023

Abstract

Urine patches are the primary sources of nitrate (NO_3^- -N) leaching from pastoral dairy farms. Since NO_3^- -N is the product of nitrification, a clear understanding of the nitrification process is a vital step toward the development of effective and efficient mitigation approaches. The first step of ammonia (NH_4^+) oxidation to hydroxylamine (NH_2OH) is catalyzed by the ammonia monooxygenase enzyme (AMO), and copper (Cu) is a co-factor in the activity of the AMO enzyme. Therefore, manipulating Cu bioavailability through the application of Cu-complexing organic compounds such as calcium lignosulphonate (LS) and co-poly acrylic-maleic acid (PA-MA) to soil could influence AMO activity and consequently limit the nitrification rate in soil. There are no published studies that have examined the effect of bioavailable Cu concentration changes on nitrification rate, ammonia-oxidizing bacteria (AOB) and archaea (AOA), and NO_3^- -N leaching. The overall aim of this thesis is to determine the significance of bioavailable Cu in the nitrification process in the context of developing novel Cu-complexing organic compounds to inhibit nitrification rate in pastoral soils.

A soil incubation study was conducted to characterize the relationship between changes in soil bioavailable Cu concentration and nitrification rate. This study was conducted using three pastoral soils (Pumice, Pallic, and Recent soils) spiked with five Cu levels (0.1, 0.3, 0.5, 1, and 3 mg kg^{-1}). Treatments of Cu-complexing compounds were separately applied to each Cu level. The treatments were urea applied at 300 mg N kg^{-1}, urea + LS at 120 mg kg^{-1}, and urea + PA-MA at 10 mg kg^{-1}. Results show that increasing the added Cu concentration from 0.1 to 3 mg kg^{-1} increased nitrification rate by 35, 22, and 33% in the Pumice, Pallic, and Recent soils, respectively. Application of LS and PA-MA significantly ($P < 0.05$) decreased nitrification rate with the mean reduction being 59 and 56%, 32 and 26%, and 39 and 38% in the Pumice, Pallic, and Recent soils, respectively at Day 8 relative to the urea-only treatment. To further extend knowledge of the relationship between bioavailable Cu and the key nitrifying microorganisms in soils, a greenhouse-based pot trial using three soils (Pumice, Pallic, and Recent soils) planted with ryegrass and treated with synthetic urine applied at 300 kg N ha^{-1} and three levels of Cu (0, 1, 10, 100 mg added Cu kg^{-1}) was established. Results show that AOB *amoA* gene abundance increased as a function of increasing added Cu from 1 to 10 mg kg^{-1} but was inhibited at 100 mg added Cu kg^{-1} in both Pallic and Recent soils. The effect of bioavailable Cu was not apparent in the Pumice soil. The increase in AOB *amoA* gene abundance positively correlated with nitrification rate in both the Pallic (r = 0.982, $P < 0.01$) and Recent soil (r =

0.943, $P < 0.01$) but not in the Pumice soil. There was no effect of increasing Cu concentration on AOA *amoA* gene abundance in all three soils. Results from both incubation and greenhouse pot trials provide strong evidence that Cu is an important trace element in the nitrification process and reducing Cu can reduce nitrification in soil. However, in order to definitively quantify this treatment effect, further field studies were necessary.

Therefore, a field lysimeter study was conducted using Pumice soil (Manawatu climate) and Pallic soil (Canterbury climate). The following treatments were investigated to reduce NO_3^- -N leaching during late-autumn application; urine only at 600 kg N ha^{-1}, urine + PA-MA at 10 kg ha^{-1}, urine + LS at 120 kg ha^{-1}, urine + a split-application of calcium lignosulphonate (2LS at same rate, initial and after a month of first application), and urine + ProGibb SG (GA at 80 g ha^{-1}) + LS (GA + LS). Another set of treatment applications, urine only, urine + GA only, and urine + GA + LS, were applied mid-winter to both soils. The GA was applied to improve the effectiveness of these organic compounds during climatic periods of poor plant growth. Results showed that there was no significant reduction in mineral N leaching associated with the late-autumn application of both PA-MA and LS for the Pumice or Pallic soils. However, the application of 2LS reduced mineral N leaching by 16 and 11% in Pumice and Pallic soils, respectively, relative to urine-only. The late-autumn inclusion of GA increased the effectiveness of LS in both soils. This was confirmed by a significant reduction of mineral N leaching by 35% from both Pumice and Pallic soils. Mid-winter application of GA + LS significantly reduced mineral N leaching only in the Pumice soil (by 20%) but not in the Pallic soil relative to urine-only. In both late-autumn and mid-winter treatments application of the different Cu-complexing treatments did not have negative effects on pasture dry matter yield in either Pumice or Pallic soils. In this lysimeter study, the mechanistic effect of PA-MA and LS on reducing bioavailable, nitrification rate and AOB/AOA *amoA* gene abundance was not investigated.

A second field lysimeter experiment was established using the Recent soil in Manawatu to explore the mechanism of Cu manipulation through the application of LS and PA-MA on nitrification rate, AOB/AOA *amoA* gene abundance, and mineral N leaching. The effect of combining organic inhibitors with GA on reducing mineral N leaching was also investigated. This study evaluated the same treatments used in the first lysimeter study and applications were again conducted at two different seasonal periods (late-autumn and mid-winter). The results showed that the effect of PA-MA and 2LS on bioavailable Cu corresponded with a reduction in nitrification rate and AOB *amoA* gene abundance. The effect of PA-MA and 2LS was associated with reduced mineral N leaching by values of 16 and 30%, respectively, relative to urine-only. The reduction in mineral N leaching induced by PA-MA and 2LS increased N uptake by 25 and 7.8% and herbage DM yield by factors of 11 and 8%, respectively, relative to the urine-only. The LS treatment did not induce a significant change of either bioavailable Cu or nitrification rate which corresponded to no significant effect on mineral N leaching. The late-autumn combination of GA + LS reduced mineral N leaching by 19% relative to urine-only, but there was no significant difference in mineral N leaching observed for the mid-winter application relative to urine-only.

The overall results of this research show that bioavailable Cu is a vital trace element in the nitrification process and for AOB functioning in soil. Therefore, reduction in bioavailable Cu through the application of Cu-complexing compounds can inhibit nitrification. In this doctoral study, the application of Cu-complexing compounds (LS and PA-MA) showed potential to inhibit nitrification rate and subsequently reduce mineral N leaching in pastoral systems, but their efficacy depends on soil characteristics. Future work is recommended to investigate the effect of LS and PA-MA application on nitrous oxide emissions. Further research is recommended to investigate the short and long terms effects of these treatments on non-target soil microbiota.

Acknowledgements

I am grateful to my supervisory team Dr Paramsothy Jeyakumar, Dr Peter Bishop, and Prof Christopher Anderson for their unconditional and endless support throughout this endeavour. To my main supervisor Dr Paramsothy Jeyakumar, thank you for your guidance, positive attitude, and ability to make this PhD journey memorable and enjoyable at the same time. Thank you for your advice in both academics and personal life. Without your patience and encouragement this would not have been achieved. To my co-supervisor Dr Peter Bishop, thank you for your knowledge, experience, and field support. All your contribution in both laboratory and field work is much appreciated. Thank you for making every challenge look easier. Thank you also to Professor Christopher Anderson for your valuable and insightful comments in shaping my arguments. Your support and encouragement were extra special.

I gratefully acknowledge the New Zealand Ministry of Foreign Affairs and Trade for funding me and making sure my stay in New Zealand is well look after. Thank you to Dr Geoff Bates and Mr Denis Collins for providing resources in conducting my research.

Many thanks to Dr Anja Schiemann, Dr Neha Jha, Dr Keren Dittmer, Dr Matthew Savoian, Ms Lesley Taylor for their technical assistance, fruitful discussions, and allowing me to use their labs. Thank you to the Soil lab technicians Mr Ian Furkert, Mr Bob Toes, Mr Ross Wallace for their technical assistance for my research. I am also grateful to Mrs Sharon Wright and Mrs Fiona Bardell for assistance in administrative issues. I deeply appreciate my office mates and colleagues in the Environmental Sciences Group for their technical assistance, discussions and providing company during my stay in New Zealand even during COVID-19 lockdowns. I am also thankful to Mrs Vaithehi Jeyakumar for her great hospitality.

Thank you to my parents, brothers, and sisters for all emotional support while I was away from home. Finally, I send my sincere thanks to my lovely wife, Lungile Sifundza and my beautiful daughter, Wenzile Sibahle Matse who came at the beginning of this PhD journey. Thank you for your patience and support throughout the journey, being away from both of you wasn't easy but we finally made it through. The long mid-night calls were always giving me encouragement to wake up every morning to push myself. Thank You!!!

Table of contents

Lists of Tables

Lists of Figures

List of Abbreviations

AOA	Ammonia-Oxidizing Archaea
AOB	Ammonia-Oxidizing Bacteria
AMO	Ammonia monooxygenase
ANOVA	Analysis of Variance
CEC	Cation Exchange Capacity
Cu	Copper
DCD	Dicyandiamide
DM	Dry Matter
DMPP	3,4-dimethypyrazole phosphate
DW	Dry Weight
GA	Gibberellic Acid
h	hour
HAO	hydroxylamine oxidoreductase
ICDD	International Centre for Diffraction Data
LS	Calcium Lignosulphonate
MP-AES	Microwave Plasma Atomic Emission Spectroscopy
MPI	Ministry of Primary Industries
N	Nitrogen
NZ	New Zealand
NIWA	National Institute of Water and Atmosphere
PA-MA	Co-poly acrylic-maleic acid
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
WFPS	Water-Filled Pore Space
WHC	Water Holding Capacity

CHAPTER 1

General Introduction

1.1 Background to nitrate leaching in New Zealand pastoral systems

The burgeoning world population has resulted in rapid global demand for dairy products (OECD and FAO 2020) and New Zealand's (NZ) dairy industry is striving to meet global demand (Christensen et al. 2019). The number of dairy cows in NZ has increased by 134% from 2.09 million in 1975/76 to 4.9 million in 2020/21 (DairyNZ 2021). To sustain production, highly intensive grazing systems have been adopted across NZ, based primarily on a pasture mixture of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.). Nitrogen (N) fertiliser is generally applied at rates of 100-190 kg N ha^{-1} yr^{-1} to meet animal foraging demand (Ministry for the Environment 2021). However, approximately 70-95% of the ingested N is excreted in urine resulting in highly concentrated but small areas known as urine patches where the N concentration can range from 200–2000 kg N ha^{-1} (Selbie et al. 2015). This is orders of magnitude higher than the pasture's N requirement (Aarons et al. 2017; Smith et al. 1985). Therefore, the excess N is leached from the soil as nitrate (NO_3^- -N) in drainage water and contaminates both ground and surface water. The increase in NO_3^- -N concentration in water sources can have detrimental effects to both human and animal health. For example, NO_3^- -N higher than 11.2 mg L^{-1} NO_3^- -N in drinking water can result in methaemoglobinaemia in babies and abortions in cattle (Fan and Steinberg 1996). While concentrations of 0.4 mg L^{-1} NO_3^- -N can expedite algal blooms and eutrophication of water bodies (Larned et al. 2016). Therefore, decreasing NO_3^- -N leaching from urine patches is important to lowering the environmental impact of the NZ dairy industry.

In an attempt to reduce NO_3^- -N leaching losses from grazed pastures, different approaches and techniques have been implemented in NZ. These include the application of nitrification inhibitors to soil (e.g.: dicyandiamide (DCD), 3,4-Dimethylpyrazole phosphate (DMPP)), the use of alternative forage species such as plantain (*Plantago lanceolatae* L.) and chicory (*Cichorium intybus* L.), and restricted grazing (Christensen et al. 2019; Di and Cameron 2005a; Di et al. 2016; Mangwe et al. 2019; Nguyen et al. 2022a). Even though the application of inhibitors has proved effective in reducing NO_3^- -N leaching, the discovery of residues in milk and dairy products has limited their application to grazed pastures (Ministry of Primary Industries 2013) and the prevalence of chemical residues in the environment is a major constraint on the use of chemicals in farm systems. While, for chicory and plantain, research conducted by Nguyen et al. (2022b) and Glassey et al. (2013) showed that persistence of these forage species, the pasture sward is significantly reduced after two growing seasons. As a result, they might need re-sowing after every two growing seasons which can be costly to farmers. Therefore, further exploration of alternative strategies to reduce the environmental effects of nitrification are necessary.

Previous studies have shown that the first process of nitrification is facilitated by ammonia oxidizers (Lin et al. 2022; Norton and Ouyang 2019). These ammonia oxidizers are able to perform nitrification because they possess the ammonia monooxygenase enzyme (AMO) encoded in the *amoA* gene (Ayub et al. 2022). The first step of ammonium (NH_4^+ -N) oxidation to hydroxylamine (NH_2OH) in the nitrification process is catalysed by the AMO enzyme (Soler-Jofra et al. 2021). There is literature evidence that copper (Cu) is the main cofactor of the AMO enzyme. For example, in a pure cell culture of *Arithrobacter arilaitensis*, He et al. (2019) demonstrated that by increasing Cu from 0.05 to 0.1 mg L^{-1}, the NH_4^+ -N oxidation and total N removal increased by 11.5 and 8.8% relative to no Cu treatment. Wagner et al. (2018) showed that <0.0015 mg L^{-1} Cu increased NH_4^+ -N oxidation in water treatment plant up to

150% within 2-3 weeks of application. Some studies have identified that Cu deficiency can affect NH_4^+ -N oxidation in wastewater treatments due to the binding of Cu with organic matter in wastewater (Gwak et al. 2020; Koike et al. 2022). Even though Cu has been reported to play a key role in nitrification, no studies have investigated the relationship between the concentration of available Cu (Cu bioavailability) and N cycling in soils and this presents a new strategy that can be explored for NZ farming systems.

Copper bioavailability in soil can be manipulated using a range of Cu-complexing organic compounds that could potentially leave no residues in the soil system. Organic compounds such as calcium lignosulphonate (LS) and co-poly acrylic- maleic acid (PA-MA) are rich in carboxylic, hydroxyl, and phenolic groups that can complex with Cu^{2+} in the soil thus reducing their availability (Feng et al. 2020; Xia et al. 2019; Yang et al. 2020). In literature, there were no studies found to have been conducted in NZ or internationally to explore the applicability, efficiency, and effectiveness of this approach in pastoral systems. However, the main challenge is that NO_3^- -N leaching in NZ grazing systems is generally more severe between late-autumn and mid-winter when plant N uptake and growth is restricted. Therefore, even if inhibitor applications are effective in reducing the oxidation of NH_4^+ -N for that specific period, poor N uptake and plant growth will limit inhibitor effectiveness in reducing the rate of N leaching. To overcome such a challenge, application of an external growth stimulant such as Gibberellic acid (GA) during periods of low plant growth can be explored. Improving N uptake and pasture growth can provide a complimentary mechanism to the inhibition effect by inhibitors in soil.

Research presented in this thesis was therefore guided by the need to develop a sustainable, low-cost approach to reduce NO_3^- -N leaching at field level by manipulating bioavailable Cu in soil and increasing N uptake in periods of low N uptake. The objective of the work is to help NZ agriculture systems to minimize environmental contamination associated with N losses from urine patches, while improving N use efficiency. This will further help dairy farmers to comply with the environmental regulations on water quality.

1.2 Research objectives

The research conducted for this thesis had a clear hypothesis, *that bioavailable Cu in soil is a main limiting factor for nitrification, and that an increase in bioavailable Cu will increase nitrification, while a reduction in bioavailable Cu, induced through the application of Cu-complexing compounds, will reduce nitrification*. *Further, increasing pasture N uptake during periods of low growth can improve the effectiveness of inhibitors in reducing* NO_3^- *-N leaching.*

The specific objectives supporting the hypothesis were:

1) To characterise the relationship between the changes in bioavailable Cu concentration and nitrification rate in dairy-grazed pastural soils.
2) To investigate the influence of bioavailable Cu concentration in soil on the abundance of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA).
3) To determine the effect of Cu-complexing organic compounds on NO_3^- -N leaching in two contrasting soil types under different climatic conditions in a field lysimeter study.
4) To investigate the effect of combining a plant growth stimulant GA with Cu-complexing organic compounds on NO_3^- -N leaching.
5) To examine the effect of reduced bioavailable Cu concentration on nitrification rate, AOB and AOA *amoA* gene abundance and NO_3^- -N leaching under field condition.

1.3 Thesis outline

This thesis consists of seven chapters, the first two Chapters comprise of a general introduction and literature review. Chapters 3-6 are research Chapters prepared as published journal articles. As a result, there might be REPETITIONS IN CONCEPTS AND ANALYTICAL PROCEDURES.

Chapter 1 is the general introduction which provides a general overview of the main research idea including the hypothesis and main objectives of the thesis.

Chapter 2 is a literature review that explores the context of the study with particular focus on (a) the source of NO_3^- -N leaching, (b) soil N cycling relating to urine deposition, (c) existing N loss mitigation strategies, (d) the significance of Cu in the nitrification process, and (e) application of GA as a complimentary treatment to organic inhibitors.

Chapter 3 describes an incubation experiment where three NZ pastoral soils (Pumice, Pallic, and Recent soils) were treated with different Cu concentrations and two Cu-complexing compounds (LS and PA-MA). This experiment aimed to characterise the relationship between bioavailable Cu and nitrification rate in the three soils.

Chapter 4 describes a greenhouse experiment examining the response of AOB and AOA to different Cu concentrations. This Chapter provides further evidence on the relationship between bioavailable Cu and nitrification rate that was elucidated in Chapter 3 in terms of AOB and AOA *amoA* gene abundance.

Chapter 5 explores the potential effect of LS and PA-MA (tested in Chapter 3) on reducing mineral N leaching in the Pumice and Pallic soils under different climatic conditions in a field lysimeter study. A further objective of this Chapter was to investigate the effect of co-treatment application of LS and GA in both late-autumn and mid-winter application.

Chapter 6 describes a controlled study which used Cu-complexing organic compounds (LS and PA-MA) to reduce bioavailable soil Cu concentrations with assessment of the effect on nitrification rate, AOB and AOA *amoA* gene abundance and the resulting NO_3^- -N leaching in a field environment using the Manawatu Recent soil. A further objective was to understand the mechanism of co-treatment of LS and GA in reducing NO_3^- -N leaching.

Chapter 7 outlines the general findings of this research work, the application of the thesis findings to farming systems, and outlines possible future research work that could build on the findings of the current study.

CHAPTER 2

Literature review

This chapter covers a thorough literature survey to address the main objectives of this thesis as presented in Chapter 1. This literature will include:

a) The source of NO_3^- -N in NZ dairy grazed pasture system

b) Soil N cycling relating to urine deposition

c) Existing NO_3^- -N leaching mitigation strategies in NZ and their limitations

d) The significance of Cu in influencing nitrification and how Cu can be manipulated to reduce NO_3^- -N leaching at field scale.

e) The use of plant growth stimulants as a complimentary mechanism to increase plant N uptake.

The reviewed literature on this research provides a thorough understanding of the above topics. This will further help to identify the key knowledge gaps that exist in literature.

2.1 Source of nitrate leaching in New Zealand dairy grazed pasture systems

Legume-ryegrass based pastoral farming is the dominant farming system in NZ dairy grazed pasture systems. To meet dairy cows' food demand, synthetic N fertilisers are applied strategically in split-applications of about 20-50 kg N ha^{-1} during spring, summer, and autumn. In addition to chemical fertilisers, some farmers apply dairy effluent constituting 5% dry matter and 140-670 mg N L^{-1} and their application is normally determined by the soil moisture content at an average rate of approximately 30 m^3 ha^{-1} (Longhurst et al. 2000; van der Weerden et al. 2016).

Apart from direct losses of the applied N fertiliser, the main challenge in grazed pasture systems is that only 5-30% is converted into milk and the rest (70-95%) is excreted in urine and dung (Oenema et al. 2005). The average N content in dung is 2.0-2.8% on dry matter basis and approximately only 20-25% of faecal N is water-soluble (Haynes and Williams 1993). However, one advantage about the N returned into the soil from dung is that: it is slowly available, and plants can utilise this or the N can become immobilised rather than being lost to the environment. The majority of the N (about 70%) returned by the dairy cows is contained in the urine and about 90% of the N in the urine is in the form of urea (Cameron et al. 2013). This urine is deposited in small and more concentrated spots known as urine patches.

2.1.1 Urine patches

Urine patches are small areas in grazed pasture resulting from the deposition of highly concentrated dairy cows' urine. On average, each cow can urinates about 0.30 to 7.83 L $event^{-1}$, averaging 2.1 L $event^{-1}$ (Saggar et al. 2004; Selbie et al. 2015) and each cow is estimated to urinate 9-14.1 events day^{-1} on average (Mangwe et al. 2019; Selbie et al. 2015). The urine N concentration in each urine patch can range between 200 to 2000 kg N ha^{-1}

(Bristow et al. 1992; Bryant et al. 2017; Selbie et al. 2015). For example, Bristow et al. (1992) found that for dairy cattle grazing white clover-ryegrass mixture, the total N concentration in urine can reach 11 g N L^{-1}.

The deposition of highly N concentrated urine onto grazed pastures results in highly localised concentrations of available N, mainly as urea. This often exceed the pasture N requirement which is expected to be between 300 and 700 kg N ha^{-1} yr^{-1}. Thus, urine patches are characterised by dark green pasture compared to other pastures in nearby surroundings. Each dairy cow urine patch has an estimated area of around 0.14-0.49 m^2 $patch^{-1}$ (Moir et al. 2011; Selbie et al. 2015), with an average of 0.24 m^2 $patch^{-1}$. Urine patches cover about 20-30% of the total pasture area per year, depending on the number of cows (Moir et al. 2011).

Figure 2.1 Dark green overgrown pasture represents urine patches in dairy grazed pasture.

Soil N surplus results in the soil when the N loading rate exceeds plant N demand, especially during periods of slow pasture growth and this creates a point source of NO_3^- -N leaching in grazed pasture systems during drainage events. According to Selbie et al. (2015), an average of 18% of the deposited urinary N is lost through NO_3^- -N leaching. However, these losses are highly dependent on many soil factors such as soil moisture, soil pH, soil organic matter content and soil type. The variability of N losses from urine patches also depends on the urine volume and frequency, urine composition and the concentration of N in the urine (Dijkstra et al. 2013; Nguyen et al. 2022a). Urine patches have been identified as the main 'hot spots' of NO_3^- -N leaching in dairy grazed pastoral systems.

2.2 Soil N cycling relating to urine deposition

2.2.1 Urea hydrolysis

About 90% of the excreted N in the urine is in the form of urea. Therefore, upon deposition into the soil, the urea in urine is immediately hydrolysed through soil urease activity to form ammonium carbonate (Gao and Zhao 2022; Mendes et al. 2017). The ammonium carbonate is further hydrolysed by urease enzyme to NH_4^+/NH_3, however some of this NH_3 is lost from soil through volatilisation as NH_3 gas (Zhongqi et al. 2016) (Equation 2.1).

$$(NH_2)_2CO + 2H_2O \rightarrow (NH_4)_2CO_3 \leftrightarrow NH_4^+ + NH_3 \uparrow + CO_2 + OH^- \qquad \textbf{Equation 2.1}$$

Dijkstra et al. (2013) reported that approximately 80% of the urine is hydrolysed within 48 h and hydroxide ions (OH^-) generated during the reaction will cause an increase in soil pH (approximately pH 8) depending on soil type. The increase in pH generally lasts for about 5 days (Gao and Zhao 2022).

2.2.2 Nitrification

Nitrification is known as the biological oxidation of NH_4^+ ions. This is a two-step process mainly carried out by the autotrophic microorganism under aerobic conditions. The first step is a rate-limited step mainly involving ammonia-oxidation. The NH_4^+ ions are first oxidised to NH_2OH facilitated by the Cu dependant AMO enzyme, then from NH_2OH to nitrite (NO_2^-) by the hydroxylamine oxidoreductase (HAO) enzyme (Bozal-Leorri et al. 2022) (Figure 2.2). The first important step of nitrification is mainly driven by the AMO enzyme found in soil AOB such as *Nitrosospira* and *Nitrosomonas* and AOA such as *Nitrosopumilus maritimus* and *Nitrososphaera viennensis* (Hayatsu et al. 2021). In general, NH_4^+ -N concentration and soil pH have been shown as the main factors in environmental differentiation of these oxidisers (Lin et al. 2021). For example, kinetic analysis has shown that AOB have lower affinity to NH_3/NH_4^+ than AOA thus AOB have high dominance in higher NH_4^+ -N conditions (Ouyang et al. 2017; Prosser and Nicol 2012). Further, the AOA have been demonstrated to have higher survival under acidic soil conditions (pH range 4.0 to 5.5) than AOB (He et al. 2020). For example, AOA *Nitrosotalea devanaterra* is acidophilic and will completely stop functioning under neutral and alkaline conditions (Lehtovirta-Morley et al. 2011).

The second process involves the conversion of NO_2^- to NO_3^- by nitrite-oxidizing bacteria (NOB) (Figure 2.2). This process is catalysed by the nitrite oxidoreductase (NOX) enzyme (Wrage et al. 2001), and this reaction happens very fast. As a result, NO_2^- does not often accumulate in the soil (Cameron et al. 2013). In general, the nitrification process occurs within 14-29 days of urine deposition. However, this process is highly dependent on several soil factors such as soil pH, temperature, moisture and aeration, and soil organic matter content (Sahrawat 2008; Zhang et al. 2023).

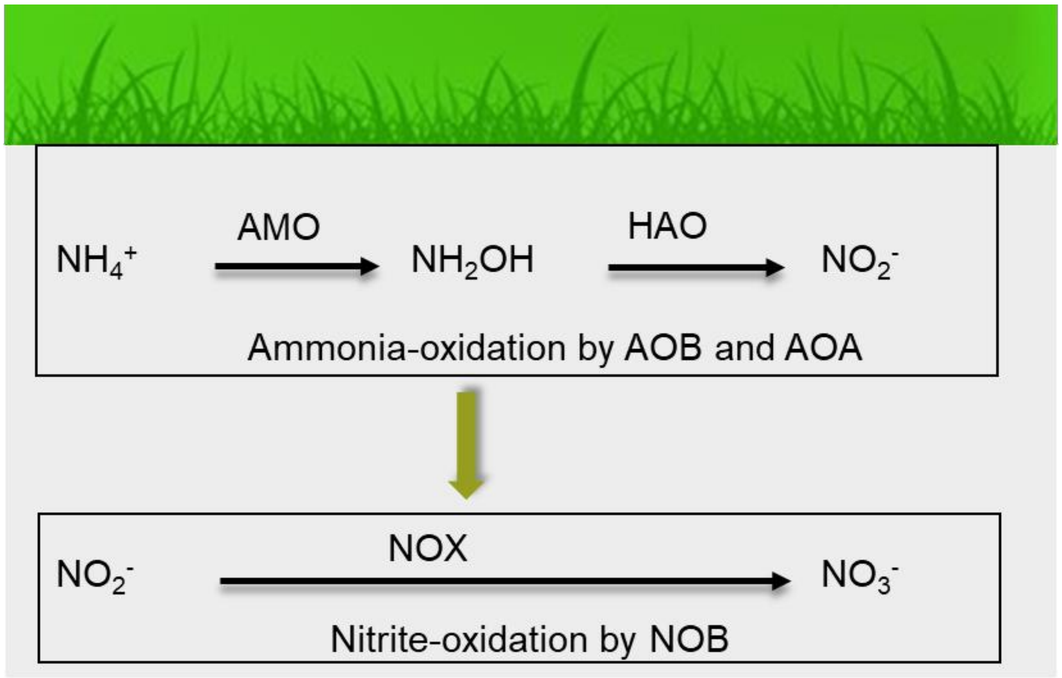


Figure 2.2 The two step process of the nitrification. AOB: ammonia oxidizing bacteria; AOA: ammonia oxidizing archaea; NOB: nitrite oxidizing bacteria; AMO: ammonia monooxygenase enzyme; HAO: hydroxylamine oxidoreductase enzyme; NOX: nitrite oxidoreductase.

2.2.3 Nitrate leaching studies in New Zealand grazed pastural systems

The predominant form of N leached from the soil is NO_3^- -N, and this is because NO_3^- -N is a negatively charged ion, which is repelled by the net negative charge of most soils, implying that it does not bind to the soil. On the other hand, NH_4^+ -N is a positively charged ion, held electrostatically by the negatively charged clay surfaces and functional groups of organic matter (Cameron et al. 2013). In dairy systems most of the NO_3^- -N is leached from urine patches. Field lysimeter studies have shown that application of urine at 600 to 1000 kg N ha^{-1} to mixed ryegrass-white clover pasture will increase autumn NO_3^- -N leaching in the range of 85 to 500 kg N ha^{-1} (Bishop and Jeyakumar 2021; Di and Cameron 2012; Malcolm et al. 2014) (Table 2.1).The magnitude of reported leaching indicates that there is an urgent need for research to find ways to mitigate NO_3^- -N losses from soil NZ grazed system.

Table 2.1 Reported NO_3^- -N leaching from individual urine application conducted in lysimeter studies of ryegrass-white clover pastures.

Urine rate (kg N ha^{-1})	Application period	Soil type/place	Drainage (mm)	NO_3^- -N leaching (kg N ha^{-1})	Reference
600	Late-autumn	Lismore stony silt loam/Hororata	115	146	(Matse et al. 2022b)
600	Late-autumn	Immature orthic pumice soil/Palmerston North	121	290	(Bishop and Jeyakumar 2021)
600	Late-autumn	Lismore stony silt loam/Canterbury	85	321	
775	Autumn	Karatau loamy sand/Taupo	348	114	(Menneer et al. 2008)
1000	Autumn	Templeton fine sandy soil/Lincoln	430-500	300-416	(Malcolm et al. 2014)
664	Autumn	Templeton sandy loam/Lincoln	400-450	113	(Woods et al. 2018)
700	Autumn	Templeton sandy loam/ Lincoln	400-450	113	
1000	Autumn	Templeton fine sandy loam/Lincoln	550	629	(Di and Cameron 2012)

Nitrate leaching is generally high during the late-autumn, winter and early spring months when the plant uptake of NO_3^- -N is low due to cooler conditions, and when drainage is occurring from soil due to water inputs exceeding the evapotranspiration demand (Cameron et al. 2013; Wild and Cameron 1980). Dry summers in NZ can result in high NO_3^- -N accumulation in the soil surface due to low plant growth and low N uptake, and this NO_3^- -N can be leached over the subsequent winter (Stout et al. 2000). Di et al. (1999) reported that application of 200 kg N ha^{-1} ammonium fertiliser (NH_4Cl) in autumn resulted in N leaching of 15-19% of input compared to 8-11% when applied in spring. A lysimeter study conducted by Bjorneberg et al. (1996) on grass/clover (*Lolium perennel/Trifolium repens*) mixtures under sandy soils revealed that the application of urea at 400 kg N ha^{-1} together with urine at 1000 kg N ha^{-1}, increased the NO_3^- -N leaching from 6 kg N ha^{-1} to 120 kg N ha^{-1} yr^{-1} when the seasons changed from summer to winter. In a study conducted by Wilcock et al. (1999), the total yield of N in the Toenepi catchment in the Waikato region increased from 0 to 35 kg ha^{-1} yr^{-1} when the seasons moved from summer to winter. This was a result of high-density animals depositing high amounts of urine during the dry summer that leached into streams during heavy winter rainfalls. The percentage of leached NO_3^- -N in urine patches depends on several factors such as soil type, plant N uptake, soil temperature, amount of accumulated N in the soil (Bishop and Jeyakumar 2021; Di and Cameron 2002a; Wei et al. 2021).

2.3 Environmental and health impacts of nitrate leaching from urine patches

Nitrate leaching from urine patches in dairy pastoral systems is a serious environmental problem. In NZ, agricultural intensification over the last decades has significantly contributed to environmental issues such as NO_3^- -N leaching. In NZ, the dairy industry is the main contributor to NO_3^- -N leaching through urine patches. Leaching of NO_3^- -N from these urine patches results to contamination of lakes, streams, rivers, and groundwater. According to the World Health Organization (WHO) and NZ standards, a NO_3^- -N concentration higher than 11.3 mg NO_3^- -N L^{-1} in drinking water is hazardous to human and animal health. Higher NO_3^- -N concentration in drinking water can lead to metamoglobinaea (blue baby syndrome) in babies and higher susceptibility to colorectal cancer (Richards et al. 2021). Apart from human health, concentrations of 0.4 mg NO_3^- -N L^{-1} can also contribute to algal blooms and eutrophication in water bodies. This can have severe effects on water quality and freshwater ecology.

2.4 Key existing nitrate leaching mitigation strategies

In the last few decades, different approaches have been developed and implemented to minimise N losses from grazed pastures. The dairy industry has been actively involved in implementing best practices and guidelines to try to reduce the environmental threat posed by N losses.

2.4.1 Nitrification inhibitors

Nitrification inhibitors are compounds which have the ability to reduce the conversion of NH_4^+ -N to NO_3^- -N in applied urine patches. The mechanism for nitrification inhibitors in reducing N losses has been associated with complete blocking of the active site of the AMO enzyme or by chelating with the Cu in soil solution thus reducing the substrate for the enzyme AMO, or by completely killing the soil microbes responsible for the nitrification process.

Different compounds have been used and shown to have potential to inhibit nitrification, however, only a few products are commercially available. Inhibitors such as [1]DMPP, [2]DCD, and Nitrapyrin have been extensively applied and reviewed (Adhikari et al. 2021; Di and Cameron 2012).

Zaman and Blennerhassett (2010) conducted a field lysimeter study in a Paparua silt loam soil under perennial ryegrass-white clover, applied with 600 kg N ha^{-1} urine during 2007-2008, and they found that application of DCD at 10 kg ha^{-1} significantly reduced NO_3^- -N leaching by 55%, and 10% in autumn and spring, respectively. Di and Cameron (2002b) reported that the application of urine at 1000 kg N ha^{-1} and DCD at 15 kg ha^{-1} significantly decreased NO_3^- -N leaching by 42% and 76% in spring and autumn, respectively. Cameron et al. (2014),

[1] 3,4-Dimethylpyrazole phosphate
[2] Dicyandiamide

investigated the effectiveness of applying DCD between April to July in a 3-year trial at a rate of 10 kg ha^{-1} to reduce NO_3^- -N leaching from Templeton silt loam soil in Canterbury which received 700 kg N ha^{-1} synthetic urine. They reported that DCD reduced the NO_3^- -N leaching by 48-69%. A field experiment conducted by McDowell and Houlbrooke (2009) showed that application of DCD at 15 kg ha^{-1} to triticale winter forage crop which received 300 kg N ha^{-1} as urine in May 2007 to May 2008, significantly reduced ($P < 0.05$) NO_3^- -N leaching by 39%.

In a field lysimeter study conducted by Di and Cameron (2012) in Waikato Horitiu silt loam soil under mixed perennial ryegrass and white clover, applied with 1000 kg N ha^{-1} during late-autumn, they reported that application of DMPP at 5 kg ha^{-1} significantly reduced NO_3^- -N leaching by 28% in late-autumn. Bishop and Jeyakumar (2021) reported that application of DMPP at 1.5 and 6 kg ha^{-1} reduced NO_3^- -N leaching by 18 and 32% in Wairakei Pumice soil and by 13 and 29% in Stony Lismore soil in late-autumn, respectively.

Even though inhibitors have proved effective and efficient in reducing N losses, the discovery of residual concentrations in the environment have limited their use. In 2012, around 19% of manufactured skim milk powder was detected with DCD residues (Ministry of Primary Industries 2013). Despite being there no international Codex limit for DCD residues in milk, companies voluntarily withdrew sales of DCD in 2013 to ensure no detectable residues in milk. Since the withdrawal of DCD sales in NZ, there has been a pause in the research and development of new products to reduce NO_3^- -N leaching. Further, the unintended consequences of inhibitors such as DMPP and Nitrapyrin in soils, plants, animals systems, animal products and surrounding environments remain unknown (Adhikari et al. 2021) and these unknown consequences have limited their adoption.

2.4.2 Use of alternative forages

Researchers have highlighted the potential of certain plants such as Plantain (*Plantago lanceolata*) and Chicory (*Cichorium intybus*) to reduce N losses from livestock systems by several potential mechanisms, including: a reduction in excreta N concentration output (Bryant et al. 2017), and/ or through affecting nitrifier activity in the soil through secretion of plant exudates (Gardiner et al. 2018). Carlton et al. (2019) reported that inclusion of 30% plantain to perennial ryegrass white clover mixture grown in Paparua fine sandy loam soil, decreased NO_3^- -N leaching by 82%, and 74% in December (summer) and February (late summer), respectively compared to 100% perennial ryegrass-white clover mixture (700 kg N ha^{-1} applied N concentration). Box et al. (2017) found that cows fed 100% plantain in late autumn 2015 and early spring 2015 excreted less urinary N concentration compared to cows feeding only pure perennial-white clover mixture. The authors stated that the reduction in urinary N concentration resulted in a 55 and 53% reduction in NO_3^- -N leaching in late autumn and early spring, respectively. On the other hand, Mangwe et al. (2019) demonstrated that dairy cows grazing 100% chicory in summer reduced urinary N concentration by 3.7-fold relative to cows grazing perennial ryegrass-white clover mixture. In an indoor feeding study conducted by Minneé et al. (2017) feeding dairy cattle a diet comprising of 40% chicory daily (dry matter intake) reduced urine N concentration by 38% relative to feeding perennial ryegrass-white clover only. Ledgard et al. (2015) reported that reduction in urine N concentration by 50% can correspond with a 65% reduction in N leaching rates.

While previous studies have shown the benefits of forage crops in reducing NO_3^- -N loss, a detailed study conducted by Stafford et al. (2016) describes how chicory and plantain can accumulate a higher Cd concentration when compared to other species. These authors found that chicory and plantain had a mean Cd concentration of 1.82 mg Cd kg^{-1} and 0.80 mg Cd

kg^{-1}, respectively, which was significantly higher than 0.103 mg kg^{-1}, and 0.035 mg kg^{-1} accumulated by ryegrass and white clover, respectively. A drawback to these two species is the potential health risk they pose through the increased accumulation of Cd in the kidneys and livers of livestock and their subsequent entry into the food chain. Further, Nguyen et al. (2022b) and Glassey et al. (2013) demonstrated that the total pasture content of plantain and chicory significantly declines after two growing seasons. As an adaptation strategy, a review by Li and Kemp (2005) suggested that farmers may need to re-establish pasture after two growing seasons. Adoption of this management practice can be costly to farmers.

2.4.3 Restricted grazing

The objective of restricted grazing is to reduce the time spent by the animals grazing outside (Schils et al. 2006) to decrease dung and urine deposition into the soil (Ledgard et al. 2006; Luo et al. 2006). Collected excreta is instead collected from hard surfaces and then applied out to the pasture at targeted rates and optimum time when the risk for N losses is minimal.

Christensen et al. (2019) reported allowing dairy cows to graze perennial ryegrass-white clover mixture for only 4 h during the day and night. This significantly reduced ($P < 0.05$) NO_3^- -N leaching (52%) when compared to allowing them to feed 7 h a day and 12 h per night on a Pallic soil. De Klein et al. (2006) found that allowing dairy cows (2.7 cows ha^{-1}) to graze perennial ryegrass-white clover mixture for only 3 h per day significantly reduced ($P < 0.05$) NO_3^- -N leaching (40%) compared to when animals graze for 24 h during winter/autumn on Pukemutu Mottled Fragic Pallic soil.

However, this system needs high financial investment in the building of infrastructure and buying supplementing feed, and widespread adoption would increase the cost of milk production in NZ (Laven and Holmes 2008). Furthermore, the management of the large quantities of farm effluent and manure in these systems is a challenge because approximately

80 % of NH_3 emissions from agriculture are produced from effluent stores (Anderson et al. 2003).

2.4.4 Other strategies

Several other studies have been conducted on different techniques such as:

- Adding salts like sodium chloride to cows' diet to increase water intake which is believed to increase the frequency of urination. This leads to deposit more urine patches with low N loading rate due to the dilution effect of urinary N (Ledgard et al. 2015; Spek et al. 2012).
- Development of devices to spread urine as animals urinate to prevent the deposition of highly concentrated urine in patches (J. Hanly and M. Hedges, personal communication, 09 December 2022).

However, all these studies are still under research, therefore there is a need to develop innovative and practical techniques that farmers can use to effectively mitigate the NO_3^- -N leaching.

2.5 Significance of Cu in the nitrification process

Copper is one essential trace element needed for enzyme functioning in the nitrification process (Wagner et al. 2019; Zhao et al. 2022). Ammonia monooxygenase enzyme encoded in the *amoA* gene of the AOB/AOA is responsible for the first process of NH_4^+ -N oxidation into NH_2OH (Ayub et al. 2022; Musiani et al. 2020). Two lines of evidence suggest that Cu is the main cofactor of the AMO enzyme. Firstly, the presence of Cu-reducing compounds inhibits nitrification by reducing AMO activity, and secondly, addition of Cu to a Cu deficient environment increases the AMO activity in pure cell culture. For example, Gwak et al. (2020)

reported that application of organic amendments into soil that can complex with Cu hinders nitrification, and further stated that this could be mitigated through the application of Cu containing amendments such as copper sulphate. Vandevivere et al. (1998) found that the presence of small amounts of Cu ligands in sewage significantly reduced the oxidation of NH_4^+ -N in sewage, which might be due to the complexation reaction of Cu in AMO with the ligands.

In an *in-vitro* experiment Ensign et al. (1993) investigated the effect of Cu addition on the function of the AMO enzyme produced by the nitrifying bacterium *Nitrosomonas europaea*. They reported that the addition of 0.13 mg ml^{-1} $CuCl_2$ to cell extracts resulted in stimulation of both ammonia dependant O_2 consumption and NO_2^- production by 5-to-15 fold. Wagner et al. (2016) found that addition of 5 μg Cu L^{-1} resulted to 14-fold NH_4^+ -N oxidation increase within 57 days of application. Furthermore, Wagner et al. (2019) reported that an initial NH_4^+ effluent concentration of 0.18 mg NH_4^+ -N L^{-1} in water treatment sand filters was significantly decreased to 0.02 mg NH_4^+ -N L^{-1} within 20 days after addition of a 1 μg Cu L^{-1} dose. However, all these studies are related to the application of Cu to water not soil.

In an analysis of concentration of Cu application, there is no exact range evidenced in the literature which exclusively stimulates or inhibits nitrification (Table 2.2). For example, Tomlinson et al. (1966), found that 4 mg Cu L^{-1} gave approximately 75% inhibition of NH_4^+ -N oxidation in pure culture of *Nitrosomonas europaea*, however, approximately 200 mg Cu L^{-1} was required to achieve the same effect in nitrifying activated sludge. At present, no studies have been conducted to analyse the effect of adding Cu-complexing organic material to reduce Cu bioavailability and subsequently reduce the nitrification process in agriculture.

Table 2.2 The effect of different Cu concentration on nitrification rate and activity of nitrifying bacteria.

Cu Concentration range (mg L^{-1})	Effect of Cu on nitrification and AMO function	Reference
0 - 0.56	In the presence of 0.06 mg Cu L^{-1}, the pure cell culture of *Nitrosomonas europaea* Increased nitrite production by 91 % compared to control.	(Loveless and Painter 1968)
0 - 0.006	Increasing Cu to less than 0.005 mg Cu L^{-1} significantly increased nitrification by approximately 14-folds	(Wagner et al. 2016)
0 - 0.5	Increasing Cu concentration from 0.05 to 0.1 mg Cu L^{-1} in cell culture of *Arthrobacter arilaitensis* (strain Y-10) significantly increased, • ammonia oxidation by 11.51% • Total nitrogen removal by 8.8% • Above 0.25 mg Cu L^{-1} inhibited growth	(He et al. 2019)
0 - 10	Increasing Cu from 0 to 10 mg Cu L^{-1} in cell culture of *Cupriavidus sp.*S1 reduced nitrification rate by 33.69%.	(Sun et al. 2016)
Extractable Cu 0 – 6	In Cecil limed soil, • 1 mg extractable Cu L^{-1} stimulated soil nitrification compared to control. • Further increase in extractable Cu inhibited nitrification.	(Cela and Sumner 2002)
0.1	*Nitrosomonas europaea* in pure cell culture increased nitrification by approximately 10% compared to control.	(Tomlinson et al. 1966)
4	*Nitrosomonas europaea* in pure cell culture inhibited nitrification by approximately 75% relative to control.	(Tomlinson et al. 1966)
200	*Nitrosomonas europaea* in sludge inhibited nitrification by 75%.	(Tomlinson et al. 1966)

Note: Cu concentration refers to added Cu unless specified.

2.6 Manipulating bioavailable Cu as an alternative strategy to reduce nitrification

Several incubation and *in-vitro* studies in literature have established that Cu is an important element in the nitrification process. However, there are no field studies which have been conducted to assess how changes in bioavailable Cu in soil can influence nitrification rate. There is a clear need to establish studies that explore how reducing bioavailable Cu can affect the functioning of the nitrifying microorganisms in soil. Reducing bioavailable Cu can reduce the available Cu for the AMO activity which is responsible for the first step thus inhibiting the oxidation of NH_4^+ -N to NO_3^- -N. However, the effectiveness and efficiency of this technique highly depends on the Cu-reducing amendment applied functional groups. Functional groups are a specific group of atoms or bonds within compound that is for the characteristic's chemical reactions of that compound (Blondel 2003). Therefore, amendments with a high number of functional groups can be more effective than compounds with less functional groups and which are highly degradable in soil. No study in literature was found to have tested this potential technique of reducing bioavailable Cu to inhibit nitrification. This could be a potential cost-effective strategy for reducing NO_3^- -N leaching in dairy systems.

Several amendments in literature have been discussed to reduce Cu bioavailability in soil. Synthetic compounds such as Ethylenediaminetetraacetic (EDTA) and Diethylenetriamine pentaacetate (DTPA) have been widely studied and applied (Chen et al. 2022). These chemical compounds can react with Cu in soil and form stable complexes with Cu (Lv et al. 2018). Several studies have demonstrated that these compounds can efficiently reduce metal concentration in soil. However, they are extremely expensive to be used in large quantities (Allen and Chen 1993). Furthermore, they have long term soil residual effects which can alter soil properties (Zupanc et al. 2014), soil microbial community (Kaurin et al. 2021), and soil mineral composition (Guo et al. 2019; Zhang et al. 2013). Therefore, this review focusses on potential organic amendments that can be applicable in agriculture with less residual effect or without causing detrimental effects to plant growth.

2.7 Cu-complexing compounds

Copper-complexing organic compounds consists of different functional groups such as carboxyl (-COOH), hydroxyl (-OH) and phenol which can form stable complexes with metals ions in the soil (Yan et al. 2013). Humic and fulvic acid are the two most studied organic compounds in agricultural systems (He et al. 2016).

Soler-Rovira et al. (2010) found that the Cu-complexing ability of humic acid increased with an increase in functional groups and emphasised that acidic functional moieties such as N, S and O in humic acid play an important role in Cu behaviour. Li et al. (2010) studied the adsorption of Cu and Zn from aqueous solution to humic acid and found that Cu adsorption greatly increased by 15.7 % when compared to the control after 3 h of incubation with 4 mg L^{-1} of humic acid (pH 5) at 20 °C. Ali et al. (2015) studied the effects of fulvic acid application on the alleviation of chromium (Cr) toxicity in wheat plants grown in sand and soil pots. They found that after 4 months, addition of fulvic at 1.5 mg L^{-1} concentration significantly (P <0.05) reduced the Cr concentration in all parts of wheat when compared to non-treated soils.

The Cu-complexing compounds formed through polymerisation of low molecular weight organic acids have also been used to reduce Cu bioavailability in soil. Qiu and Mao (2013) found that the removal efficiency of Cu^{2+} from an aqueous solution was increased as much as 98% by using the copolymer of maleic acid and acrylic acid (PMA-100) at 10 mg L^{-1} concentration. The authors observed that the binding affinity of PMA-100 with trace metal ions decreased in the order $Cu^{2+} > Zn^{2+} > Ni^{2+} > Mn^{2+}$, indicating that Cu^{2+} has the highest affinity towards PMA-100. Xia et al. (2019) compared the removal efficiencies of carboxy-alkylthiosuccinic acid (CETSA), a co-polymer of maleic acid and acrylic acid (MA/AA) and ethylenediamine tetraacetic acid (EDTA) in the amelioration of Cd^{2+}, Pb^{2+}, and Zn^{2+} toxicity at different application concentrations. They found CETSA to show the greatest efficiency of the three absorbents in reducing heavy metal at a concentration of 100 mg L^{-1}. Suanon et al. (2016) reported that application of N, N-Bis (Carboxymethyl) glutamic acid at a concentration of 100 mg L^{-1} resulted in free Cu ion removal efficiency of 81.2% from sewage sludge at equilibrium (pH=3.3). These studies provide evidence that the application of organic compounds can influence the availability of Cu in the soil.

2.7.1 Possible unintended consequences of using organic compounds

The following consequences might result from the application of organic compounds:

1) Copper deficiency in NZ soils is an existing concern and farmers spend money on providing livestock with Cu supplements. Therefore, reducing bioavailable Cu in the soil might further worsen the Cu deficiency in pasture.
2) Applied organic compounds might complex with other micronutrients in the soil thus reducing plant growth, pasture nutrition uptake, and pasture yield.
3) These organic compounds might have residual toxic effects on plant growth and the microbial community in the soil which might affect nutrient cycling in the soil.

2.8 Gibberellic acid

Since the NO_3^- -N leaching is a serious issue during periods of low plant N uptake and growth due to cooler temperatures, there is a need to complement the effect of inhibitors in pasture systems. Some studies have suggested that exogenous application of a plant growth stimulant, GA, can help to increase plant N uptake in periods of low plant growth (Matthew et al. 2009;

Parsons et al. 2013). Gibberellic acid is a plant stimulant first discovered in Japan (1935) and has been identified to be responsible for plant stem and leaves elongation (Matthew et al. 2009). Several studies have shown increased growth and dry matter accumulation induced by GA application in pasture (Table 2.3). For example, the application of GA at 20 g GA ha^{-1} increased pasture DM yield by 13-107% relative to control in five different experimental sites (Zaman et al. 2014). Parsons et al. (2013) and Zaman et al. (2014) have reported that the application of GA is more effective when applied in cooler temperatures than in summer periods.

Although several studies have demonstrated the potential of GA to increase plant growth, there are no studies that have investigated the use of GA as a complimentary plant stimulant with Cu-complexing compounds used to reduce NO_3^- -N leaching in pasture systems. This lack of information in literature presents an opportunity for the current research study to explore how GA can be applied to improve the potential of organic compounds to reduce NO_3^- -N leaching in pastoral systems.

Table 2.3 Effect of GA application on pasture growth.

Pasture	Soil type (Place)	GA rate (g GA ha^{-1})	Effect of GA on pasture DM yield relative to control (% increase)	Reference
Perennial ryegrass-white clover mixture	Wakanui silt loam (Canterbury)	8	45-74	(Bryant et al. 2016)
Perennial ryegrass-white clover mixture	Silt loam (Lincoln)	20	18.31	(Zaman et al. 2014)
	Lismore Silt loam (Ashburton)	20	106.67	
	Pukemutu Pallic soil (Alexandra)	20	31.69	
	Whakapara silt loam (Whangarei)	20	24.92	
	Brunt silt loam (Matamata)	20	12.96	
Perennial ryegrass-white clover mixture	Paparua sandy loam (Canterbury)	24	39	(van Rossum et al. 2013)

2.9 Summary and knowledge gaps

Literature evidence shows that urine patches in dairy-grazed pasture soils are the main factor of NO_3^- -N leaching and a source of serious environmental problems such as water pollution. Even though several mitigation strategies have been applied to reduce NO_3^- -N leaching from urine patches, there is no fully adopted strategy implemented at the field level. Therefore, there is still an urgent need to underpin new strategies that can be adopted by farmers at the field level to reduce NO_3^- -N leaching while also increasing their production. This literature survey indicated that NO_3^- -N is a product of the nitrification process, initiated by the oxidation of NH_4^+ -N to NH_2OH. Previous studies have demonstrated that this first step of NH_4^+ -N oxidation is catalysed by the AMO enzyme and Cu is the main co-factor in its functioning. However, studies have only analysed the effect of Cu on nitrification in water treatment plants, not in soils. Specifically, no studies have investigated the significance of Cu in influencing the nitrification process and the AOB/AOA *amoA* genes in NZ pastoral soils. The literature review also identified a need to characterise the relationship between Cu and the nitrification process in soils.

Since Cu is a critical element for the functioning of the AMO enzyme, the complexation of Cu with Cu-complexing organic compounds in soil may limit NH_4^+ -N oxidation. Available literature suggests that the presence of Cu ligands in water treatment plants has reduced NH_4^+ oxidation. However, no studies have explored this strategy of applying Cu-complexing compounds to limit nitrification rate in agricultural systems. Literature has demonstrated that several organic compounds can be used to reduce bioavailable Cu in soils. Among these, the application of compounds with low-cost and no or low residual effects, such as LS and PA-MA, is more desirable. Therefore, LS and PA-MA can be applied to the soil as Cu-complexing compounds. However, there is no study found in the literature investigating the effect of reducing bioavailable Cu through the application of LS and PA-MA as a strategy to minimise NO_3^- -N leaching in urine patches.

The review of literature has also shown that the effectiveness of inhibitors in the soil is reduced by poor plant growth due to cool winter temperatures. The reduction in plant N uptake results in available N in the soil which becomes susceptible to leaching during drainage events. To overcome this challenge, literature shows that the application of a plant stimulant such as GA could enhance plant N uptake even under cool temperatures. However, no study has examined the co-treatment application of GA with organic compounds as a complementary strategy in terms of reducing NO_3^- -N leaching. It is therefore important to investigate the effect of combining GA and organic compounds to achieve effective N use efficiency.

2.10 Research questions

The literature survey has identified key knowledge gaps that limit understanding of how manipulating bioavailable Cu can be applied as a strategy to reduce NO_3^- -N leaching in dairy pastoral systems. The overall aim of this research is to investigate the significance of Cu in the nitrification process and determine how manipulating bioavailable Cu through the application of LS and PA-MA can influence the nitrification process and the abundance of ammonia-oxidizing microorganisms in soil. The research work in this PhD thesis has been designed to address three identified knowledge gaps.

The first knowledge gap is lack of understanding in the relationship between bioavailable Cu, nitrification rate, and the AOB/AOA *amoA* gene abundance in the soil. The following specific questions have been identified to address this research gap:

1. How does increasing bioavailable Cu in soil influence the nitrification rate in soil?
2. Do soil characteristics influence bioavailable Cu concentration?

3. Does increasing bioavailable Cu in soil influence the AOB/AOA *amoA* gene abundance?
4. Which ammonia-oxidizing microorganisms are impacted by increasing bioavailable Cu in soil?
5. Does the application of LS and PA-MA reduce bioavailable Cu in soil?
6. Does bioavailable Cu reduction influence the nitrification rate and AOB/AOA *amoA* gene abundance in soil?

The second knowledge gap relates to the need to investigate the effect of reducing bioavailable Cu concentration as a strategy to minimise NO_3^- -N leaching in urine patches. The following questions have been identified to understand this research gap.

1. Can the application of LS and PA-MA reduce bioavailable Cu in a field environment?
2. Can the reduction in bioavailable Cu have an impact on the nitrification rate and AOB/AOA *amoA* gene abundance under field conditions?
3. Does the reduction in bioavailable Cu by LS and PA-MA correspond with a reduction in NO_3^- -N leaching under field conditions?
4. Is the effect of LS and PA-MA on reducing NO_3^- -N leaching the same under different soils and climatic conditions?
5. What is the effect of LS and PA-MA on pasture growth?

The third knowledge gap is the limited studies that investigated the co-treatment application of GA with LS as a complementary strategy to enhance the effectiveness of LS in reducing NO_3^- -N leaching. The following questions have been identified to address this research gap:

1. Does the external application of a plant stimulant increase pasture N uptake?
2. Is there any difference in pasture N uptake between GA and LS combined compared to a single application of either GA or LS?

3. In which season does the combination of GA and LS perform exceptionally well?
4. How does the combination of GA and LS perform in different soils and climatic conditions?

The experimental Chapters in this research have been executed to provide more understanding of the above research gaps and further explore possible answers (where possible) to the specific questions.

CHAPTER 3

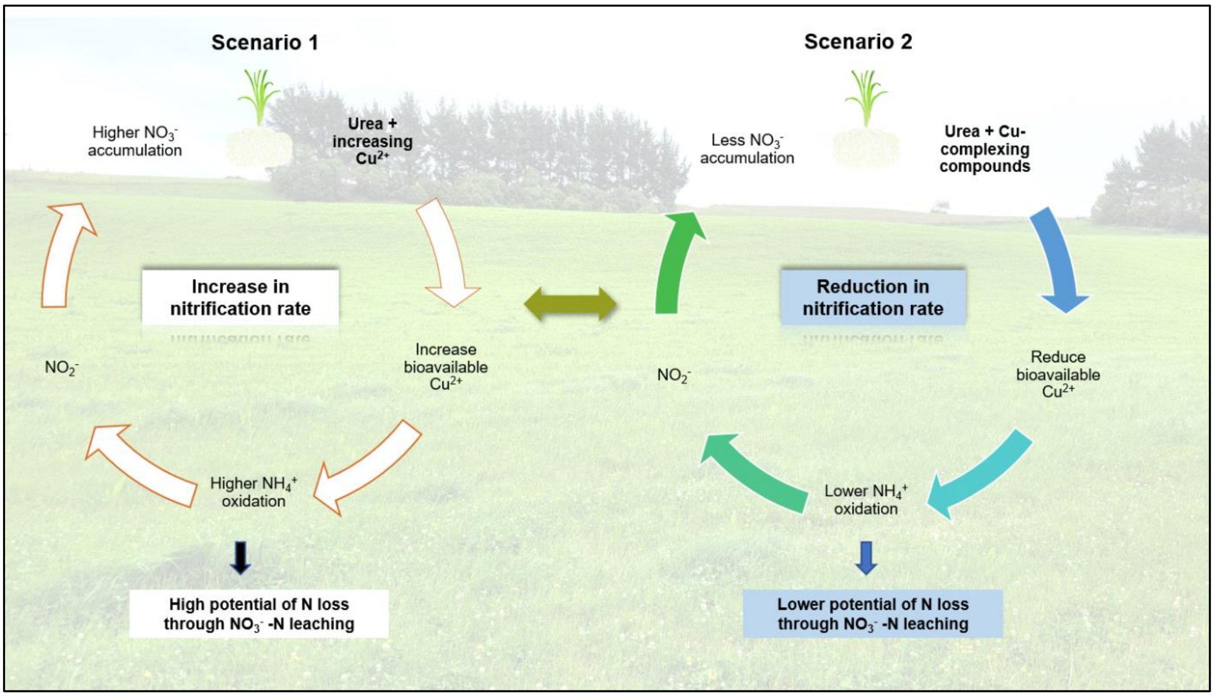
Bioavailable Cu can influence nitrification rate in New Zealand dairy farm soils

*This chapter was published in the **Journal of Soils and Sediments** in 2021. Citation:*

Matse, D.T., Jeyakumar, P., Bishop, P., and Anderson, C. W. N., (2022). Bioavailable Copper can influence nitrification rate in New Zealand farm soils. Journal of Soils and Sediments. 22(3), 916-930. doi: 10.1007/s11368-021-03113-8.

The literature survey in Chapter 2 demonstrated that the AMO enzyme is responsible for the first step of NH_4^+ -N oxidation to NH_2OH, and Cu is the main-cofactor in AMO enzyme activity. However, there is limited literature that explains the relationship between Cu and nitrification rate in agricultural soils. Therefore, Chapter 3 aims to characterise the relationship between bioavailable Cu concentration and nitrification rate in different soil types. Manipulating soil bioavailable Cu concentration using different organic compounds is also examined in this Chapter.

Graphical abstract



Abstract

The AMO enzyme catalyses the first step of NH_4^+ oxidation into NH_2OH, and Cu is the main co-factor in its functioning. It is hypothesised that bioavailable Cu in soil is a limiting factor for nitrification and that an increase in bioavailable Cu will increase nitrification, while a reduction in bioavailable Cu, induced through the application of Cu-complexing compounds, will reduce nitrification. Hence, this study aimed to characterise the relationship between bioavailable Cu concentration and nitrification rate in dairy grazed pasture systems. An incubation study was undertaken using three contrasting soils (Pumice, Pallic, and Recent soils) and organic compounds. The three soils were spiked with five levels of Cu (0.1, 0.3, 0.5, 1, and 3 mg kg^{-1}) and incubated for 14 days before amendment with treatments. Four treatments were separately applied to each Cu level: urea only (300 mg N kg^{-1}), dicyandiamide (DCD) (10 mg kg^{-1}) + urea, calcium lignosulphonate (LS) (120 mg kg^{-1}) + urea, and co-poly acrylic-maleic acid (PA-MA) (10 mg kg^{-1}) + urea. After 4 and 8 Days of incubation, the soil was extracted and analysed for bioavailable Cu and mineral N (NO_3^- -N, NH_4^+ -N) to establish the effect of bioavailable Cu on nitrification rate. The dose response of nitrification rate to Cu was also computed. The nitrification rates at Day 8 significantly ($P < 0.05$) increased by 35, 22, and 33% in the Pumice, Pallic, and Recent soils, respectively when the soil added Cu increased from 0.1 to 3 mg kg^{-1}. Application of LS and PA-MA significantly ($P < 0.05$) decreased nitrification rate with the mean reduction being 59 and 56%, 32 and 26%, and 39 and 38% in the Pumice, Pallic, and Recent soils, respectively at Day 8 relative to the urea-only treatment. These results indicated that increasing bioavailable Cu significantly increased nitrification rate and suggested that the application of Cu-complexing compounds to soil could potentially mitigate the positive effect of soil Cu on nitrification rate and underpin new advances in the development of nitrification inhibitors.

Key words: Nitrification; Cu-complexing; Bioavailable Cu; Organic compounds.

3.1 Introduction

New Zealand dairy production is based on a pastoral system where animals graze N-rich pastures mainly composed of white clover *(Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.). Excess protein N ingested by dairy cows is excreted in urine (Kebreab et al. 2001) and this results in urine patches. The N load in urine patches on an average NZ dairy farm ranges from 200 to 2000 kg N ha^{-1} (Di and Cameron 2002a; Selbie et al. 2015) and this high application rate can exceed the N requirements of pasture (Cai and Akiyama 2016; Smith et al. 1985). This excess N can leach through the soil as NO_3^--N in drainage water and contaminates both ground and surface water (Bishop and Jeyakumar 2021). Urine patches are therefore hot spots of N losses in grazed pasture systems and previous research has shown that urine patches are the dominant source of N leaching in the NZ environment (Ledgard et al. 2009). To reduce NO_3^--N leaching a range of mitigation approaches have been proposed, including; the application of nitrification inhibitors to soil (e.g. DCD and DMPP), the use of low protein N forage species such as plantain (*Plantago lanceolatae* L.) and chicory (*Cichorium intybus* L.), and restricted grazing (Carlton et al. 2019; Christensen et al. 2019; Dai et al. 2013; Mangwe et al. 2019). However, these mitigations have encountered problems in their widespread application to farm systems. For example, while inhibitors have proved effective in reducing NO_3^--N leaching, the discovery of residues in milk and dairy products has effectively excluded their use in grazed pastures (Marsden et al. 2015; Pal et al. 2016). For chicory and plantain, research conducted by Stafford et al. (2016) showed that these two forages accumulate significantly higher cadmium (Cd) concentrations than ryegrass and clover, from even low Cd soils, and this has raised questions over the potential risk of increased transfer of Cd to animals grazing these forage crops. Current approaches therefore have several constraints, and further exploration of mitigation strategies to reduce NO_3^--N leaching is necessary.

Nitrification is a critical process in the N cycle which involves the oxidation of NH_4^+ -N into NO_3^- -N via NO_2^-. The NH_4^+ -N oxidation in nitrification is performed by two distinct nitrifying groups known as AOB and AOA (Gwak et al. 2020; Prosser and Nicol 2012). During nitrification NH_4^+ -N is first oxidised to NH_2OH by the AMO enzyme. Hydroxylamine is then oxidized to NO_2^- by the hydroxylamine oxidoreductase enzyme and to NO_3^- -N by the nitrite oxidoreductase enzyme (Preena et al. 2021). Nitrification in soil is dependent on AMO activity which is responsible for the first step of this reaction (Arp et al. 2002; Li et al. 2018; Tao et al. 2017). Literature evidence shows that the enzyme AMO contains an active Cu site and therefore Cu is a main cofactor for AMO activity (Gorman-Lewis et al. 2019; Wagner et al. 2019). Ensign et al. (1993) reported that the addition of 0.13 mg mL^{-1} $CuCl_2$ to cell culture of *Nitrosomonas europaea* resulted in the stimulation of both NH_4^+ -N reduction and NO_2^- production by 5-to-15 fold. Wagner et al. (2016) demonstrated that addition of up to 0.005 mg L^{-1} Cu stimulated nitrification in a drinking water production plant. Further, other studies have observed that Cu deficiency in wastewater treatments results to low NH_4^+ -N oxidation (Bédard and Knowles 1989; Vandevivere et al. 1998).

Several compounds such as ethylenediaminetetraacetic acid (EDTA) and diethylenetriamine pentaacetate (DTPA) have been shown to reduce the concentration of soil soluble and extractable Cu (often referred to as bioavailable Cu) in soils by forming complexes with Cu (Pinto et al. 2014; Xu et al. 2020). However, studies have identified these compounds as expensive and residual levels in soil are associated with environmental concerns (Jez and Lestan 2016). Organic compounds such as humic acid and co-polymers of maleic and acrylic acid have also been demonstrated to complex with Cu in soil (dos Santos et al. 2020; He et al. 2016) via carboxyl (-COOH) and hydroxyl (-OH) functional groups (Xia et al. 2019; Yan et al. 2013; Yang and Hodson 2019). The hypothesis of this study was that these compounds could be used to reduce the bioavailable Cu concentration in soil and therefore inhibit AMO enzymic activity, and consequently nitrification rate. However, limited studies have examined the effect of bioavailable Cu concentration on nitrification rate in soils, and no previous research has explored the potential for the use of Cu-complexing organic compounds to mitigate N loss in NZ pastoral soils.

The current research work was therefore conducted to examine (a) the effect of organic compounds on soil bioavailable Cu concentration, (b) the effect of bioavailable Cu on nitrification rate, and therefore (c) the effect of organic compound amendments on nitrification rates in pastoral soils.

3.2 Materials and Methods

Two incubation experiments were conducted to achieve the objectives of this work. The first incubation was a preliminary experiment (without Cu addition) to determine the effect of Cu- complexing compounds on the bioavailable Cu concentration in two soils (Pumice and Pallic soils). The second incubation (with Cu addition) investigated the effect of Cu and Cu-complexing compounds on nitrification rate using three soils (Pumice, Pallic, and Recent soils).

3.2.1 Effect of organic compounds on native soil bioavailable Cu

3.2.1.1 Soil collection and characterization

Bulk samples of topsoil were collected (0-20 cm depth) from two dairy farms located in the Wairakei (38°33'0.60"S, 176°13'43.97"E) and Canterbury (43°34'13.15"S, 171°55'47.33"E) regions of NZ. The deeper soil sampling depth represented the average effective rooting depth of some pasture species like white clover in different soils (Talbot et al. 2021). These soils were representative of the Orthic Pumice Soil (Wairakei) and Pallic Firm Brown (Canterbury) soil orders of NZ (Typic Udivitrand and Typic Dystrustept, respectively in the US Soil

Taxonomy Classification; (Hewitt 2010)). Field moist soil was sieved immediately after collection through a <2-mm stainless sieve and stored at <4 °C before further use. Soil physio-chemical parameters were measured on a sub-sample of fresh soil (Table 3.1). Gravimetric soil water content was calculated after oven-drying at 105 °C for 48 h. Soil pH was determined at a soil: water suspension ratio of 1:2.5 using a pH meter (acumen 910, Fisher Scientific Ltd, Pittsburgh, PA, USA). Extractable aluminium (Al) and iron (Fe) were measured using the acid ammonium oxalate extraction method (Blakemore 1987) with analysis performed using MP-AES (4200 MP-AES, Agilent, USA). Soil cation exchange capacity (CEC) was measured using the semi-micro leaching method (Blakemore 1987) with analysis using MP-AES (4200 MP-AES, Agilent, USA). The total soil N and carbon (C) content was quantified on ring-ground soil using a Vario MACRO cube CHNS elemental Analyser (Elementa Anlysensysteme Gmbh, Hanau, Germany).

Table 3.1 Selected chemical properties of Pumice, Pallic, and Recent soils used in the study.

Soil parameters	Pumice soil	Pallic soil	Recent soil
Soil pH (H_2O)	5.80 ±0.03	5.00 ±0.03	5.80 ±0.03
%Fe	0.31 ±0.01	0.43 ±0.01	0.20 ±0.01
%Al	0.97 ±0.03	0.38 ±0.01	0.06 ±0.01
%C	3.77 ±0.12	4.12 ±0.02	0.53 ±0.03
%N	0.22 ±0.01	0.37 ±0.01	0.08 ±0.01
CEC[1] (cmol$_c$ kg^{-1})	21.10 ±0.59	9.10 ±0.59	11.90 ±0.59
NH_4^+ (mg kg^{-1})	1.50 ±0.50	16.05 ±3.03	1.04 ±0.38
NO_3^- (mg kg^{-1})	71.55 ±3.71	200.26 ±10.41	28.98 ±3.61
Bioavailable Cu (mg kg^{-1})	0.27 ±0.01	0.15 ±0.01	0.13 ±0.01
WHC[2] (%)	80.03 ±0.01	45.34 ±0.01	31.02 ±0.01

[1]Cation Exchange Capacity (CEC), [2]Water Holding Capacity (WHC), and numbers after ± are standard deviation (*n* = *5*).

3.2.2 Treatment application and soil analysis

Ten grams of field moist soil was added into replicate 50 ml centrifuge tubes and treated with one of five chemicals to complex with Cu: control (water), DCD at 10 mg kg^{-1}, LS at 120 mg kg^{-1}, poly maleic acid (PMA) at 10 mg kg^{-1}, and PA-MA at 10 mg kg^{-1}. In this study we used 120 mg kg^{-1} of LS based on results from a previous study by Bishop and Jeyakumar (2021). They found that 120 kg LS ha^{-1} was the potential application rate. All application rates were calculated on a dry weight basis. Each treatment was replicated three times, and in total 30 centrifuge tubes were used for this experiment, considering two sampling time periods. The treated soils were incubated at <4°C to minimise microbial activity such as mineralisation of organic matter, which could potentially use Cu in soil during this process. The soil was maintained at 60% water capacity throughout the incubation period. Sampling was performed at Days 3 and 7 after the application of treatments by extracting the whole 10 g soil in the centrifuge tube with 30 ml 0.05 M $CaCl_2$. The Cu concentration in the extract solution (bioavailable Cu) was quantified using graphite furnace atomic absorption spectrophotometry (900Z PerkinElmer, PinAAcle).

3.2.3 Effect of Cu and Cu-complexing compounds on nitrification rate

3.2.3.1 Soils

Three dominant NZ pasture soil types with contrasting soil characteristics (Pumice, Pallic, and Recent soils) were selected for this second incubation. In addition to the soils used in the preliminary incubation, Manawatu Recent soil (Dystric Fluventic Eutrudept according to US Soil Taxonomy Classification; (Hewitt 2010)) was collected from the dairy No.1 farm, Massey University, Palmerston North (40°23'0.95"S, 175°36'36.16"E). Soil chemical analysis were undertaken as described in section 3.2.1.1.

3.2.3.2 Treatments

Subsamples of field moist soil (10 g) were separately spiked with a calculated amount of copper sulphate ($CuSO_4.5H_2O$) in replicate 50 ml centrifuge tubes to achieve 5 Cu treatments; 0.1, 0.3, 0.5, 1, and 3 mg Cu kg^{-1} with thirty samples (5 treatments*3 replicates*2 sampling times) per treatment in each soil. This Cu concentrations were proposed based on literature survey (Table 2.2) (Cela and Sumner 2002; He et al. 2019; Tomlinson et al. 1966). The treated soils were then incubated for 14 days at 25 °C to equilibrate Cu with the soil matrix. Each Cu treatment for each soil was subsequently amended with five different treatments; control (only Cu), N applied as urea at 300 mg N kg^{-1}, DCD at 10 mg kg^{-1} + 300 mg N kg^{-1} as urea, LS at 120 mg kg^{-1} + 300 mg N kg^{-1} as urea, and PA-MA at 10 mg kg^{-1} + 300 mg N kg^{-1} as urea. DCD was used as a standard reference material for nitrification reduction (Di and Cameron 2002b; Di et al. 2007). The tubes were loosely closed and further incubated for 8 days at 25 °C, and bioavailable Cu and mineral N was measured at Days 4 and 8.

3.2.3.3 Bioavailable Cu and mineral N extraction

For each 10 g soil in an incubation tube, 30 ml of 0.05 M $CaCl_2$ was added, and bioavailable Cu extracted in an end-over-end shaker for 2 h. The solution was centrifuged at 1100 g for 10 min and the supernatant was filtered through Whatman 42 filter papers. The residual soil after centrifuging was further extracted for NH_4^+ -N/NO_3^- -N measurement. Briefly, 30 ml of 2 M KCl was added and NH_4^+ -N/NO_3^- -N was extracted with the same procedure used for bioavailable Cu. Both extractants were stored at <4 °C and analysed within three days. Bioavailable Cu was analysed using graphite furnace atomic absorption spectrophotometry (900Z PerkinElmer, PinAAcle). Soil extracted NH_4^+ -N and NO_3^- -N concentrations were analysed in the $CaCl_2$ and KCl extract supernatant using Technicon autoanalyzer (Blakemore

1987). Concentrations in each extract were summed to calculate the extractable concentration of NH_4^+ -N and NO_3^- -N.

3.2.3.4 Net nitrification rate calculation

Net nitrification rate was calculated using equation 3.1 based on the work of Chen et al. (2015):

$$NH_4^+ - N\ kg^{-1}\ day^{-1} = \frac{(NH_4^+ - N)_{d4} - (NH_4^+ - N)_{d8}}{t} \qquad \textbf{Equation 3.1}$$

Where $(NH_4^+ -N)_{d4}$ is the extracted NH_4^+ -N concentration at Day 4, $(NH_4^+ -N)_{d8}$ is the extracted NH_4^+ -N concentration at Day 8, and t is the number of days between the two sampling dates.

3.2.3.5 Dose response curve

The dose response of nitrification rate on bioavailable Cu and added Cu was assessed using the dose response software of origin 9.0 according to equation 3.2 outlined by Dawson et al. (2012):

$$y = A1 + \frac{A2 - A1}{1 + e^{(logx0 - x) * c}} \qquad \textbf{Equation 3.2}$$

Where y is the % increase in nitrification rate; A1 is the bottom asymptote (minimum value); A2 is the top asymptote (maximum value); logxo is the log to base 10 of the Cu concentration, x is the EC_{50}, and c is the fitting parameter.

3.2.3.6 Quality control measures

All laboratory equipment was cleaned with 2% HCl overnight, followed by deionized water, and dried before use. Two certified standard reference materials were used for quality control: National Institute of Standards and Technology SRM 2710a, Montana soil (3420 ±0.005 mg Cu kg^{-1}); and SRM 1573a, tomato leaves (4.70 ± 0.14 mg Cu kg^{-1}). The mean Cu concentration in the Montana soil and Tomato leaves was within 93.8-97.0% and 91.8-95.6%, of the certified values, respectively.

3.2.4 Statistical analysis

All results presented in this work were analysed and tested for normality using SPSS ver. 24.0 (SPSS Inc., USA) statistical software and significant differences between treatment means were performed using Turkey test. Linear regression analysis was performed using SPSS regression model. Graphs were drawn using SigmaPlot® ver.14.0 (Systat Software Inc., San Jose, CA, USA).

3.3 Results

3.3.1 Effect of organic compounds on reducing native soil bioavailable Cu

Incubation with DCD, LS, PMA, and PA-MA significantly ($P < 0.05$) reduced the bioavailable Cu concentration of the Pumice soil by 8, 17, 26, and 31%, respectively at Day 3, and by 12, 66, 52, and 61%, respectively at Day 7 when compared to the control treatment (Figure 3.1). Similarly, for the Pallic soil, application of DCD, LS, PMA, and PA-MA significantly ($P < 0.05$) reduced the bioavailable Cu concentration by 9, 43, 41, and 30%, respectively at Day 3 and by 10, 66, 48, and 52%, respectively at Day 7 when compared to control (Figure 3.1). Based on the results from this experiment, LS and PA-MA were selected for further testing of their potential to inhibit nitrification through reducing bioavailable Cu concentration.

3.3.2 Reduction in bioavailable Cu concentration during the nitrification experiment

The control treatment (Cu only) showed significantly ($P < 0.05$) higher levels of bioavailable Cu concentration in soil solution compared to the other treatments at each Cu treatment level in the Recent soil, and when greater than 0.5 mg Cu kg^{-1} was added to the Pumice and Pallic soils (Figure 3.2). Addition of urea significantly ($P < 0.05$) reduced the bioavailable Cu concentration relative to the control for an added Cu concentration of 0.3 mg kg^{-1} and greater in the Pallic and Recent soils, and 0.5 mg kg^{-1} and greater in the Pumice soil (Figure 3.2). When compared to the urea-only treatment, application of DCD induced a further significant ($P < 0.05$) decrease in bioavailable Cu concentration for all three soils on both Days 4 and 8 when the added Cu concentration was greater than 1 mg Cu kg^{-1}. Generally, application of LS, and PA-MA induced a significant ($P < 0.05$) decrease in bioavailable Cu concentration for the 1 and 3 mg added Cu kg^{-1} treatments in all three soils in comparison to urea-treated soils, with the order of decrease being LS > PA-MA on both Days 4 and 8. Overall, significant differences due to treatment effect were observed for the higher added Cu concentration levels in all three soils.

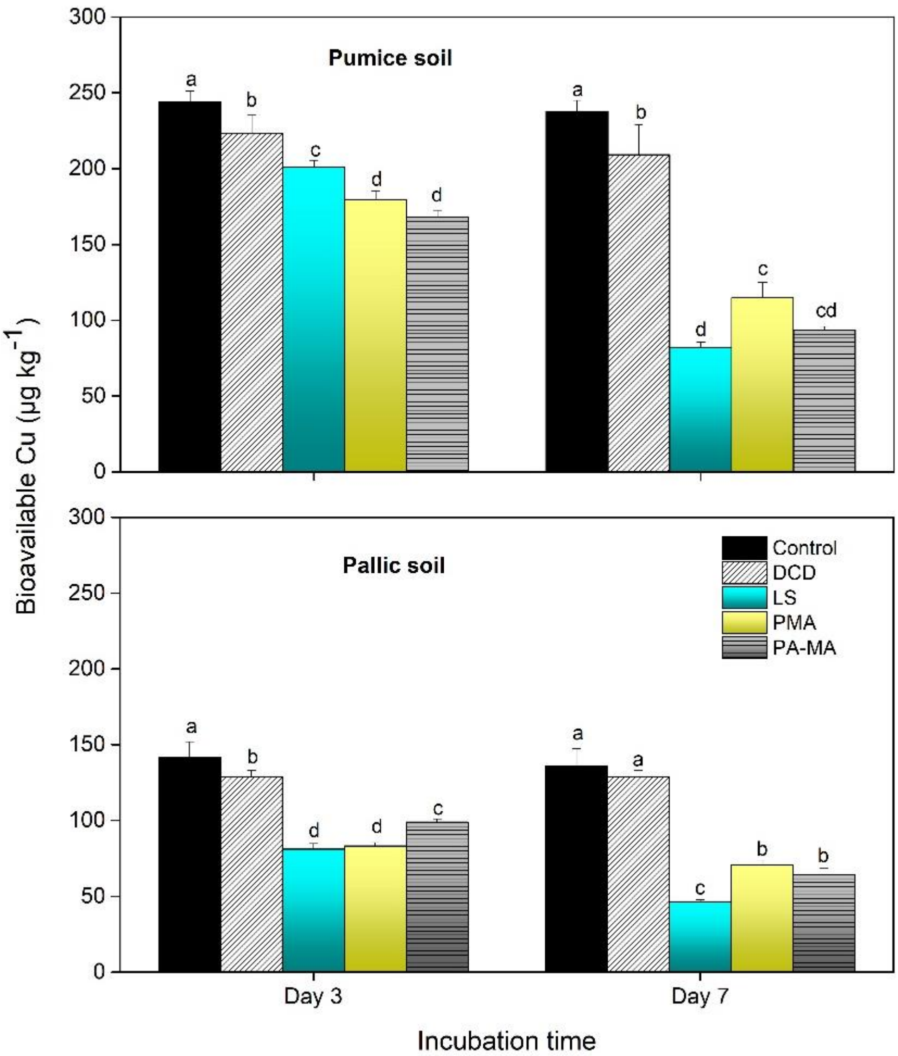


Figure 3.1 Effect of DCD and organic compounds on the bioavailable Cu concentration in the Pumice and Pallic soil. Vertical error bars indicate standard deviation of means (*n = 3*). Different letters indicate significant differences among treatments with same sampling day.

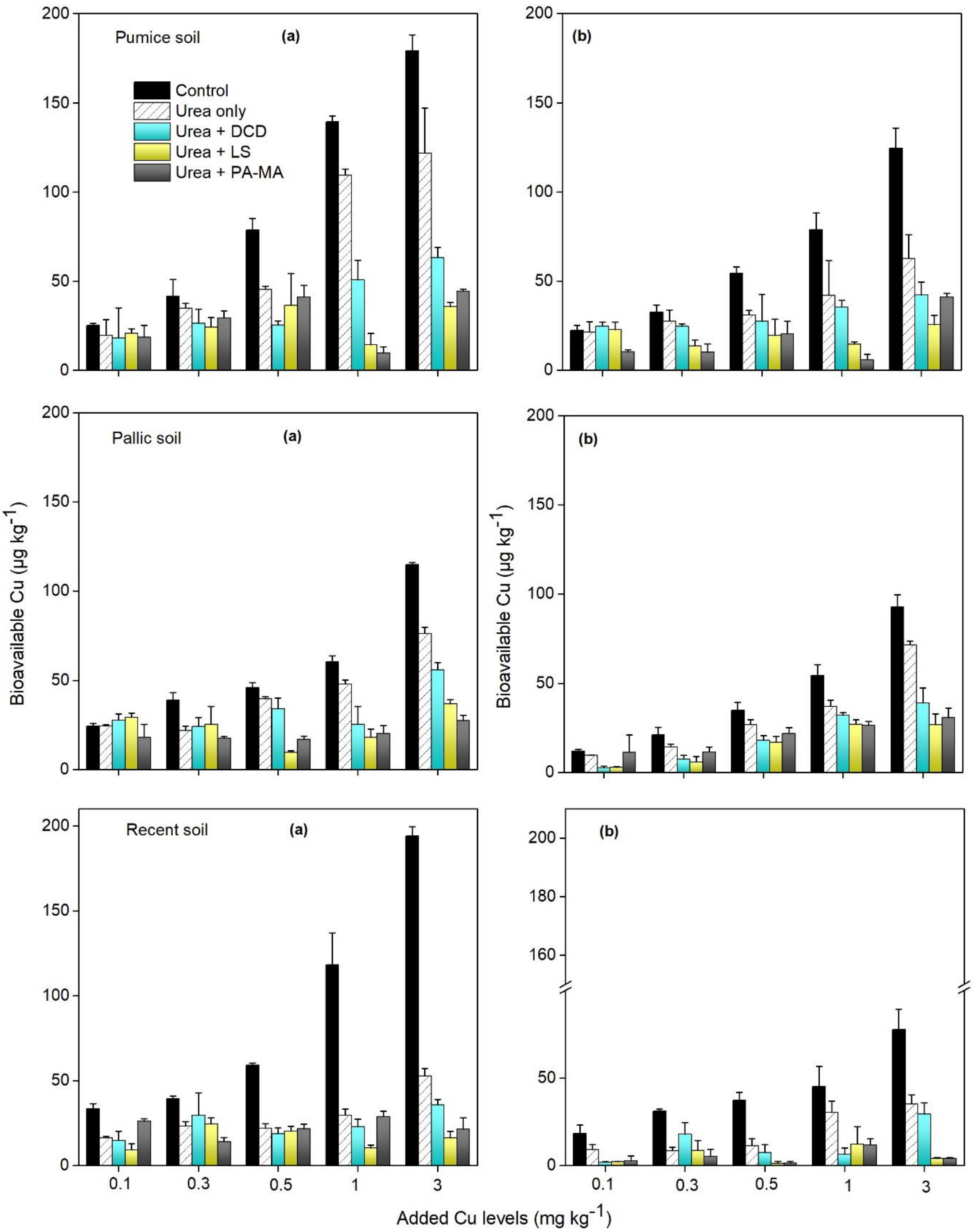


Figure 3.2 Bioavailable Cu concentration for the Pumice, Pallic, and Recent soils on Day 4 (a) and Day 8 (b) as a function of added DCD and organic compounds after urea application. Vertical error bars indicate standard deviation of mean (*n* = *3*).

3.3.3 Effect of adding Cu and HMWOAs on mineral N concentrations

3.3.3.1 NH_4^+ - N concentrations

The increasing Cu concentration in the Pumice soil was associated with a significant decrease in the NH_4^+ -N concentration for the urea-only treatments at Day 4 (Figure 3.3). However, there was no significant ($P > 0.05$) decrease in NH_4^+ -N concentrations in the Pallic and Recent soils for the urea-only treatments as a function of increasing Cu concentrations at Day 4. At Day 8 there was a significant reduction ($P < 0.05$) in the extracted NH_4^+ -N concentration in all three soils amended with urea only, for an added Cu concentration of 0.1-3 mg kg^{-1}.

At Day 4 there was no significant difference in NH_4^+ -N concentration between the DCD, LS, and PA-MA treatments and the urea-only treatment in the Recent soil, although for the Pallic and Pumice soils a significantly ($P < 0.05$) greater NH_4^+ -N concentration was recorded for these treatments at Day 4 relative to urea-only for soils where the added Cu concentration was 0.5 mg kg^{-1} and greater (Figure 3.3). DCD significantly ($P < 0.05$) increased the NH_4^+ -N concentration in extracts by an average of 114, 41, and 52% in the Pumice, Pallic, and Recent soils, respectively, at Day 8 over the urea-only treatment. The increase in NH_4^+ -N concentration induced by the LS and PA-MA treatments relative to urea at Day 8 was lower than for DCD, but significant ($P < 0.05$) with average values of 59 and 56% for Pumice, 32 and 26% for Pallic, and 39 and 38% for the Recent soil, respectively. Overall, DCD resulted in a significantly ($P < 0.05$) higher NH_4^+ -N concentration relative to LS and PA-MA in the Pumice soil, but no significant differences were observed in the Pallic and Recent soils.

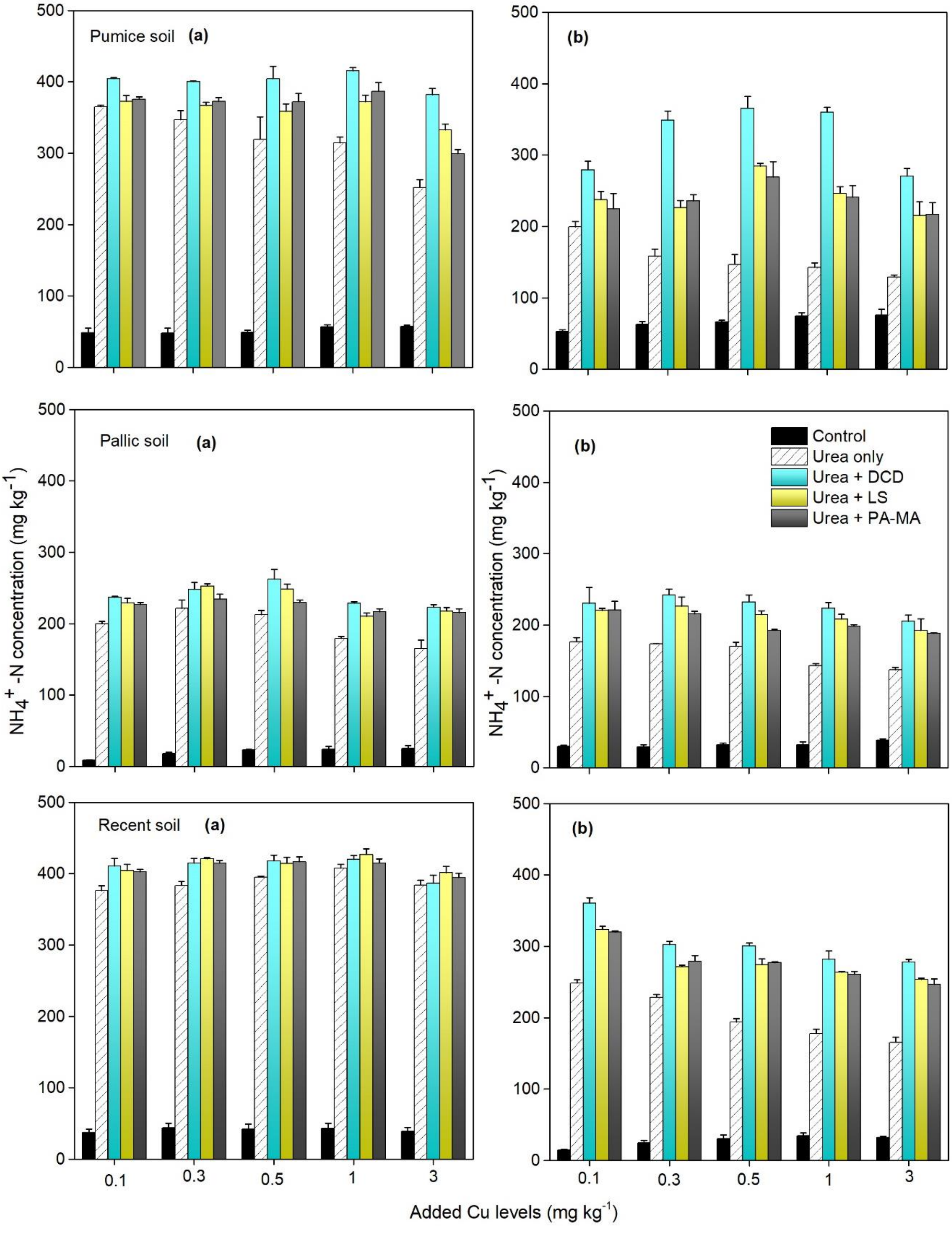


Figure 3.3 NH_4^+-N concentration in extract from the Pumice, Pallic, and Recent soils on (a) Day 4 and (b) Day 8 as a function of added Cu, DCD, and organic compounds. Vertical error bars indicate standard deviation of means (*n = 3*).

3.3.3.2 NO_3^- -N concentration

Application of Cu to all three soils (Pumice, Pallic, and Recent soils) had no significant effect on the NO_3^- -N concentration at Day 4 for the urea-only treatment. Similarly, no applied treatments induced a significant change in the extracted NO_3^- -N concentration at Day 4 relative to the urea-only treatment (Figure 3.4). At Day 8 there was no significant effect of Cu on the NO_3^- -N for the Pallic soil, but in the Recent and Pumice soils increasing Cu concentration was associated with a significant increase in the NO_3^- -N concentration for the urea-only treatments. The NO_3^- -N concentration increased by 36 and 61 mg kg^{-1}, respectively, in the Recent and Pumice soils as a function of increasing Cu concentration. Application of DCD significantly ($P < 0.05$) reduced the NO_3^- -N concentration by an average of 45, 16, and 62% in the Pumice, Pallic, and Recent soils, respectively, at Day 8 relative to the urea-only treatment. The LS and PA-MA treatments also significantly ($P < 0.05$) reduced the extract NO_3^- -N concentrations by an average of 17 and 17% in the Pumice soil, 10 and 10% in the Pallic soil, and by 28 and 31% in the Recent soil, respectively, compared to the urea-only (Figure 3.4). The NO_3^- -N concentration significantly increased from Day 4 to Day 8 in the Pumice soil, but not in the Pallic or Recent soil.

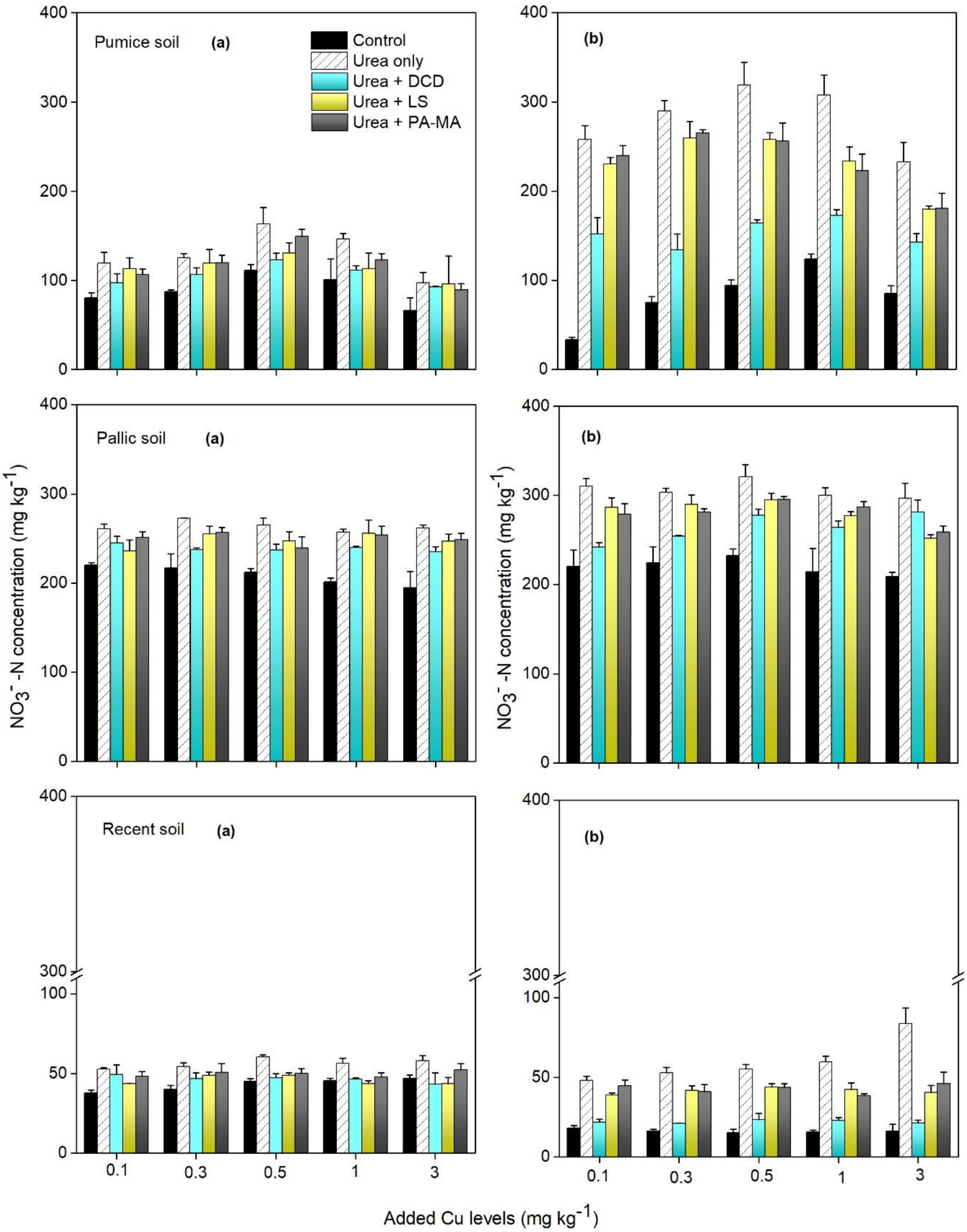


Figure 3.4 NO_3^- -N concentration in extract from the Pumice, Pallic and Recent soils on (a) Day 4 and (b) Day 8 as a function of added Cu, DCD and organic compounds. Vertical Error bars indicate standard deviation of means (*n = 3*).

3.3.3.3 Dose response model

Results from the dose result experiment confirm that nitrification rate increased as a function of the Cu concentration in soil (Figure 3.5). The bioavailable Cu concentration corresponding to a 50% increase nitrification rate (EC_{50}) for the Pumice, Pallic, and Recent soils was 0.027 mg kg^{-1} (R^2 = 0.898), 0.036 mg kg^{-1} (R^2 = 0.991), and 0.010 mg kg^{-1} (R^2 = 0.957), respectively (Figure 3.5 a-c). The corresponding EC_{50} values for the total added Cu concentration were 0.293 mg kg^{-1} (R^2 = 0.933), 0.697 mg kg^{-1} (R^2 = 0.999), and 0.430 mg kg^{-1} (R^2 = 0.985), respectively (Figure 3.5 d-f).

3.4 Discussion

3.4.1 Effect of increasing bioavailable Cu concentration and application of Cu-complexing compounds in influencing nitrification rate

Bioavailability has been widely defined by different researchers (Alexander 2000; Ruby et al. 1996). In this study bioavailable Cu is defined as the fraction of the total soil Cu concentration that exists in an available and extractable form. This includes the free Cu^{2+} ion species that is readily available for microbe adsorption or utilisation (Petruzzelli 1989). Therefore, in this work, the results are described in terms of bioavailable Cu concentration, not total Cu concentration.

This work aimed to investigate the effect of organic compounds on reducing the concentration of bioavailable Cu in the soil and to explore the relationship between the soil concentration of bioavailable Cu and nitrification rate. These findings show that the application of LS, PA-MA, and PMA to soil significantly (P <0.05) reduced the bioavailable Cu concentration relative to the control treatment in both incubations (Figure 3.1 and Figure 3.2).

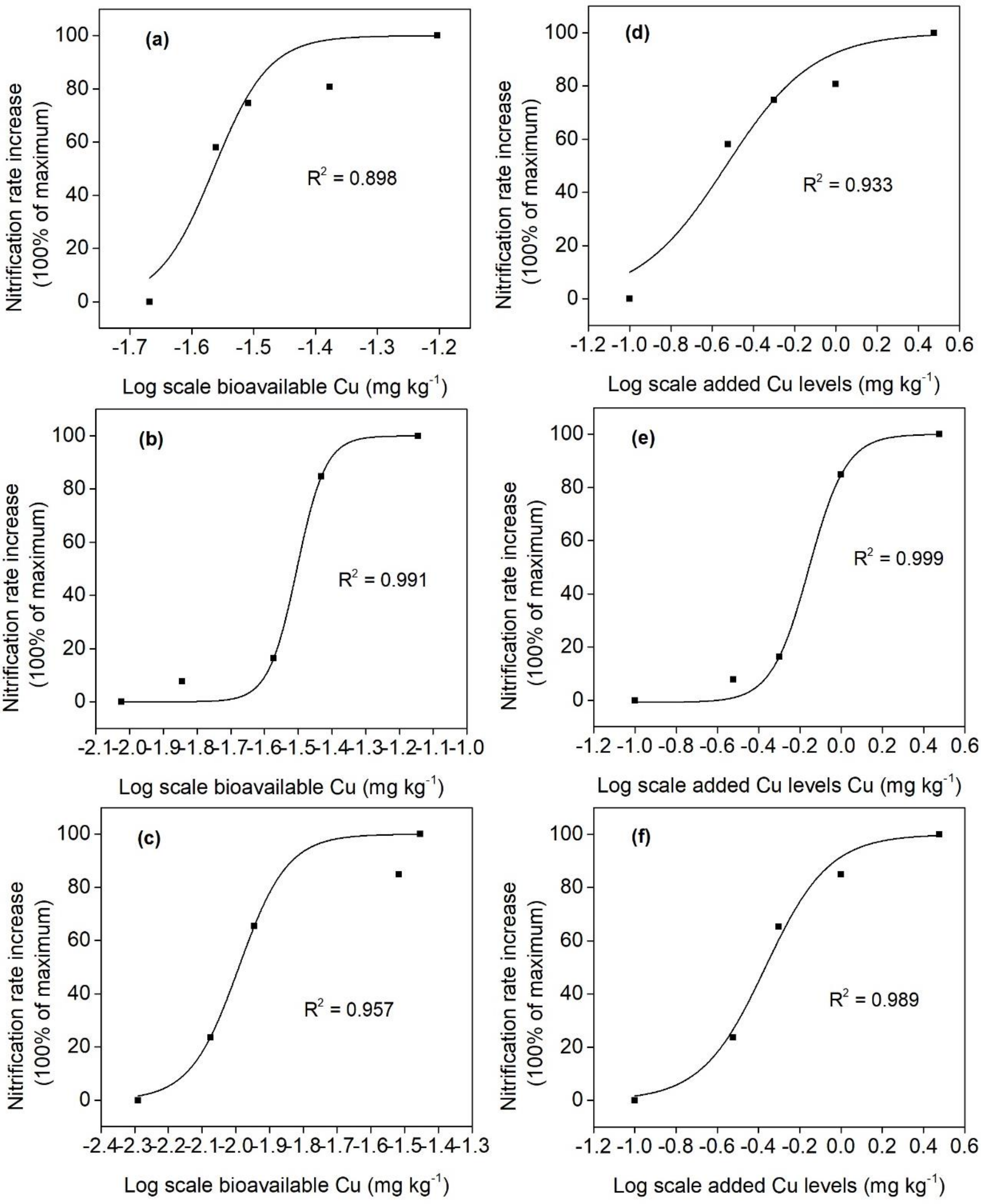


Figure 3.5 Relationship of nitrification rate increase with bioavailable Cu concentrations on Pumice (a), Pallic (b), and Recent soils (c), and added Cu concentrations levels on Pumice (d), Pallic (e), and Recent soils (d).

These findings agree with other studies which have reported a reduction in soil metal concentration in soil solution due to the application of these organic compounds (Qi et al. 2017; Tang et al. 2019; Zhao et al. 2019). For example, Suanon et al. (2016) reported that the application of N,N-Bis (Carboxymethyl) glutamic acid reduced solution Cu in sewage sludge by 81.2%. Li et al. (2010) studied the adsorption of Cu in aqueous solution onto humic acid and found that Cu adsorption was increased approximately 2-fold, 3 h after application of 4 mg humic acid L^{-1} at 20 °C, pH=5. It is proposed that the reduction in bioavailable Cu induced by LS, PA-MA, and PMA was associated with the presence of carboxyl (-COOH) and hydroxyl (-OH) functional groups in the organic compounds which can complex with Cu and thereby reduce its bioavailability in the soil.

Copper has been defined as an essential trace element required for NH_4^+ -N oxidation by AOA and AOB due to the presence of essential mono- and dinuclear Cu centers within the key enzyme AMO (Lieberman and Rosenzweig 2005). In this doctoral research, soil NH_4^+ -N concentration was used to quantify the effect of Cu on nitrification rate due to the greater stability of NH_4^+ -N in soil; NO_3^- -N is highly susceptible to reduction by denitrifying bacteria. Takeda et al. (2021) reported that denitrification increases with soil NO_3^- -N availability. The results showed a significant ($P < 0.05$) increase in nitrification rate for all urea-only treated soils, and this increase in nitrification rate was inferred to be associated with an increase in bioavailable Cu concentration in the soil (Figure 3.2). It is therefore proposed that the presence of Cu stimulates AMO microbial activity which subsequently resulted in increased nitrification rate. These results agree with other studies that have analysed the effect of Cu on increasing NH_4^+ -N oxidation in pure cell culture. Zhang and Edwards (2010) reported that an increase in solution Cu concentration from 0 to 20 μg L^{-1} increased NH_4^+ oxidation from 45 to 60%. He et al. (2019) reported that increasing Cu concentration from 0.05 to 0.1 mg L^{-1} significantly ($P < 0.05$) increased NH_4^+ -N and total N removal efficiency of strain Y-10 by 11.5 and 8.81%

in pure culture, respectively. Wagner et al. (2016) found that addition of up to 0.005 mg Cu L^{-1} resulted in a 14-fold increase in NH_4^+ -N and NO_2^- oxidation in drinking water. These results demonstrate that the bioavailable Cu concentration necessary to cause a 50% increase in nitrification rate was lower in the Recent soil (0.010 mg kg^{-1}) than in the Pumice soil (0.026 mg kg^{-1}) and Pallic (0.036 mg kg^{-1}) soil (Figure 3.5 a-c). The lower EC_{50} value in the Recent soil meant bioavailable Cu was more effective in stimulating nitrifying bacteria on a unit concentration basis than in the other soils. This difference between the Recent soil and the other two soils might be associated with the differences in soil properties (Table 3.1). The higher percent C and Fe in the Pumice and Pallic soils, might have also complexed with Cu in soil solution (Zhang et al. 2021). The complexing of bioavailable Cu with organic matter and Fe-oxides might have increased the EC_{50} value (Figure 3.5).

To further explore the effect of added Cu on nitrification, the net nitrification rate, expressed as NH_4^+ -N lost per day, was calculated (Figure 3.6). Addition of 0.1 to 3 mg Cu kg^{-1} soil significantly increased the net nitrification rate in the urea-only treatment by 43-71% in the Pallic soil and 21-80% in the Recent soil (Figure 3.6). For the Pumice soil there was a nominal increase of 13% across this range of added Cu. Net nitrification rate in the Pallic soil was significantly lower than in the Recent and Pumice soils for all treatments (Figure 3.6) and this could be due to the low soil pH in the Pallic soil (5.0) compared to the Recent (5.8) and Pumice (5.8) soils. Mével and Prieur (2000) demonstrated that the abundance and activity of nitrifying bacteria is soil pH dependent. They found optimal nitrification potential at pH 7.5-8, with maximum growth across a pH range of 6.0-6.5. Zhang et al. (2012b) found that nitrification activity of *Bacillus methylotrophicus* strain L7 significantly increased when the pH was above 6.0 but was restricted at pH below 5.5.

In this study, it was observed that the urea-only treatment had a significantly ($P < 0.05$) lower bioavailable Cu concentration in all soil types in comparison to the control (Figure 3.2) and it is proposed that this can be explained in terms of a change in microbial activity after the addition of urea. Chen et al. (2015) showed that application of urea to soil induces an increase in populations of ammonia oxidizers. These authors found that addition of 200 mg kg^{-1} urea to a soil incubation resulted in an approximately 1.2- and 3.1-fold increase in AOA and AOB *amoA* gene copies compared to control. An increase in *amoA* gene copies results in increased utilisation and complexation of Cu by enzyme activity associated with nitrifying bacteria which may reduce the bioavailable concentration of Cu in soil. This theory is supported by the observation of a positive relationship between net nitrification rate and bioavailable Cu concentration in the Pallic soils ($R^2 = 0.625$, $P > 0.05$) and Recent ($R^2 = 0.706$, $P < 0.01$) amended with urea only (Figure 3.7).

The current study demonstrates that the application of DCD, LS, and PA-MA treatments significantly ($P < 0.05$) reduced nitrification rate in all soils relative to the urea-only treated soil at Day 8 (Figure 3.3) and this corresponded with lower net nitrification rates (Table 3.2). Numerous studies have reported the effectiveness of DCD as a nitrification inhibitor in both incubation and field studies (Di and Cameron 2005b; Guo et al. 2014). These results show that the addition of DCD to soil significantly ($P < 0.05$) reduced bioavailable Cu concentration, and this provides experimental evidence of the chelating effect of DCD on Cu (Figure 3.1 and Figure 3.2), supporting the hypothesis that nitrification rates are affected by changes in soil bioavailable Cu concentration.

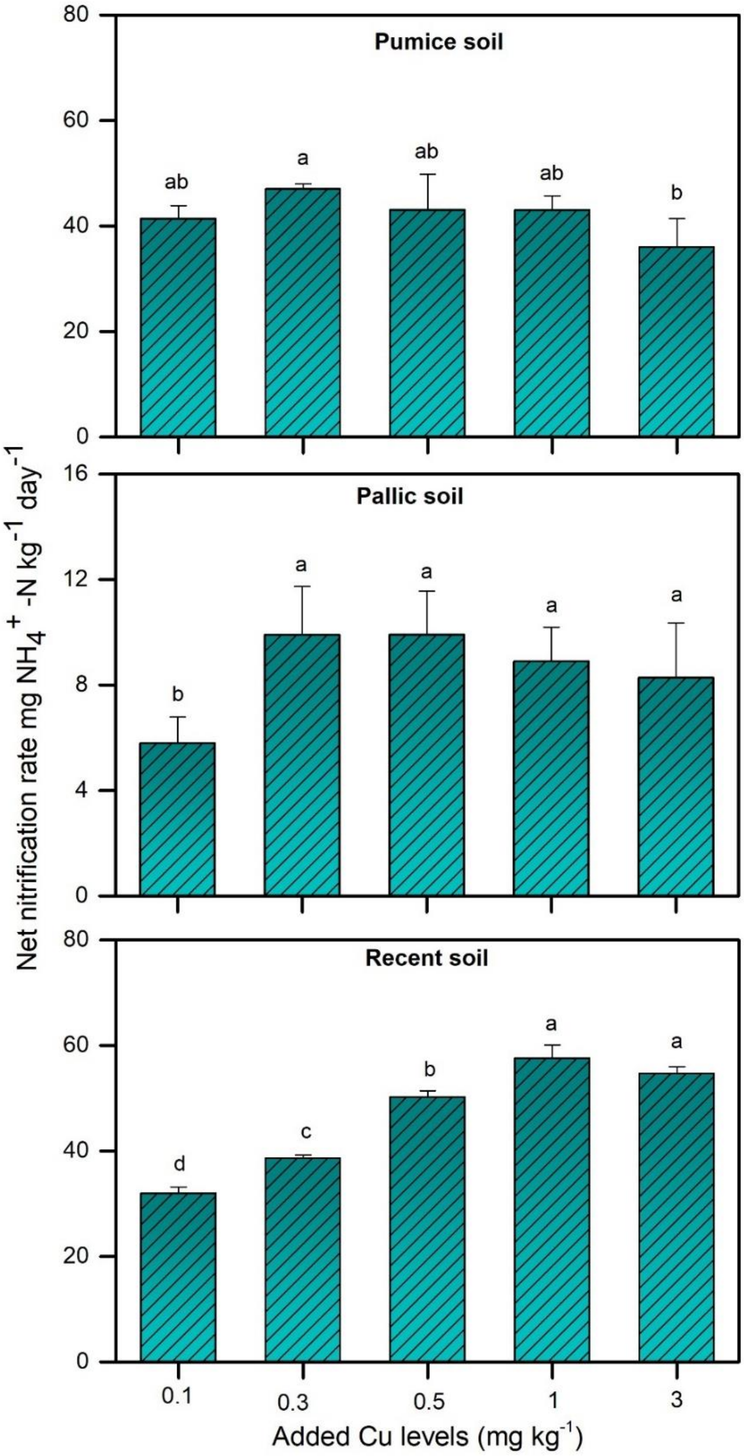


Figure 3.6 Effect of increasing Cu concentration on net nitrification rate in the urea-only treated soil. Vertical errors bars indicate standard deviation of means *(n = 3*). Different letters following each other are significantly different at $P < 0.05$.

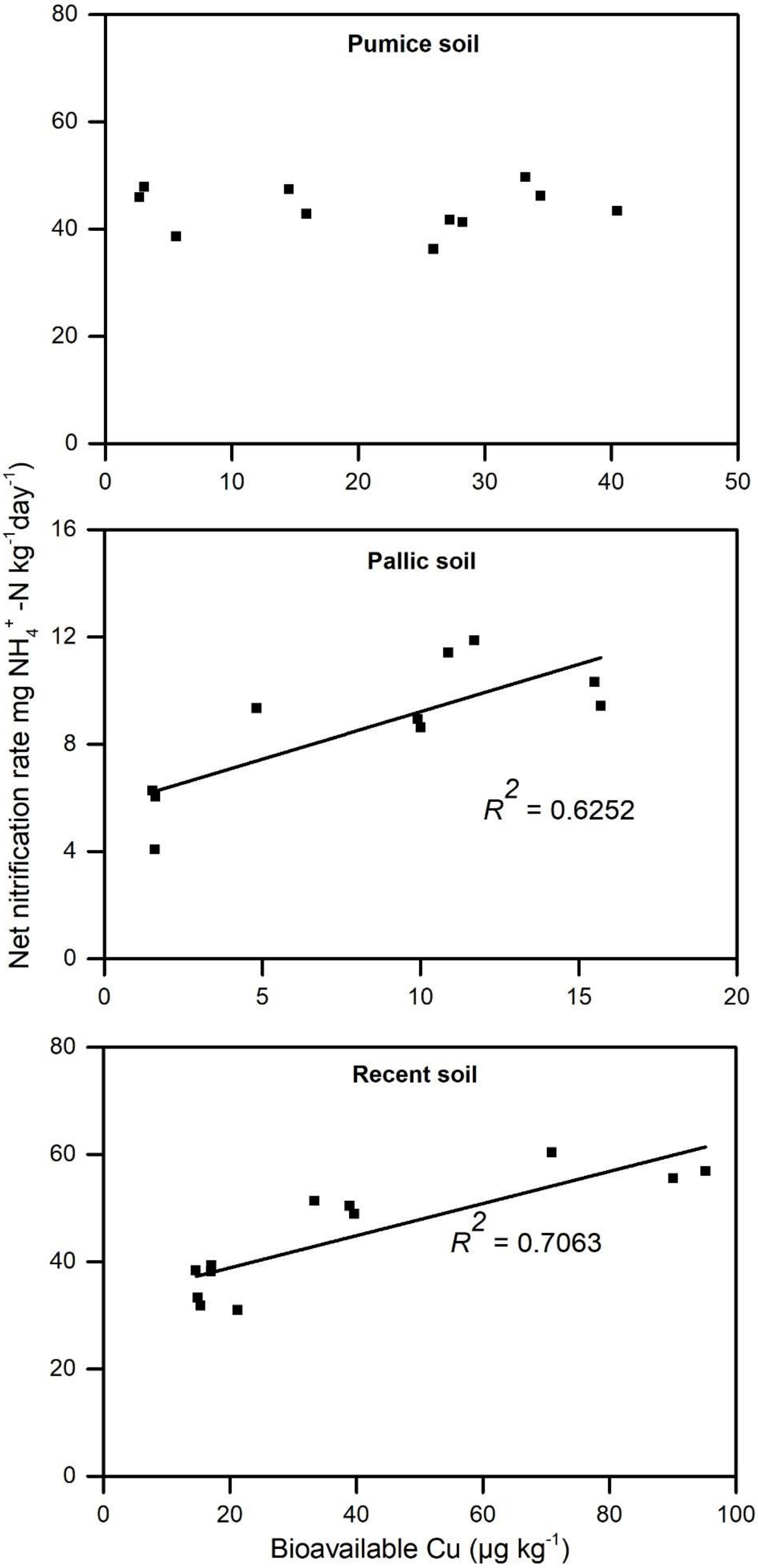


Figure 3.7 Correlation of bioavailable Cu concentration with net nitrification rate in the urea-only treatment for the Pumice, Pallic, and Recent soils.

Table 3.2 Effect of Cu, DCD, and Cu-complexing organic compounds soil amendments on net nitrification rate.

Treatments	Net nitrification rate (mg NH_4^+-N kg^{-1} day^{-1})				
	0.1 mg Cu kg^{-1}	**0.3 mg Cu kg^{-1}**	**0.5 mg Cu kg^{-1}**	**1 mg Cu kg^{-1}**	**3 mg Cu kg^{-1}**
Pumice soil					
Urea-only	41.40 ± 2.5 a	47.02 ± 1.0 a	43.08 ± 6.7 a	43.04 ± 2.7 a	36.02 ± 1.4 a
Urea + DCD	31.40 ± 3.5 a	12.88 ± 3.1 c	9.63 ± 1.9 c	13.85 ± 1.7 c	27.88 ± 1.5 b
Urea + LS	33.88 ± 4.8 a	35.19 ± 1.5 b	18.60 ± 2.6 bc	31.49 ± 3.5 b	29.36 ± 2.8 b
Urea + PA-MA	37.73 ± 6.4 a	34.26 ± 1.6 b	25.70 ± 2.8 b	36.48 ± 5.2 ab	20.56 ± 3.6 c
Pallic soil					
Urea only	5.79 ± 1.6 a	9.89 ± 0.8 a	9.90 ± 1.7 a	8.89 ± 1.3 a	8.27 ± 2.3 a
Urea + DCD	3.14 ± 0.9 ab	1.66 ± 0.4 c	7.45 ± 1.2 a	1.72 ± 0.1 c	4.32 ± 0.5 a
Urea + LS	2.13 ± 1.9 b	6.49 ± 1.1 b	8.43 ± 1.4 a	1.71 ± 0.2 c	6.30 ± 1.6 a
Urea + PA-MA	2.43 ± 0.2 ab	4.53 ± 0.7 b	9.26 ± 0.6 a	4.60 ± 1.5 b	6.78 ± 1.3 a
Recent soil					
Urea-only	31.98 ± 1.2 a	38.64 ± 0.6 a	50.20 ± 1.2 a	57.61 ± 2.5 a	54.69 ± 1.3 a
Urea + DCD	12.58 ± 2.6 c	28.17 ± 2.7 c	29.31 ± 2.5 b	34.63 ± 4.0 b	27.29 ± 2.7 c
Urea + LS	20.22 ± 2.6 b	37.37 ± 0.5 ab	35.08 ± 4.2 b	40.70 ± 2.2 b	36.99 ± 2.6 b
Urea + PA-MA	20.63 ± 1.2 b	33.98 ± 1.9 b	34.82 ±1.7 b	38.52 ± 2.5 b	36.85 ± 1.1 b

Note: Numbers after ± are standard deviation of three replicates. Values in each column, followed by different letters within a column for each soil, are significantly different at $P < 0.05$.

Similarly, the reduction in nitrification rate associated with LS and PA-MA treatments was mainly due to a reduction in the bioavailable Cu concentration in the soil induced by these soil amendments: complexation of Cu induced Cu deficiency, and consequentially affected AMO activity for nitrification. This hypothesis has literature precedent. Copper deficiency has been shown to be a limiting factor in nitrification in wastewater treatments plants due to complexation of Cu with organic matter (Wagner et al. 2016). Reyes et al. (2020) found that adding 1 µM of chelator TETA to *Nitrososphaera viennensis* culture significantly reduced NH_4^+ -N oxidation, NO_2^- production and cell growth due to a reduction in available Cu^{2+}. Gwak et al. (2020) found that addition of 50 µM histidine decreased the growth rate of soil AOA strain *Nitrososphaera viennensis* by 56-82% compared to a control without histidine. All these studies support the theory of an effect of bioavailable Cu on AOA and AOB functioning in the nitrification process.

3.5 Conclusion

This study provides a strong link between nitrification rate and bioavailable Cu concentration in soils. Dose response shows that increasing Cu concentration in soil significantly increases nitrification rate. Application of DCD, LS, and PA-MA to all soils significantly reduced the bioavailable Cu concentration and this induced deficiency and significantly reduced the nitrification rate for amended soils. It is proposed that these treatments limited the activity of the AMO enzyme, which depends on Cu as co-factor. The results of this study enhance understanding of the mechanism of nitrification and could underpin the ongoing development of nitrification inhibitors. Further work is needed to field test the nitrification response to variable rates of LS and PA-MA.

CHAPTER 4

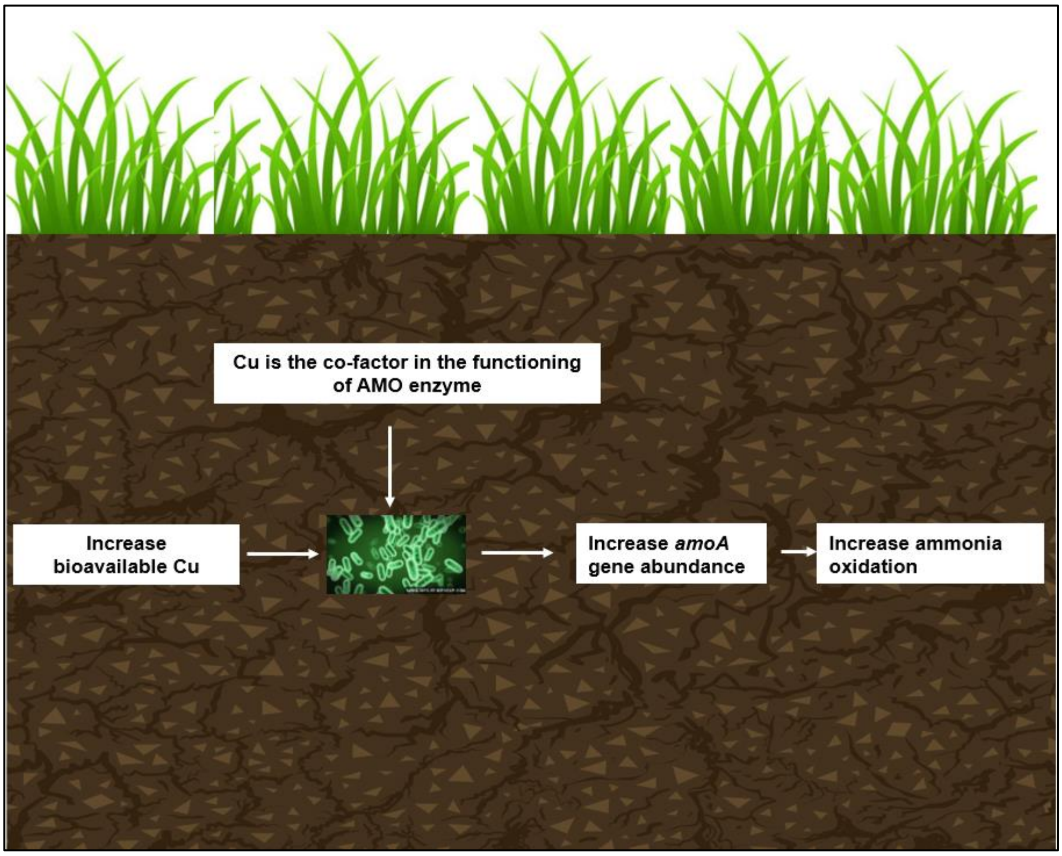
Copper induces Nitrification by Ammonia-Oxidizing Bacteria and Archaea in Pastoral Soils

*This chapter was published in the **Journal of Environmental Quality** in 2022. Citation:*

Matse, D. T., Jeyakumar, P., Bishop, P., & Anderson, C. W. N., (2023). Copper induces nitrification by ammonia-oxidizing bacteria and archaea in pastoral soils. Journal of Environmental Quality. 52, 49-63. doi: 10.1002/jeq2.20440.

The results presented in Chapter 3 showed that increasing bioavailable soil Cu increased nitrification, but did not perform microbial profiling. Therefore, in Chapter 4, a greenhouse pot trial demonstrates the effect of increasing bioavailable Cu concentration on nitrification rate, AOB/AOB population, and AOB/AOA *amoA* gene abundance in the same soils used in Chapter 3.

Graphical abstract



Abstract

Copper (Cu) is the main co-factor in the functioning of the AMO enzyme, which is responsible for the first step of ammonia oxidation. This study reports a greenhouse-based pot experiment that examines the response of AOB and AOA to different bioavailable Cu concentrations in three pastoral soils (Pumice, Pallic, and Recent soils) planted with ryegrass (*Lolium perenne* L.). Five treatments were used: control (no urine and Cu), urine-only at 300 mg N kg^{-1} soil (Cu0), urine + 1 mg Cu kg^{-1} soil (Cu1), urine + 10 mg Cu kg^{-1} soil (Cu10), and urine + 100 mg Cu kg^{-1} soil (Cu100). Pots were destructively sampled at day 0, 1, 7, 15, and 25 after urine application. AOB/AOA *amoA* gene abundance was analysed by real-time quantitative polymerase chain reaction at Days 1 and 15. AOB *amoA* gene abundance increased 2.1- and 2.5-fold in the Pallic soil, and 10.0- and 22.6-fold in the Recent soil for the Cu10 compared with Cu0 on Days 1 and 15, respectively. In contrast, the Cu100 was associated with a reduction in AOB *amoA* gene abundance in the Pallic and Recent soils but not in the Pumice soil. This may be due to the influence of soil cation exchange capacity differences on the bioavailable Cu. Bioavailable Cu in the Pallic and Recent soils influenced nitrification and AOB *amoA* gene abundance, as evidenced by the strong positive correlation between bioavailable Cu, nitrification, and AOB *amoA*. However, bioavailable Cu did not influence the nitrification and AOA *amoA* gene abundance increase.

Key words: Bioavailable copper; Nitrification; Ammonia-oxidizing bacteria; Ammonia-oxidizing archaea.

4.1 Introduction

Grazing livestock inefficiently utilise ingested N (Kebreab et al. 2001). About 80% of ingested N is excreted with urine and several studies have shown that a significant proportion of N can be lost from the soil via NO_3^- -N leaching before it can be taken up by plants (Di and Cameron 2002a; Di and Cameron 2016). Leached NO_3^- -N can trigger environmental problems such as water and air pollution (Rex et al. 2021; Richards et al. 2021; Yu et al. 2019), and science-led strategies to improve NO_3^- -N management are actively sought to decrease the impact of N losses.

Nitrification is a naturally-occurring process that describes the microbial oxidation of NH_4^+ -N to NO_3^- -N via NO_2^-. The first step of the nitrification process is performed by AOB and AOA (Carey et al. 2016; Prosser and Nicol 2012). There are unique biological characteristics between AOB and AOA that are mostly influenced by environmental conditions (Lu et al. 2020; Prosser et al. 2020). For example, oxidizing bacteria have low affinity of NH_4^+ -N, and several studies have reported AOB to be dominant in high NH_4^+ -N soils (Carey et al. 2016; Jia and Conrad 2009). In contrast, AOA are dominant in low NH_4^+ -N conditions because of their high affinity to NH_4^+ -N (Nicol et al. 2008; Rütting et al. 2021; Ying et al. 2010). Both AOB and AOA possess the AMO enzyme encoded in the *amoA* gene, which is responsible for catalysing the first and often rate limiting step of NH_4^+ -N oxidation into NH_2OH (Principi et al. 2009).

Previous studies demonstrated that the AMO enzyme contains a Cu-active site, making Cu a co-factor in the functioning of the enzyme (Hooper et al. 1997; McCarty 1999). For example, Vandevivere et al. (1998) showed that application of 5 mg Cu L^{-1} as $CuSO_4$ improved NH_4^+ -N oxidation in sewage sludge, while Wagner et al. (2016) found that increasing the Cu dose from 0.05 to 5 µg Cu L^{-1} significantly increased NH_4^+ -N oxidation during water treatment. However, several studies present contrasting results on the effect of Cu on NH_4^+ -N oxidation (Loveless and Painter 1968; Wagner et al. 2016). Cela and Sumner (2002) demonstrated that a water-extractable Cu concentration above 3.8 mg kg^{-1} severely inhibited nitrification in three soils. He et al. (2018) reported that a Cu concentration above 100 mg Cu kg^{-1} significantly reduced AOB *amoA* gene transcripts in an incubation study with a Fluvo-aquic soil (Shondong, China) relative to a control. Scientific literature, therefore, indicates that there is a lack of clear evidence on the relationship between Cu concentrations in soils and AOB/AOA functioning genes.

In Chapter 3 Cu is identified as an important trace element in the process of nitrification in three pastoral soils (Pumice, Pallic, and Recent soils). The data demonstrated that reducing the Cu concentration in these soils negatively affected the nitrification rate in the soil. However, relative changes in the profile of ammonia nitrifiers were not determined. Therefore, to provide direct evidence of the significance of Cu on microbial nitrification processes, the current study was conducted to evaluate the effect of Cu on bacterial and archaeal population, and AOB/AOA *amoA* gene expression. In this study, *amoA* gene abundance has been used to quantify the AMO enzyme activity responsible for the first step of ammonia oxidation. To my knowledge, no study has previously explored the relationship between bioavailable Cu and AOB/AOA in pastoral soils.

The present study examines the response of bacterial and archaeal total population, and AOB/AOA *amoA* gene abundance, to different Cu concentrations in pastoral soils. The objective was to quantify the effect of bioavailable Cu on AOA/AOB and nitrification rate in the context of research programmes that are developing nitrification inhibitors for dairy pastoral systems. The aim of this work is to provide new insights into the relationship between Cu and ammonia nitrifiers in pastoral soils.

4.2 Materials and Methods

4.2.1 Soil collection and characterization

Bulk topsoil samples of Pumice, Pallic, and Recent soils were sampled to a depth of 20 cm. The soils were representative of the Orthic Pumice soil, Pallic Firm Brown, and Manawatu Recent soil in terms of the NZ soil orders (Typic Dystrustept, Typic Dystrustept, and Dystric Fluventic Eutrudept, respectively, according to the U.S. Soil Taxonomy classification; (Hewitt 2010)). Pumice soil was collected from a farm near Stratford in the Taranaki region (39°20'9"S, 174°18'20"E), Pallic soil was collected from the Canterbury region (43°34'13.15"S, 171°55'47.33"E), and Recent soil was collected from the Dairy 1 farm located at Massey University (40°23'0.95"S, 175°36'36.16"E). Soil samples were sieved through <2 mm stainless steel sieve and divided into two portions. The first portion was air-dried for soil pH and cation exchange capacity determination. The second portion was stored fresh at <4 °C for less than a week to minimise any changes that might occur before use in the pot experiment described in this paper. Sub-samples were analysed for mineral N, moisture, and water holding capacity. All soil chemical properties were analysed using the methods described in section 3.2.1.1. A summary of chemical parameters is presented in Table 4.1. These soils are the dominant soils in NZ under dairy pastoral system and were used in this study because they present contrasting soil properties.

Table 4.1 Soil chemical characteristics

Parameter	Pumice soil	Pallic soil	Recent soil
Soil pH	5.8	5.2	5.8
NH_4^+ -N (mg kg^{-1})	1.68	3.02	2.22
NO_3^- -N (mg kg^{-1})	21.40	38.01	24.29
Bioavailable Cu (mg kg^{-1})	0.28	0.19	0.11
Exchangeable cations (cmol$_c$ kg^{-1})			
Ca	3.4	3.5	4.5
Mg	0.76	0.58	1.15
K	0.33	0.46	0.44
Na	0.09	0.08	0.11
^{a}CEC (cmol$_c$ kg^{-1})	20	13	11
^{b}BS (%)	27	36	56
^{c}WHC (%)	80.6	45.9	31.5

Note: ^{a}CEC = Cation Exchange Capacity; ^{b}BS = Base Saturation; ^{c}WHC = water holding capacity

4.2.2 Treatments and application

This study was conducted using five treatments with three replicates of five sets in each soil: control (no urine and no Cu), urine-only at 300 mg N kg^{-1} (Cu0), urine + 1 mg Cu kg^{-1} (Cu1), urine + 10 mg Cu kg^{-1} (Cu10), and urine + 100 mg Cu kg^{-1} (Cu100). The applied Cu concentration ranges were selected based on conditions of deficiency, sufficiency, and toxicity with respect to the bioavailable Cu concentration in soil (Cela and Sumner 2002). Hydrated copper sulphate ($CuSO_4.5H_2O$) was used as the Cu source, with application rate calculated using the dry weight (DW) of the different soils to achieve the required concentrations. Field moist soil (0.5 kg) was spiked with the specified treatment and filled into each pot (11.5 cm top internal diameter x 10.4 cm slopping height). Soil spiking and mixing were done as described by Ubeynarayana et al. (2021). Soils were incubated for 3 weeks at 25 °C to equilibrate the Cu with the soil matrix. The soil was maintained at 70% water-filled pore space throughout the incubation period by weighing pots every after 2 days and maintaining the moisture content with deionised water.

Pots were transferred into the greenhouse at the Massey University Plant Growth Unit after 21 days of soil incubation and were arranged in a randomized complete block design. Perennial ryegrass (*Lolium perenne* L. 'Maxsyn NEA4') seeds (20 seeds pot^{-1}) were planted to model the dominant pasture cover for NZ dairy soils. Ryegrass was thinned to maintain 15 plants pot^{-1} after 7 days of germination. Ryegrass was then allowed to establish for four weeks before urine application.

4.2.3 Synthetic urine preparation and application

Synthetic urine was prepared using the formulation described by Clough et al. (1998): urea applied at 11.7 g L^{-1}, glycine at 2.90 g L^{-1}, $KHCO_3$ at 13.98 g L^{-1}, K_2SO4 at 1.38 g L^{-1}, and KCl at 5.04 g L^{-1}. Four weeks after ryegrass establishment, a calculated amount of synthetic urine was applied at an equivalent rate of 300 mg N kg^{-1} to all treatments except the no-urine control. Daily watering was done after weighing each pot to ensure 70% water-filled pore space during the ryegrass growth period. Average day and night temperatures were recorded over the time period from synthetic urine application to last harvest (Figure 4.1).

4.2.4 Plant and soil sampling

Destructive pot sampling was done in the lab at Day 0 (before urine application), and at Day 1, 7, 15, and 25 after urine application (three replicates sampled at each time point). A total of 225 samples were collected throughout the experimental period (5 treatments x 3 replicates x 3 soils x 5 times). Plants were gently pulled from the soil by hand, and rhizosphere soil around the roots was removed by shaking before roots were washed several times using tap water. The ryegrass shoots and roots were separated using a set of stainless steel scissors (Matse et al. 2020). The DW of shoot and root biomass was recorded after oven-drying at 65 °C for 72 h. During sampling, the field moist soil from each pot was homogenised and subsampled into two parts. The first part was immediately stored at -20 °C for soil DNA extraction. The second part was kept at <4 °C for NH_4^+ -N and NO_3^- -N, and bioavailable Cu analysis.

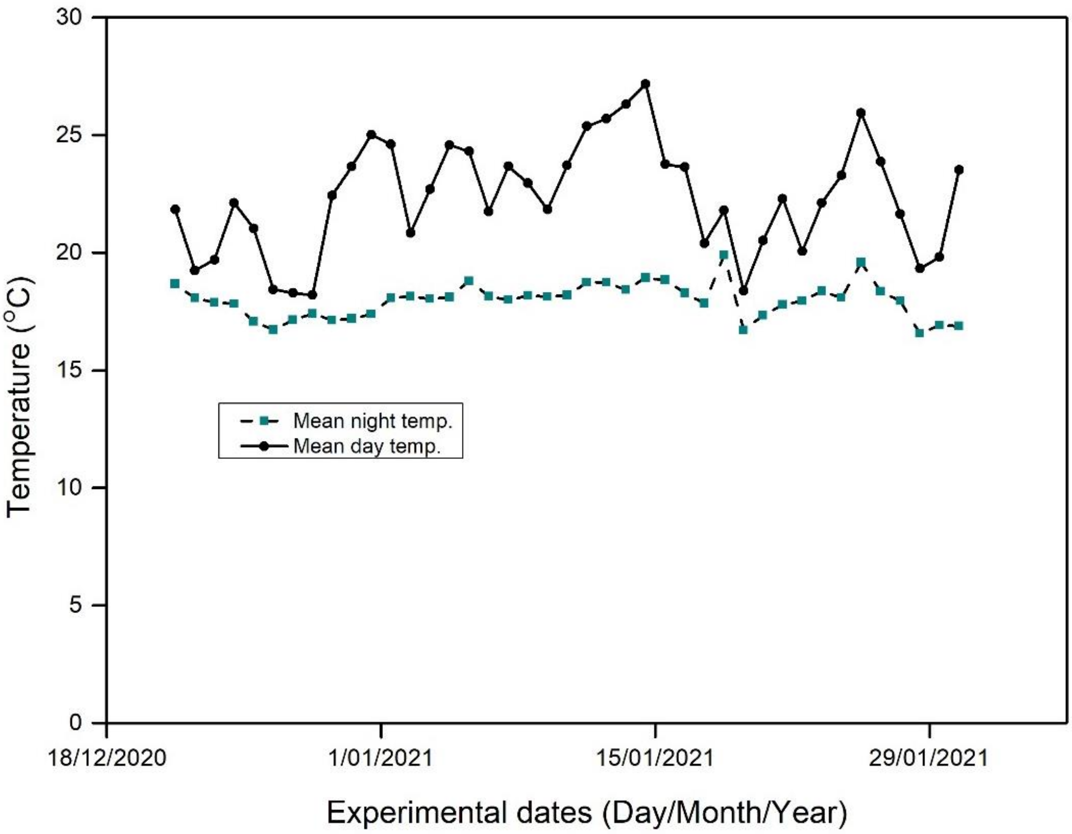


Figure 4.1 The average day and night temperatures during the glasshouse experiment.

4.2.5 Soil analysis

Soil bioavailable Cu concentration was measured through extraction of 5 g field moist soil with 30 ml, 0.05 $CaCl_2$ in an end-over-end shaker for 2 h. The resulting extraction was centrifuged at 1,100 g for 10 min, then filtered through Whatman 42 filter papers before analysis of bioavailable concentration Cu using microwave plasma atomic emission spectroscopy (4200 MP-AES, Agilent).

Soil mineral NH_4^+ -N and NO_3^- -N concentration was measured through extracting 5 g field moist soil with 30 ml, 2 M KCl in an end-over-end shaker for 1 h. The resulting extraction was centrifuged at 1,100 g for 10 min then filtered through Whatman 42 filter papers before analysis of soil mineral NH_4^+-N and NO_3^--N concentration using a Technicon autoanalyzer (Blakemore 1987).

4.2.6 Quality control measures

Two certified standard reference materials (SRM 2710a, Montana soil and SRM 1573a, tomato leaves) were analysed during all sample runs. Recovery concentrations of the tomato leaves and Montana soil ranged from 92.4 to 98% and 93.4 to 98.6%, respectively, of certified Cu concentration values. Further, blanks and samples of known concentrations were included after ten samples in each run. In addition, the instrument itself triplicated each sample analysis as a default setting.

4.2.7 Soil DNA extraction and quantitative polymerase chain reaction (qPCR)

Dai et al. (2013) indicated that the ammonia oxidiser activities peak approximately 15 d after treatment application. Therefore, soil sampling was done only on Day 1 and 15 for the microbial gene analysis. Soil DNA was extracted from 0.25 g of soil using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. DNA quantity was confirmed by the ratio of absorbance at 260 and 280 nm using a NanoDrop ® ND-1000 (Thermo Fisher Scientific Inc., USA). The quality of the DNA was further confirmed using gel electrophoresis (1.5% TAE-Agarose gel, Tris-acetate-EDTA).

Quantitative polymerase chain reaction (qPCR) of AOB *amoA*, AOA *amoA*, bacterial 16S rRNA, and archaeal 16S rRNA was performed on a LightCycler® 480II (Roche, software release 1.5.1.62 SP3) based on the fluorescence intensity during amplification using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories Inc., USA). Each DNA soil sample was analysed in duplicate during the qPCR reaction. The primers used, sequences, and qPCR conditions are outlined in Table 4.2 and Table 4.3. The qPCR reaction mixture (total of 10 µL) contained 1 µL of 10-fold diluted DNA samples, 1x SsoFast™ EvaGreen® Supermix, and 0.52 µL of each forward and reverse primer. The qPCR standard curves were performed using DNA samples with known concentration serially diluted ranging from 10^2 to 10^7. The qPCR efficiency for each primer pair ranged between 89 and 98%. Each DNA soil sample was analysed in duplicate during the qPCR reaction. Each qPCR 96-plate run contained triplicates of non-template control samples for each primer pair. The copy of target gene per gram of soil was calculated according to Behrens et al. (2008).

Table 4.2 The different primers and primer sequence used in the qPCR reaction.

Target group	**Primers**	**Length of amplicon (bp)**	**Sequence (5'-3'**	**Annealing temp (°C)**	**Reference**
AOB-*amoA*	*amoA*-1F *amoA*-2R	490	GGGGHTTYTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	54	(Rotthauwe et al. 1997)
AOA-*amoA*	Arch-*amoA*F Arch-a*moA*R	635	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	54	(Francis et al. 2005)
Bacterial 16S rRNA	27F 519R	530	AGAGTTTGATCMTGGCTCAG GWATTACCGCGGCKGCTG	56	(Mao et al. 2012)
Archaea 16S rRNA	A364AF A934BR	554	CGGGGYGCASCAGGCGCGAA GTGCTCCCCCGCCAATTCCT	56	(Blöchl et al. 1997; Großkopf et al. 1998)

Table 4.3 The different primer's reaction conditions.

Target group	Primers	Sequence (5'-3'	Thermal cycling
AOB-*amoA*	*amoA*-1F	GGGGHTTYTACTGGTGGT	94°C-120 s, 94°C-20 s, 40 cycles;
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC	54°C-30 s, 72°C-30 s, 72°C-180 s
AOA-*amoA*	Arch-*amoA*F	STAATGGTCTGGCTTAGACG	94°C-120 s, 94°C-20 s, 40 cycles;
	Arch-a*moA*R	GCGGCCATCCATCTGTATGT	54°C-30 s, 72°C-30 s, 72°C-180 s
Bacterial 16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	94°C-120 s, 94°C-20 s, 40 cycles;
	519R	GWATTACCGCGGCKGCTG	56°C-30 s, 72°C-30 s, 72°C-180 s
Archaea 16S rRNA	A364AF	CGGGGYGCASCAGGCGCGAA	94°C-120 s, 94°C-20 s, 40 cycles;
	A934BR	GTGCTCCCCCGCCAATTCCT	56°C-30 s, 72°C-30 s, 72°C-180 s

4.2.8 Statistical analysis

All results presented in this work were tested for normality and analysis of variance (ANOVA) followed by Turkey's post hoc test to determine significant differences between applied treatments means using Minitab (Version 19. Minitab Inc., USA).

4.3 RESULTS

4.3.1 Changes in bioavailable Cu concentration

There was a trend toward increasing bioavailable Cu concentration in soil as a function of the applied Cu (Figure 4.2). For the Pumice soil, the Cu1 treatment did not induce significant changes in bioavailable Cu relative to Cu0, while the Cu10 treatment showed a nominal increase with time. The Cu100 treatment increased bioavailable Cu across all samplings relative to the Cu0 treatment. For the Pallic soil, no clear changes were induced by the application of the Cu1 treatment relative to Cu0. The Cu10 and Cu100 treatments increased bioavailable Cu at all sampling days relative to Cu0. In the Recent soil, all Cu treatments (Cu1, Cu10, and Cu100) showed increased bioavailable Cu for all sampling days relative to the Cu0 treatment (Figure 4.2).

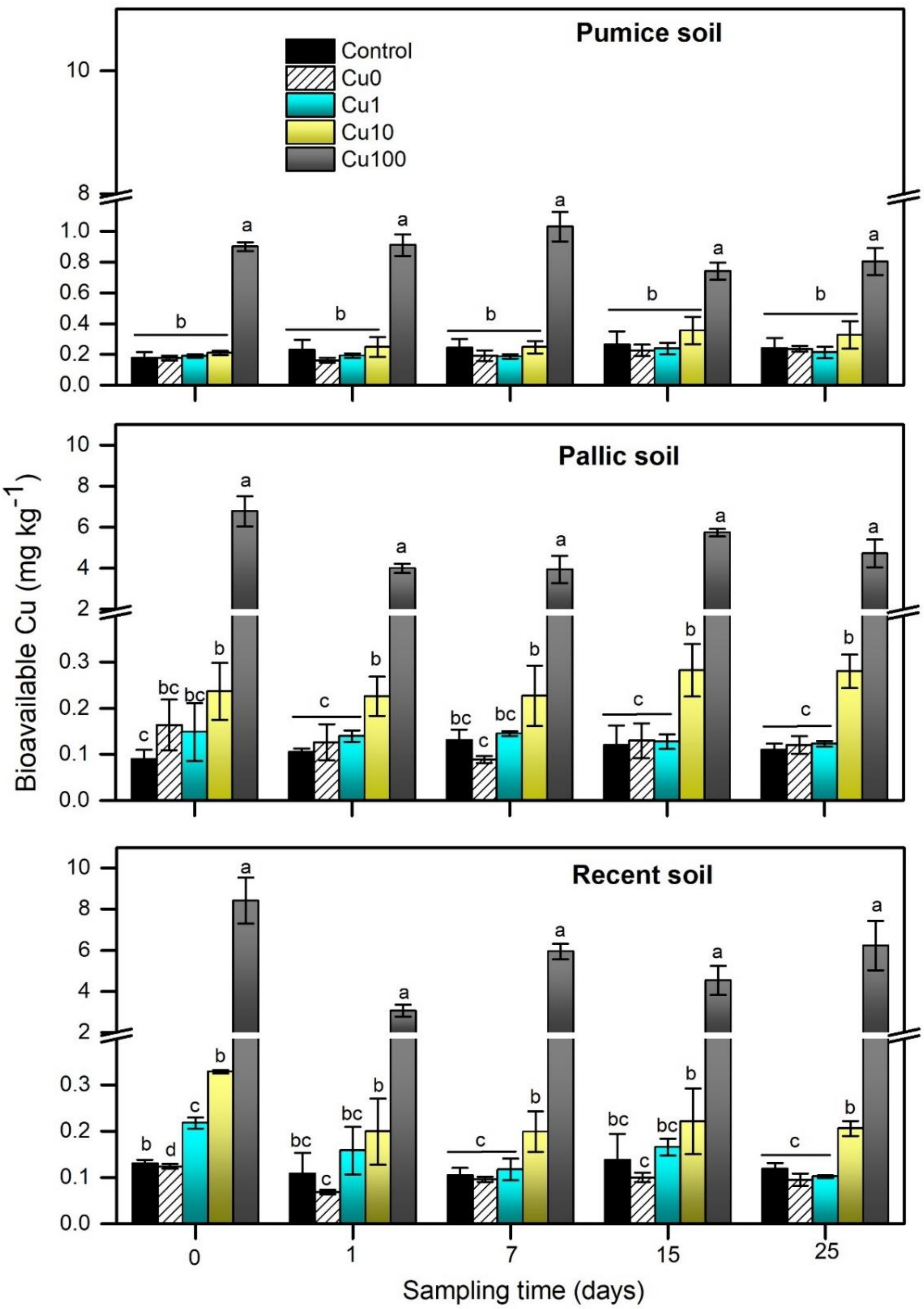


Figure 4.2 Bioavailable Cu concentration in Pumice, Pallic, and Recent soils as a function of treatments and sampling time. Error bars indicate standard deviation of mean (*n* = *3*). Control = no urine and Cu: Cu0 = urine only at 300 mg N kg^{-1} soil: Cu1 = urine + 1 mg Cu kg^{-1} soil: Cu10 = urine + 10 mg Cu kg^{-1} soil: Cu100 = urine + 100 mg Cu kg^{-1} soil.

4.3.2 Soil NH_4^+ -N and NO_3^- -N concentration

Copper had no effect on the NH_4^+ -N concentration for the Pumice soil but a variable effect on the NH_4^+ -N concentration for the Pallic and Recent soils (Figure 4.3). For the Pumice soil, there was no significant change in NH_4^+ -N concentration associated with any treatment, with the exception of the Cu100 treatment on Day 7, which was significantly lower than the Cu0 treatment. For the Pallic soil, there was no significant change in the NH_4^+ -N concentration induced by the Cu1 treatment at any sampling time relative to the Cu0 treatment. However, the Cu10 treatment reduced the NH_4^+ -N concentration by a factor of 23 and 40% compared with Cu0 on Days 1 and 7, respectively (Figure 4.3). The Cu100 treatment was associated with an increase in NH_4^+ -N concentration by factors of 18 and 106% relative to the Cu0 at Days 1 and 7, respectively. For the Recent soil, the Cu1 treatment reduced the NH_4^+ -N concentration by a factor of 24 and 45% relative to the Cu0 treatment, at Days 1 and 7, respectively. There was a similar reduction for the Cu10 treatment, by 32 and 39% at Days 1 and 7, respectively. In the Cu100-treated soil, there was a nominal increase in NH_4^+ -N relative to the Cu0 treatment but this was only apparent on Day 1 (Figure 4.3).

The NO_3^- -N concentration was influenced by Cu treatment in all three soils. For the Pumice soil, there was a reduction in NO_3^- -N concentration induced by the application of Cu1 and Cu10 at Days 7 and 15 (Figure 4.3). There was no consistent effect of the Cu100 treatment on soil NO_3^- -N concentration in the Pumice soil relative to the Cu0 treatment. For the Pallic soil, there was no significant effect of the Cu1 treatment on soil NO_3^- -N concentration relative to the Cu0 treatment at any sampling time, however the Cu10 treatment increased the NO_3^- -N concentration by values of 205 and 37% at Days 1 and 7, respectively. The Cu100 treatment showed a nominal reduction in NO_3^- -N concentration at Days 7 and 15 compared with Cu0. For the Recent soil, the Cu1 treatment increased the NO_3^- -N concentration by 38 and 18% on both Days 1 and 7, respectively, relative to Cu0. The Cu10 treatment increased the NO_3^- -N concentration by 145 and 48% (relative to Cu0) at Days 1 and 7, respectively (Figure 4.3). In contrast, the Cu100 treatment reduced the NO_3^- -N concentration by factors of 27 and 31% at Days 1 and 7, respectively.

4.3.3 Bacterial and archaeal population in soil

The total bacterial and archaeal population in the soil was analysed based on the abundance of 16S rRNA (Figure 4.4). There was no effect of the Cu1 treatment on bacterial population when compared to the Cu0 treatments in any of the three soils at either Day 1 or 15. Similarly, there was no significant increase in bacterial population that could be attributed to Cu10 and Cu100 for the Pumice soil. However, the bacterial population in the Cu10 soil was increased by 1.4- and 1.5-fold in the Pallic soil, and 1.6- and 3.3-fold in the Recent soil, for Days 1 and 15, respectively relative to the Cu0 treatment.

The archaeal population in the control treatment was higher in all three soils relative to the Cu0 treatment irrespective of sampling time (Figure 4.4). Application of urine to the Cu1 and Cu10 treatments did not change the archaeal population relative to Cu0 treatments at any sampling time for any of the three soils. The Cu100 treatment showed higher archaeal population in the Pumice and Recent soils relative to the lower Cu treatments at both sampling times, although this increase was not apparent for the Pallic soil.

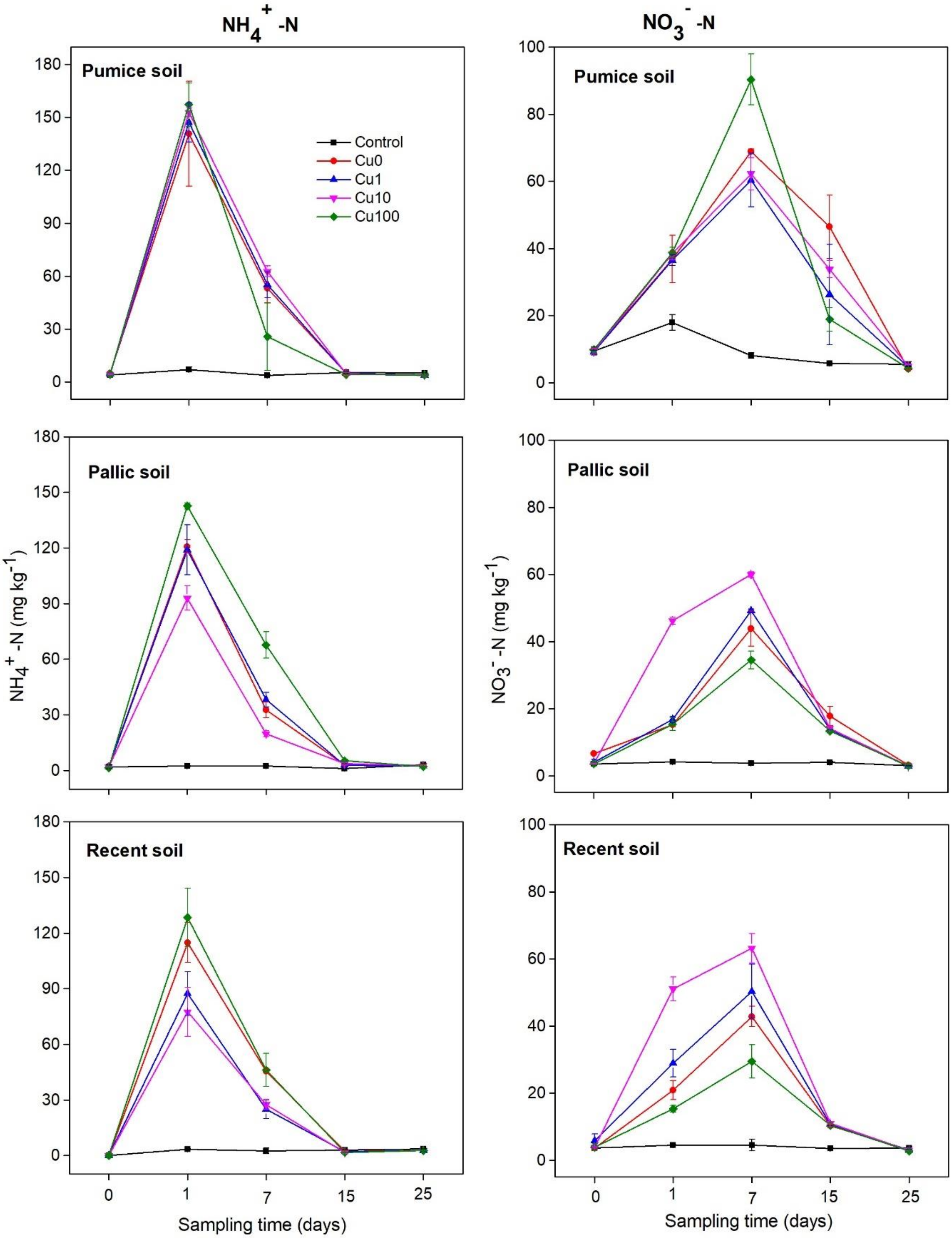


Figure 4.3 NH_4^+-N and NO_3^- -N concentration for Pumice, Pallic, and Recent soils as a function of treatments and sampling time. Error bars indicate standard deviation of mean (*n = 3*). Control = no urine and Cu: Cu0 = urine only at 300 mg N kg^{-1} soil: Cu1 = urine + 1 mg Cu kg^{-1} soil: Cu10 = urine + 10 mg Cu kg^{-1} soil: Cu100 = urine + 100 mg Cu kg^{-1} soil.

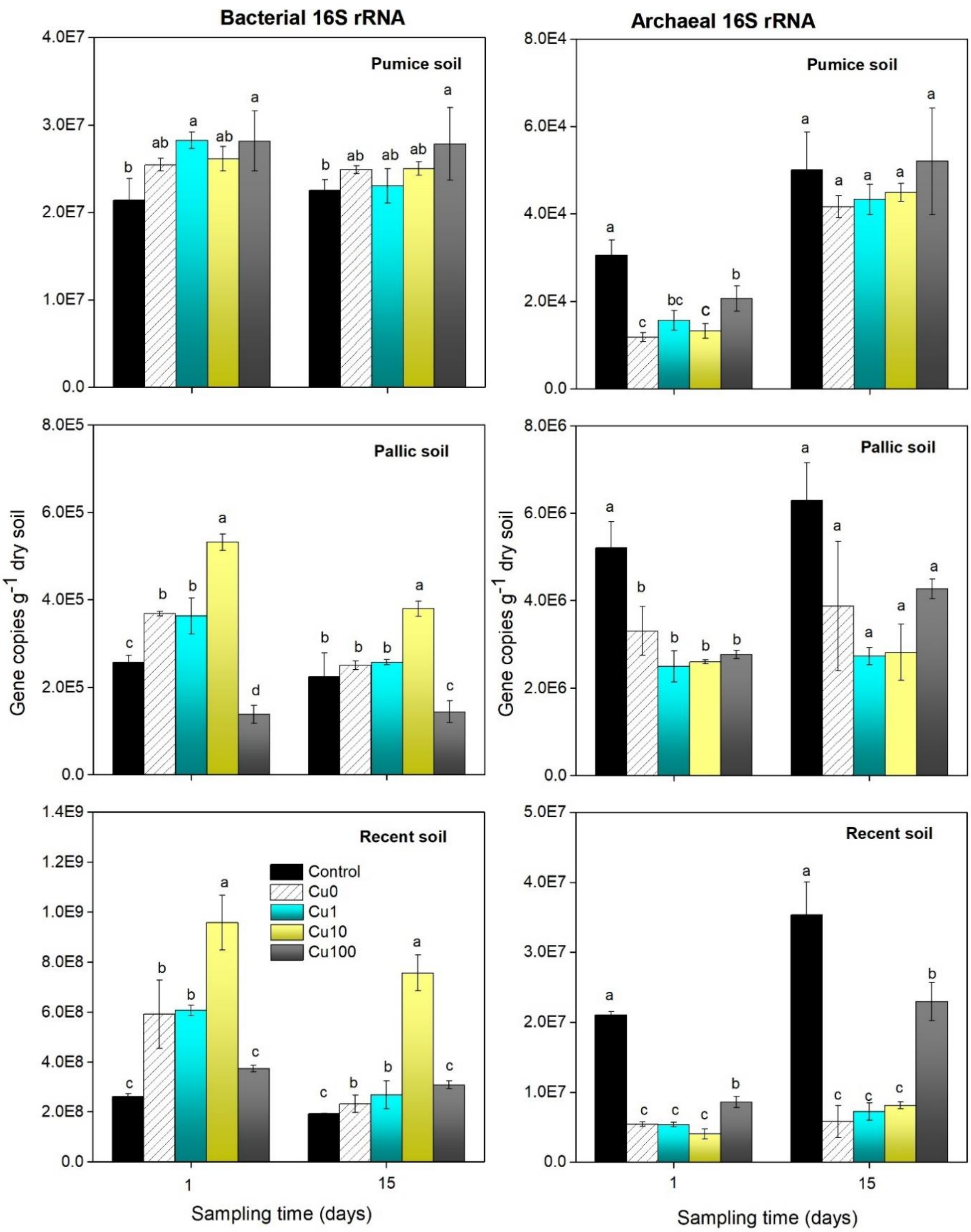


Figure 4.4 Abundance of bacterial and archaeal 16S rRNA for Pumice, Pallic, and Recent soils as a function of treatments and sampling time. Different small letters in the same sampling day represent significant difference (P < 0.05). Vertical error bars represent standard deviation of mean (n = 3). Control = no urine and Cu: Cu0 = urine only at 300 mg N kg^{-1} soil: Cu1 = urine + 1 mg Cu kg^{-1} soil: Cu10 = urine + 10 mg Cu kg^{-1} soil: Cu100 = urine + 100 mg Cu kg^{-1} soil.

4.3.4 AOB/AOA amoA gene abundance in soil

There were no significant differences in AOB *amoA* gene abundance between the Cu0 and Cu1 treatment for all three soils at all sampling times (Figure 4.5). However, for the Cu10 treatment, AOB *amoA* gene abundance increased 2.1- and 2.5-fold in the Pallic soil and 10.0- and 22.6-fold in the Recent soil relative to the Cu0 treatment on Days 1 and 15, respectively (Figure 4.5). This increase was not apparent for the Pumice soil. After application of the Cu100 treatment, AOB *amoA* abundance increased 1.2- and 1.2-fold in the Pumice soil relative to the Cu0 treatment on Days 1 and 15, respectively. In contrast, for the Cu100 treatment, AOB *amoA* gene abundance reduced 2.6- and 2.5-fold in the Pallic soil and 7.4- and 1.3-fold in the Recent soil relative to the Cu0 on Days 1 and 15, respectively.

Generally, there was an increase in AOA *amoA* gene abundance in the control treatment (relative to the urine treatments) in all three soils for both sampling times (Figure 4.5). There were no significant changes in AOA *amoA* gene abundance associated with the Cu1 or Cu10 treatments on Days 1 and 15 for all three soils relative to Cu0 treatment, but there was an increase in abundance for the Pallic and Recent soils at the Cu100 treatment level on Days 1 and 15 (Figure 4.5). There was no effect of Cu100 treatment on AOA *amoA* gene abundance for the Pumice soil.

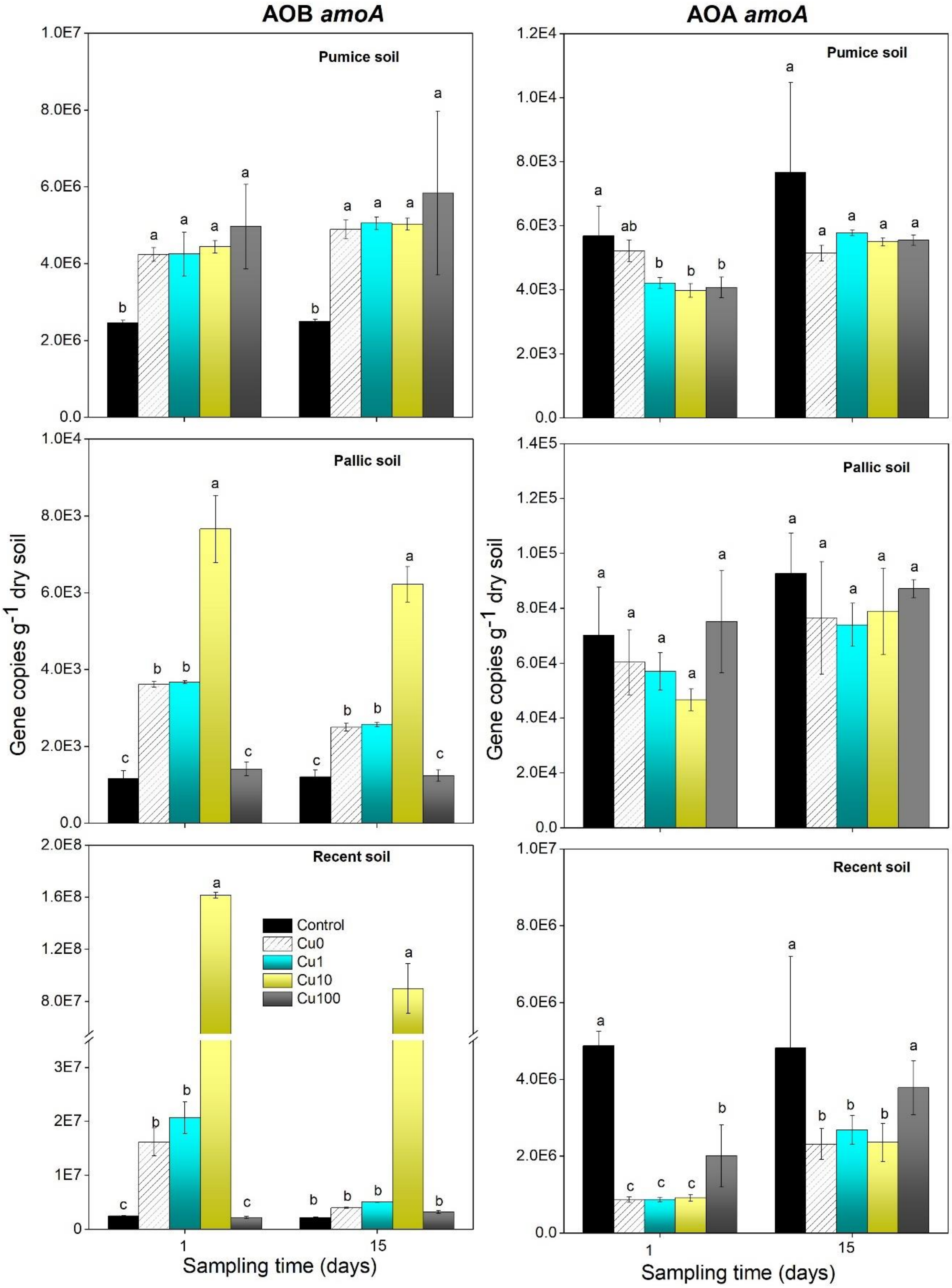


Figure 4.5 Abundance of AOB/AOA *amoA* gene for Pumice, Pallic, and Recent soils as a function of treatments and sampling time. Different small letters in the same sampling day represent significant difference ($P < 0.05$). Vertical error bars represent standard deviation of mean ($n = 3$). Control = no urine and Cu: Cu0 = urine only at 300 mg N kg^{-1} soil: Cu1 = urine + 1 mg Cu kg^{-1} soil: Cu10 = urine + 10 mg Cu kg^{-1} soil: Cu100 = urine + 100 mg Cu kg^{-1} soil.

4.3.5 Perennial ryegrass growth

There was a trend towards increasing shoot DW as a function of time and treatment for all three soils. For the Cu0, Cu1, and Cu2 treatments, there were no significant differences in mean DW between for the Pumice and Pallic soils (Figure 4.6). The Cu1 and Cu10 treatments increased the shoot DW by an average of 64 and 83%, respectively, for the Recent soil relative to Cu0. For the Cu100 treatment, there was no significant effect in DW for the Pumice soil relative to Cu0 treatment. There was a general trend for reduced DW associated with the Cu100 treatment relative to the lower Cu levels for the Pallic and Recent soils. An increase in shoot DW due to the Cu100 treatment relative to Cu0 was only recorded for the Recent soil on Day 25 (Figure 4.6).

Root DW tended to increase for the Cu1 and Cu10 treated soils relative to the Cu0 treatment, but DW gains were mitigated by the Cu100 treatment (Figure 4.6). An increase in root DW due to the Cu1 treatment was recorded in all samplings for the Recent soil, with no clear changes for the Pumice and Pallic soils. Similarly, there were no changes in root DW for the Cu10 treatment recorded for the Pumice and Pallic soils, but there was a significant increase in the Recent soil after Day 1. Generally, there was no effect in root DW induced by the Cu100 treatment in the Pumice soil. However, there was reduction in root DW induced by the Cu100 treatment for the Pallic and Recent soils relative to the Cu0 treatment.

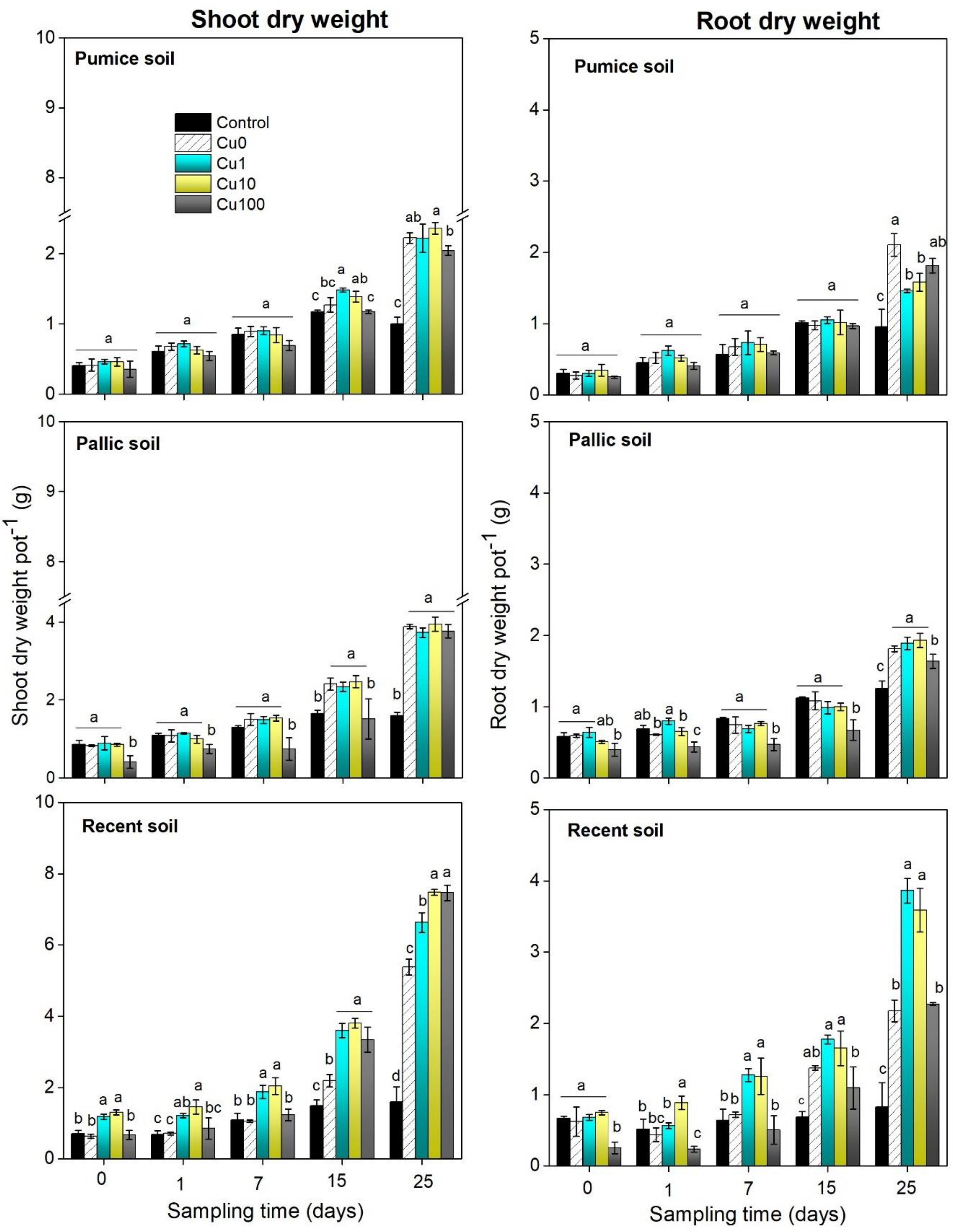


Figure 4.6 Shoot and root dry weight for the Pumice, Pallic, and Recent soils as a function of treatments and sampling time. Vertical error bars indicate standard deviation of mean (*n = 3*). Control = no urine and Cu: Cu0 = urine only at 300 mg N kg^{-1} soil: Cu1 = urine + 1 mg Cu kg^{-1} soil: Cu10 = urine + 10 mg Cu kg^{-1} soil: Cu100 = urine + 100 mg Cu kg^{-1} soil.

4.4 Discussion

4.4.1 Dynamics of the NH_4^+ -N and NO_3^- -N

Following urine application to the soils of this study there was an increase in NH_4^+ -N concentration that is attributed to the rapid hydrolysis of urea in the urine by the urease enzyme (Cameron et al. 2013). Within 15 days after urine application, there was rapid oxidation of NH_4^+ -N to NO_3^- -N, which resulted in accumulation of NO_3^- -N in the soil (Figure 4.3). These observations are consistent with results reported in previous studies (Duan et al. 2019; Hink et al. 2018; Williams and Haynes 2000). The rapid nitrification rate observed in this study, may have been influenced by optimal environmental conditions during this experimental period such as temperature, soil moisture and N availability (Cameron et al. 2013). It is proposed that the decline in NO_3^- -N after 15 days is associated with N uptake by grass.

4.4.2 Effect of Cu application on NH_4^+ -N oxidation to NO_3^- -N

Significant changes in the NH_4^+ -N and NO_3^- -N concentrations in soil were induced by Cu treatments, with the change varying between soils. The lower Cu treatment induced a reduction in NH_4^+ -N in the Recent soil, whereas the Cu10 treatment induced a significant reduction in both the Recent and Pallic soils (Figure 4.3). The reduction in NH_4^+ -N corresponded with an increase in NO_3^- -N concentration in soil; this demonstrates that there was an increase in the oxidation of NH_4^+ -N to NO_3^- -N. There was correlation between the increase in NO_3^- -N with an increase in the bioavailable Cu concentration in both soils (Pallic soil, $r = 0.748$, $P < 0.05$ [Appendix 1 Table A1.2] and Recent soil, $r = 0.937$, $P < 0.01$ [Appendix 1 Table A1.3]). To further analyse the effect of the different Cu concentrations on changes in mineral N, the ratio of NO_3^- -N/NH_4^+ -N was calculated (Chen et al. 2021) (Figure 4.7). The ratio of NO_3^- -N/NH_4^+ -N quantified for the Cu10 treatment was greater in the Pallic and Recent soils, providing strong evidence that this treatment had a significant effect on nitrification rate in these two soils.

Copper has been reported in various pure cell incubation and water treatment studies to play a significant role in ammonia oxidation (Gwak et al. 2020; Matse et al. 2022a; Wagner et al. 2019). For example, in Chapter 3 it is demonstrated that increasing the Cu concentration from 0.1 to 3 mg Cu kg^{-1} significantly increased the soil nitrification rate. Results from the current Chapter therefore provide strong evidence that the change in NH_4^+ -N in the Pallic and Recent soils was influenced by the Cu concentration in the soil. In the Pumice soil, there was no change in NH_4^+ -N concentration induced by the Cu1 or Cu 10 treatments, suggesting that these Cu treatments did not effect the bioavailable Cu concentration in the Pumice soil.

Increasing the applied Cu concentration to 100 mg kg^{-1} (Cu100 treatment) induced a significant increase in bioavailable Cu concentration in all three soils (Figure 4.2), and this was associated with a higher NH_4^+ -N concentration in the Pallic and Recent soils (Figure 4.3). The higher NH_4^+ -N concentration corresponded with a lower NO_3^- -N concentration, demonstrating that the high Cu level reduced nitrification in both soils, which may be due to Cu inducing toxicity to nitrifying microbes. The ratio of NO_3^- -N/NH_4^+ -N showed a reduction for the Cu100 treatment, providing evidence that this treatment had a toxicity effect to the nitrifying microbes (Figure 4.7). However, this reduction in nitrification and ratio of NO_3^- -N/NH_4^+ -N was not observed in the Pumice soil, where the absolute concentration of bioavailable Cu, while significantly greater than the control, was 6-8 times lower than in the Pallic and Recent soils. The difference in bioavailable Cu between the Pumice soil and the other two soils may be associated with differences in soil properties. The higher cation exchange capacity (CEC) of the Pumice soil compared with the Recent and Pallic soils (Table 4.1) may have led to greater adsorption of added Cu through formation of organo-metal complexes reducing the concentration of Cu in soil solution (Gao et al. 1997; Rieuwerts et al. 1998). In Chapter 3, it is reported that this Pumice soil was high in percentage Al and Fe oxides. These soil components may have complexed with Cu, reducing the bioavailable Cu concentration (Rieuwerts 2007).

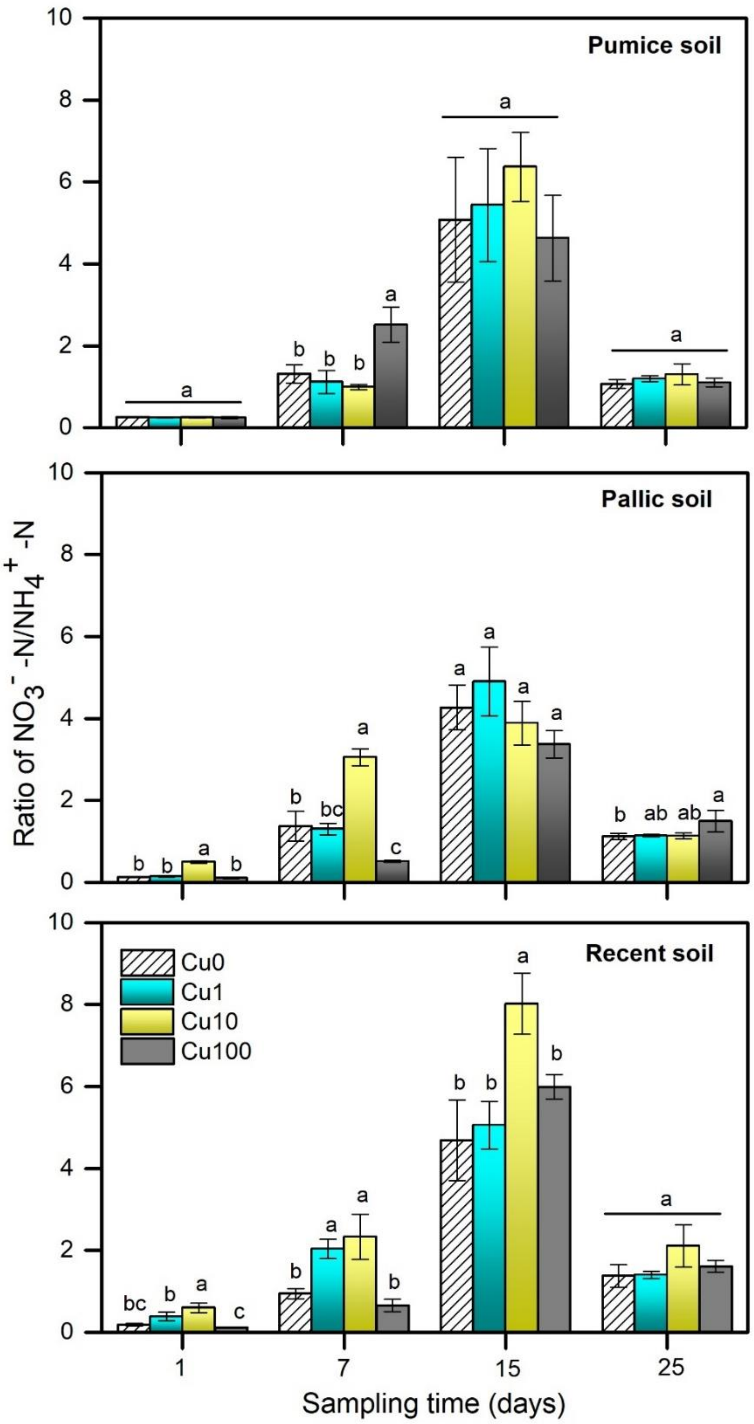


Figure 4.7 Ratio of NO_3^- -N/NH_4^+ -N for Pumice, Pallic, and Recent soils as a function of treatments and sampling time. Error bars indicate standard deviation of mean (*n* = *3*). The control treatment was excluded because no urine was added in this treatment and day 0 was before urine addition. Cu0 = urine only at 300 mg N kg^{-1} soil: Cu1 = urine + 1 mg Cu kg^{-1} soil: Cu10 = urine + 10 mg Cu kg^{-1} soil: Cu100 = urine + 100 mg Cu kg^{-1} soil.

4.4.3 Changes in microbial population and AOB/AOA amoA gene abundance

Results from this Chapter show that bacterial populations were dominant in the Pumice and Recent soils, but that archaeal populations were dominant in the Pallic soil (Figure 4.4). The low soil pH of the Pallic soil (5.2) compared to the Pumice (5.8) and Recent soil (5.8) may have been one of the key factors that increased the dominance of AOA over AOB in this soil. Dominance of AOA over AOB under conditions of low soil pH has been reported in several other studies (Gubry-Rangin et al. 2010; Waggoner et al. 2021; Zhang et al. 2012a). However, literature does not clearly describe whether numerical dominance at genomic level has an effect at the functional level.

An increase in the AOA population was observed when the NH_4^+ -N concentration was low (control) (Figure 4.4). This trend has been observed in other studies (Di et al. 2010; Huérfano et al. 2022; Ouyang et al. 2017; Waggoner et al. 2021) and has been associated with the ability of AOA to thrive under conditions of low NH_4^+ -N availability. In the present study, AOB abundance dominated at higher NH_4^+ -N availability (Day 1). This can be associated with the greater tolerance of AOB to high NH_4^+ -N concentration, which may be inhibitory to AOA (Ouyang et al. 2017), or due to the greater competition for substrate by AOB.

In terms of the Cu effect across the different soils, these results show that the Cu10 treatment significantly increased AOB *amoA* gene abundance in both the Pallic and Recent soils relative to all other applied treatments. This can be attributed to a beneficial increase in bioavailable Cu concentration in both of these soils. The greater AOB *amoA* abundance for the Pallic and Recent soils at the Cu10 treatment levels corresponded with a reduction in NH_4^+ -N and significantly higher NO_3^- -N recorded on Days 1 and 7 (Figure 4.3), demonstrating that nitrification in these soils was Cu limited. This data provides strong evidence that bioavailable Cu plays a significant role in influencing AOB *amoA* abundance in soil through a correlation between bioavailable Cu and AOB *amoA* (Pallic soil $r = 0.702$, $P < 0.05$ [Appendix 1 Table A1.2] and Recent soil $r = 0.940$, $P < 0.01$ [Appendix 1 Table A1.3]). With respect to AOA *amoA* gene abundance, there were no significant changes associated with the application of the Cu1 and Cu10 treated soils relative to the Cu0 treatment. Therefore, it can be concluded that the AOA *amoA* was not the dominant nitrifier responsible for nitrification rate at these Cu levels.

Soil Cu at the Cu100 treatment level inhibited AOB *amoA* abundance in the Pallic and Recent soils. The inhibition of Cu to AOB *amoA* in the present study was substantiated by the higher NH_4^+ -N concentration and lower NO_3^- -N in the Cu100 treatment (Figure 4.3), indicative of low nitrification taking place for this treatment. However, the toxicity effect of the Cu100 treatment was not observed in the Pumice soil. This was associated with the low bioavailable Cu concentration recorded in the Pumice soil (Figure 4.2) relative to the other two soils.

The AOA *amoA* gene showed greater abundance under the Cu100 treatment for both the Pallic and Recent soils. This behaviour was reported by He et al. (2018), where the AOA *amoA* gene showed greater dominance in a high Cu environment (100 mg kg^{-1} added Cu) than the AOB *amoA* gene. The greater tolerance of AOA to higher Cu concentration is associated with greater cell wall membrane rigidity than AOB, making this barrier less permeable to ions (Kandler and König 1998).

4.4.4 Effect of treatments on perennial ryegrass growth

The presence of Cu in soil at the Cu1 or Cu10 treatment level increased ryegrass shoot and root DW in the Recent soil (Figure 4.6); this can be attributed to the increase in bioavailable Cu in the soil (Figure 4.2). Copper is an important micronutrient in plant growth that is responsible for various metabolic processes and for the synthesis of chlorophyll (Rehman et al. 2019). Similar results were reported by Kumar et al. (1990), where application of 5 mg Cu kg^{-1} to soil resulted in a significant increase in shoot and root DW yield and increased N uptake by wheat (*Triticum aestivum* L.) plants. In the Pumice and Pallic soils, the Cu1 and Cu10 treatments did not increase shoot and root DW. This was because the concentration of Cu added in these treatments was insufficient to stimulate plant growth (no deficiency) or because the Cu concentration in soil was already at a level sufficient to support plant growth (sufficiency). These data show that the Cu concentration in the Recent soil is deficient for plant growth.

The reduction in root growth for the Recent and Pallic soils (Figure 4.6), associated with the highest Cu treatment (Cu100), suggests this level of applied Cu was toxic to ryegrass. Previous studies reported plant Cu toxicity at a similar treatment level. Yan et al. (2006) reported that 100 mg kg^{-1} added Cu reduced average rice (*Oryza sativa* L.) grain yield and straw height by 17.37 and 13.74%, respectively, relative to a control treatment. Xu et al. (2006) also found that application of 100 mg Cu kg^{-1} significantly decreased rice growth and grain by 22.13 and 10.76%, respectively. Copper growth inhibition effect was more apparent in ryegrass roots than shoots, possibly due to the limited translocation of Cu from root to shoots reported by Bolan et al. (2003). However, no Cu induced toxicity was apparent for the Pumice soil; this can be attribute to the limited increase in bioavailable Cu concentration for this soil compared with the other two soils.

4.5 Conclusion

The results obtained from the present study demonstrated that a bioavailable Cu concentration of up to 0.24 mg kg^{-1} in the Pallic soil and 0.33 mg kg^{-1} in the Recent soil increased nitrification rate. However, a bioavailable Cu concentration above 6 mg Cu kg^{-1} (Cu100 treatment) inhibited nitrifying bacteria and reduced nitrification rate in both these soils. For the Pumice soil, a bioavailable Cu concentration of up 0.8 mg Cu kg^{-1} did not induce an increase in nitrification rate and AOB/AOA *amoA* gene abundance. These results showed that AOA *amoA* is more resistant to Cu than AOB *amoA* due to the greater abundance of AOA *amoA* at higher bioavailable Cu concentrations. Bioavailable Cu was the main factor in the Pallic and Recent soils influencing both nitrification and AOB *amoA* gene abundance as evidenced by the strong positive correlation between bioavailable concentration, nitrification, and AOB *amoA*. The results from this study will help expedite the development of new inhibitors to reduce NO_3^- -N leaching in pastoral dairy systems.

CHAPTER 5

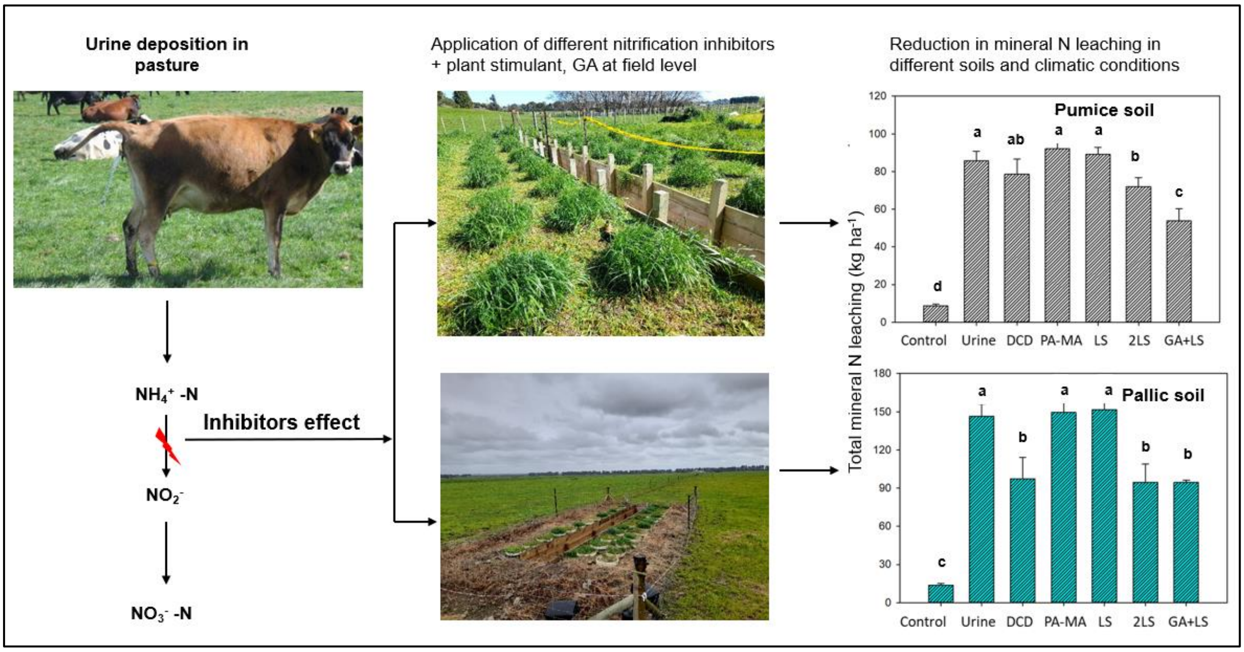
Nitrate leaching mitigation options in two dairy pastoral soils and climatic conditions in New Zealand

*This chapter was published in the **Plants Journal** in 2022. Citation:*

Matse, D. T., Jeyakumar, P., Bishop, P., & Anderson, C. W. N., (2022). Nitrate leaching mitigation options in two dairy pastoral soils and climatic conditions in New Zealand. Plants. 11(8): 24-30. doi: 10.3390/plants11182430.

Results in Chapter 3 showed that the organic compounds of LS and PA-MA have the potential to inhibit nitrification rate under controlled conditions. The next important step is to examine the potential application of these treatments in different soils under different climate and field management conditions. Therefore, a field lysimeter study discussed in Chapter 5 investigated the application of LS, PA-MA, and co-treatment of LS with GA on reducing NO_3^- -N leaching in the Pumice soil under a Manawatu climate and Pallic soil under a Canterbury climate conditions.

Graphical abstract



Abstract

This lysimeter study investigated the effects of late-autumn application of DCD, PA-MA, LS, a split-application of calcium lignosulphonate (2LS), and a combination of GA and LS (GA + LS) to reduce N leaching losses in lysimeter field sites at Manawatu (using Orthic Pumice soil) and Canterbury (using Pallic Orthic brown soil), NZ. The study was conducted between May and December 2020 (beginning in late-autumn). In a second application, urine-only, GA-only and GA + LS treatments were applied in July 2020 (mid-winter) on both sites. Results showed that late-autumn application of DCD, 2LS and GA + LS reduced mineral N leaching by 8, 16, and 35% in the Pumice soil, and by 34, 11, and 35% in the Pallic soil, respectively when compared to urine-only. There was no significant increase in cumulative herbage N uptake and yield between urine-treated lysimeters at both sites. Mid-winter application of GA and GA + LS reduced mineral N leaching by 23 and 20%, respectively, in the Pumice soil relative to the urine-only treated lysimeters. On the contrary there was no significant reduction in the Pallic soil. These results demonstrate the potential application of these treatments to different soils under different climate and management conditions.

Keywords: Organic inhibitors; Gibberellic acid; Urine patches; Nitrate leaching

5.1 Introduction

Nitrogen leaching from agricultural systems is a global environmental concern. In NZ, pastoral dairy farming is mainly characterised by dairy cows feeding outside all year round in pastures mainly dominated by perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens L.*) (Talbot et al. 2021). However, dairy cows only utilise a fraction (5-30%) of ingested N from these pastures, and a higher proportion (70-95%) of N is excreted with the urine resulting into small areas of highly concentrated N in pastures known as urine patches (Oenema et al. 2005). The urinary N concentration at each urine patch ranges from 200 to 2000 kg N ha^{-1} (Di and Cameron 2002a; Selbie et al. 2015) and usually this N rate exceeds plant N uptake. Therefore, the residual N becomes susceptible to leaching as NO_3^- -N into water sources. Nitrate concentrations greater than 11.2 mg L^{-1} NO_3^- -N in both surface and drinking water are deemed harmful to both human and animal health (Fan and Steinberg 1996). While concentrations above 0.4 mg L^{-1} NO_3^- -N may accelerate algal blooms and eutrophication of water bodies (Larned et al. 2016), thus reducing water quality. Decreasing the amount of NO_3^- -N leaching from urine patches is therefore important for lowering environmental impacts.

Different approaches have been developed and implemented to minimise N losses from grazed pastures (Malcolm et al. 2022; Meng et al. 2021). The use of nitrification inhibitors such as DCD and DMPP have been shown to reduce urine patch NO_3^- -N leaching in soils with high risk of leaching (Di and Cameron 2016; Meng et al. 2021). Reductions ranging from 10 to 76% relative to untreated urine patches have been shown in lysimeter studies (Di and Cameron 2012; Zaman and Nguyen 2012). Nitrification inhibitors have been shown (Duff et al. 2022; Shi et al. 2017) to reduce NO_3^- -N leaching through reducing the first step of the nitrification process: the oxidation of NH_4^+ -N to NH_2OH. However, in practice, a range of regulatory and technical constraints have limited their widespread use. Companies voluntarily withdrew the sales of

DCD in NZ following the detection of DCD residues in export milk powder in 2012 (Ministry of Primary Industries 2013). While, for DMPP, efficiency is known to be highly influenced by site conditions such as soil properties and climate, which implies that widespread deployment is difficult (Barth et al. 2001; Nair et al. 2021). Therefore, there is still a need to develop new inhibitors to reduce the environmental consequences associated with dairy farming.

In Chapter 3, it was observed that organic compounds have the ability to inhibit nitrification. The results demonstrated that the application of LS and PA-MA can slow nitrification, and the effect was associated with a reduction in soil bioavailable Cu. Calcium lignosulphonate is derived from the wood pulp industry and contains high levels of phenolic groups, while PA-MA is an acrylic acid-maleic acid copolymer solution. These compounds have shown a great potential to inhibit nitrification in a controlled environment and reduce the potential for leaching (Chapter 3). The research described in this Chapter is the first field study conducted to evaluate the effectiveness of LS and PA-MA in reducing NO_3^- -N leaching from urine patches under a wide range of soils and climatic conditions.

The main challenge in reducing NO_3^- -N leaching in NZ is that the peak NO_3^- -N leaching period in grazed pastural systems coincides with slow N uptake due to low temperatures (i.e., winter season). To overcome this shortfall in N uptake, Parsons et al. (2013) proposed the application of a plant growth stimulant, GA, to enhance plant growth and subsequent pasture N uptake. However, only Woods et al. (2016) examined the potential effect of GA in reducing N leaching. The study found that GA application to Italian ryegrass did not significantly reduce the amount of total NO_3^- -N leaching. This suggests that GA alone is not an effective treatment in reducing N leaching. Instead, an additional inhibitor might need to be applied with GA.

To address the challenges, the current study was conducted to determine the potential effect of nitrification inhibitors and to increase plant growth on NO_3^- -N leaching from dairy cow urine patches in different soils, environment, and management conditions. The hypothesise of this work is that (1) application of nitrification inhibitors might reduce nitrification in the soil, thus decreasing NO_3^- -N leaching and (2) the application of GA will reduce the excess of NO_3^- -N in the soil by increasing the N utilisation of pasture during periods of low N uptake, thus limiting N leaching.

5.2 Materials and Methods

5.2.1 Experimental sites and soils

This field lysimeter research was conducted at two different geographic locations: Massey University, Palmerston North, Manawatu (40°23'0.95"S175°36'36.16"E) in the North Island, and Hororata, Canterbury (43°34'13.15"S, 171°55'47.33"E) in the South Island of NZ. The Manawatu lysimeter soil columns contained intact Orthic Pumice soil (Hewitt 2010) collected from Wairakei, and transported to the Manawatu lysimeter facility. The Orthic Pumice soil has low bulk density and is well-drained with high plant-available water holding capacity (150-200 mm). The Canterbury lysimeter soil columns were intact Lismore Stony silt loam (Pallic Orthic Brown soil) (Hewitt 2010) collected from Hororata. This soil is characterised by an average bulk density and low plant-available water holding capacity (40-50 mm) and consists of a shallow layer of fine soil at the top surface, below which the gravel content increases significantly. This profile makes the Pallic orthic brown soil free draining. The soils selected for this study are representative of soils supporting the highest dairy cow numbers in NZ: stock at Canterbury and Waikato dairy farms represent 19.7 and 22.4% of total dairy cows in NZ, respectively (DairyNZ 2021). In addition, these soils present different properties in terms of water holding capacities which can influence the rate of leaching.

5.2.2 Lysimeters collection and pastures

The research described in this Chapter was undertaken in a lysimeter facility established in May 2019 at both locations. The lysimeter facility at each experimental site consisted of forty-four (44) undisturbed monolith lysimeters made from polyvinyl chloride (PVC) tubes with an internal diameter of 500 mm and a depth of 600 mm. Monolith soil columns at both locations were collected following the procedure outlined by Di et al. (2007) and installed in a trench facility. A soft wax coat was used between the walls of the PVC casing and the soil to prevent edge flow effects (Bishop and Jeyakumar 2021).

The pasture at the Pumice soil lysimeter facility was Italian ryegrass and at the Pallic soil lysimeter facility it was perennial ryegrass and white clover. These two pasture compositions followed farmer practice in the respective area. The soil in each lysimeter was analysed for soil fertility parameters before the application of treatments (Table 5.1). Based on the initial soil fertility results, the Manawatu site Pumice soil was low in magnesium (Mg) thus 10 g of Nitrophoska fertiliser (12:5.2:14 + S, Mg, and trace elements) was applied to each lysimeter at the Manawatu facility only on 06 March 2020.

Table 5.1 Selected soil basic properties analysed prior to treatment application.

Parameter	**Pumice soil (Manawatu site)**	**Pallic soil (Canterbury site)**
pH	5.85	5.12
% N	0.20	0.36
% C	3.68	4.20
% Al	0.85	0.30
% Fe	0.30	0.38
Exchangeable Cations (cmol$_c$ kg^{-1})		
Ca	28	1
K	1.71	0.22
Mg	0.32	0.90
Na	0.12	0.11
[1]CEC	22.1	9.4
[2]WHC (%)	80.6	45.9

[1]CEC=Cation Exchange Capacity; [2]WHC=Water Holding Capacity

5.2.3 Experimental Design

At each experimental site, two sets of experiments were conducted at two different seasonal periods (late-autumn and mid-winter). The late-autumn treatment used twenty-eight (28) lysimeters at each experimental site with the aim to reduce NO_3^- -N leaching during the wet and cold periods of the year (autumn-winter-spring) (Di et al. 2007). The mid-winter treatment application used sixteen (16) lysimeters at each site and it was aimed to test the effectiveness of GA and its combination with LS on growth during the winter period. Previous studies have indicated that GA can perform better in increasing yield when applied in winter temperatures (Williams and Arnold 1964). The experimental design was a completely randomised block design.

5.2.4 Treatments application

To simulate urine application by dairy cows, synthetic urine was prepared by dissolving urea (11 g L^{-1}), glycine (2.90 g L^{-1}), $KHCO_3$ (13.98 g L^{-1}), KCl (5.04 g L^{-1}), and K_2SO_4 (1.38 g L^{-1}) in water (Clough et al. 1998) producing a final N concentration of 6 g N L^{-1}. Prior to urine application in each period, the grass was cut to 50 mm above the soil surface and lysimeter leachate was collected in both experimental sites to determine leachate NO_3^- -N concentration, to ensure low background NO_3^- -N levels (Appendix Table A2.1 and A2.2). Urine was applied at a rate of 2 L per lysimeter (equivalent 10 L m^{-2} or 600 kg N ha^{-1}). Control lysimeters received an equal volume of water (2L).

5.2.4.1 Experiment 1-Late-autumn treatments application

The first treatment application (late-autumn) was made on 09 June 2020 for the Pumice soil, and 27 May 2020 for the Pallic soil. Seven treatments outlined in Table 5.2 were applied at each experimental soil. In this study, DCD was used as a reference material in terms of reducing

NO_3^- -N leaching. At both experimental soils, treatments were applied as a surface spray to each designated lysimeter, 4 h following the urine application. In all lysimeters, 5 mm of water was applied after treatment application to wash applied treatments from pasture canopy and to help distribute treatments in the soil (Zaman and Nguyen 2012).

Table 5.2 Description of late-autumn treatments applied in the Pumice soil lysimeters on 09 June 2020 and in the Pallic soil lysimeters on 27 May 2020.

Late-autumn treatments	Urine N rate (kg N ha^{-1})	Replicates
Control (water)	Nil	4
Urine only	600	4
Urine + DCD at 10 kg ha^{-1}	600	4
Urine + PA-MA at 10 kg ha^{-1}	600	4
Urine + LS at 120 kg ha^{-1}	600	4
Urine + split-application of LS (2LS) at same rate initial and after a month of first application	600	4
Urine + GA (ProGibb SG at 80 g ha^{-1}) + LS at 120 kg ha^{-1}	600	4

5.2.4.2 Experiment 2- Mid-winter treatments application

The second treatment application (mid-winter with air temperature less than 10 °C) was on 29 July 2020 for the Pumice soil and on 26 August 2020 for the Pallic soil. The treatments as outlined in Table 5.3 were applied as discussed in section 5.2.4.1.

Table 5.3 Description of mid-winter treatments applied in the Pumice soil lysimeters on 29 July 2020 and in the Pallic soil lysimeters on 26 August 2020.

Late-autumn treatments	Urine N rate (kg N ha^{-1})	Replicates
Control (water)	Nil	4
Urine only	600	4
Urine + GA (ProGibb SG at 80 g ha^{-1})	600	4
Urine + GA (ProGibb SG at 80 g ha^{-1}) + LS at 120 kg ha^{-1}	600	4

5.2.5 Drainage water collection and analysis

Drainage water from each lysimeter was collected in 20 L black plastic containers connected to the base of each lysimeter via a drainage pipe. Drainage water was collected after 48 h and 72 h for the Manawatu and Canterbury sites, respectively, after each heavy rainfall event (>20 mm). The drainage water collection interval was informed by results obtained in a preliminary study which showed that collection period of drainage sample does not have influence on either NH_4^+ -N or NO_3^- -N concentration (Appendix 2 Figure A2.1). The drainage water volume was measured and a sub-sample of approximately 30 ml was collected, filtered, and stored at <4 °C prior to analyses. All samples were analysed within one week of collection for mineral N (NH_4^+ -N and NO_3^- -N) using Technicon autoanalyzer (Blakemore 1987).

5.2.6 Dry matter (DM) yield

The timing of herbage harvest from lysimeters was based the 2-3 leaf stage of pasture growth. This resulted in five harvests at the Manawatu site and four at Canterbury for the late-autumn treatments. For mid-winter treatments, there were four harvests from Manawatu and three from Canterbury. During harvest, herbage was cut to a height of 50 mm and the dry weight was recorded after samples were oven-dried at 65 °C for a week.

5.2.7 Herbage analysis

Oven-dried herbage was homogenised using a Foss Cyclotech mill (Thermo Fisher Scientific, Waltham, MA, USA) and passed through a 1 mm sieve. A sub-sample of ground biomass (0.1 g) was analysed for N concentration using the Kjeldahl N method (McKenzie and Wallace 1954). Total dry weight herbage Cu concentration and macronutrient uptake were determined on a 0.1 g sub-sample of ground herbage digested in 10 ml of 70% nitric acid (HNO_3). Sample digests were diluted to 25 ml and filtered through Whatman 42 filter paper before quantification using Microwave Plasma Atomic Emission Spectroscopy (MPAES-4200, Agilent, USA).

5.2.8 Soil mineral N analysis

A stainless-steel corer with an internal diameter of 30 mm was used to collect six soil cores in each lysimeter to 0-600 mm depth at the end of the experimental period for both experimental soils. Field moist soil cores from each lysimeter were composited, mixed manually and then sieved through a 2 mm sieve before a 5 g sub-sample was taken for mineral N analysis. Soil samples were extracted using 30 ml, 2 M KCl on an end-over shaker for 1 h. The tubes were then centrifuged at 1100 g for 10 min and filtered through Whatman 42 filter paper. Samples were analysed for mineral N (NH_4^+ -N and NO_3^- -N) using a Technicon autoanalyzer (Blakemore 1987). The autoanalyzer used two colorimetric methods: NH_4^+ -N was determined using an indophenol method based on the reaction of NH_3 with hypochlorite and phe-nol/salicylate catalysed by nitroprusside. Nitrate-N was determined using the reduction of nitrate to nitrite by hydrazine followed by the reaction of nitrite with N-(1-naphthyl)-ethylenediamine dihydrochloride to form an azo dye. The resulting colours produced were measured using individual colorimeter and voltage outputs were converted to concentration using a computerised data aquations system (USB-1208FS analog to digital converter and DAQami™ software, Measurement Computing Corporation, USA) (Maynard et al. 1993).

5.2.9 Climatic data

Climatic data for the experimental period at both sites were downloaded from the National Institute of Water and Atmosphere (NIWA) database (cliflo.niwa.co.nz). The NIWA data for the Manawatu site was sourced from station number 21963 which was approximately 300 m from site, whereas for the Canterbury site it was from station number 4701 located approximately 500 m from experimental site. Rainfall was also measured onsite using installed manual rain gauges and recordings were taken after every rainfall event.

5.2.10 Statistical analysis and quality control

Data normality test and statistical analyses were done using Minitab (Version 19. Minitab Inc., USA). The treatment comparison effects were analysed using ANOVA and significant (P < 0.05) differences between means were determined using Tukey post-hoc test. The percentage of N recovered from applied urine N during the study was calculated using the equation 5.1 (Zvomuya et al. 2003).

$$\%\, N\ recovered\ \ from\ applied\ urine\ N\ = \frac{N_{UR} - N_0}{N_U} \times 100 \qquad \textbf{Equation 5.1}$$

Where N_{UR} and N_0 represents cumulative N output (leached, soil residual N, and herbage N uptake) in urine treated lysimeters and control, respectively and N_U represents applied urine N concentration (kg N ha^{-1}).

To ensure the accuracy of analytical determinations, Standard reference material SRM 2710a, Tomato leaves, and SRM 1573, Montana soil, were analysed for Cu during all sample runs. Analysed concentrations of the tomato leaves and Montana soil were within 92.4-98% and 93.4-98.6%, respectively of certified values.

5.3 Results

5.3.1 Rainfall and Temperature

Total rainfall for the Manawatu site lysimeters was 805 mm, with 333 mm drainage water collected during the experimental period (09 June 2020 to 15 December 2020) (Figure 5.1a).

Average daily soil temperatures (0-10 cm) for the Manawatu site were below 7 °C for 15 days between July and August and increased to approximately 20 °C in December.

Total rainfall for the Canterbury lysimeters was 314 mm, with 114.9 mm of drainage collected (27 May 2020 to 16 December 2020) (Figure 5.1b). At the Canterbury site, average soil temperatures were below 5 °C from June to August, increasing to about 14 °C in December.

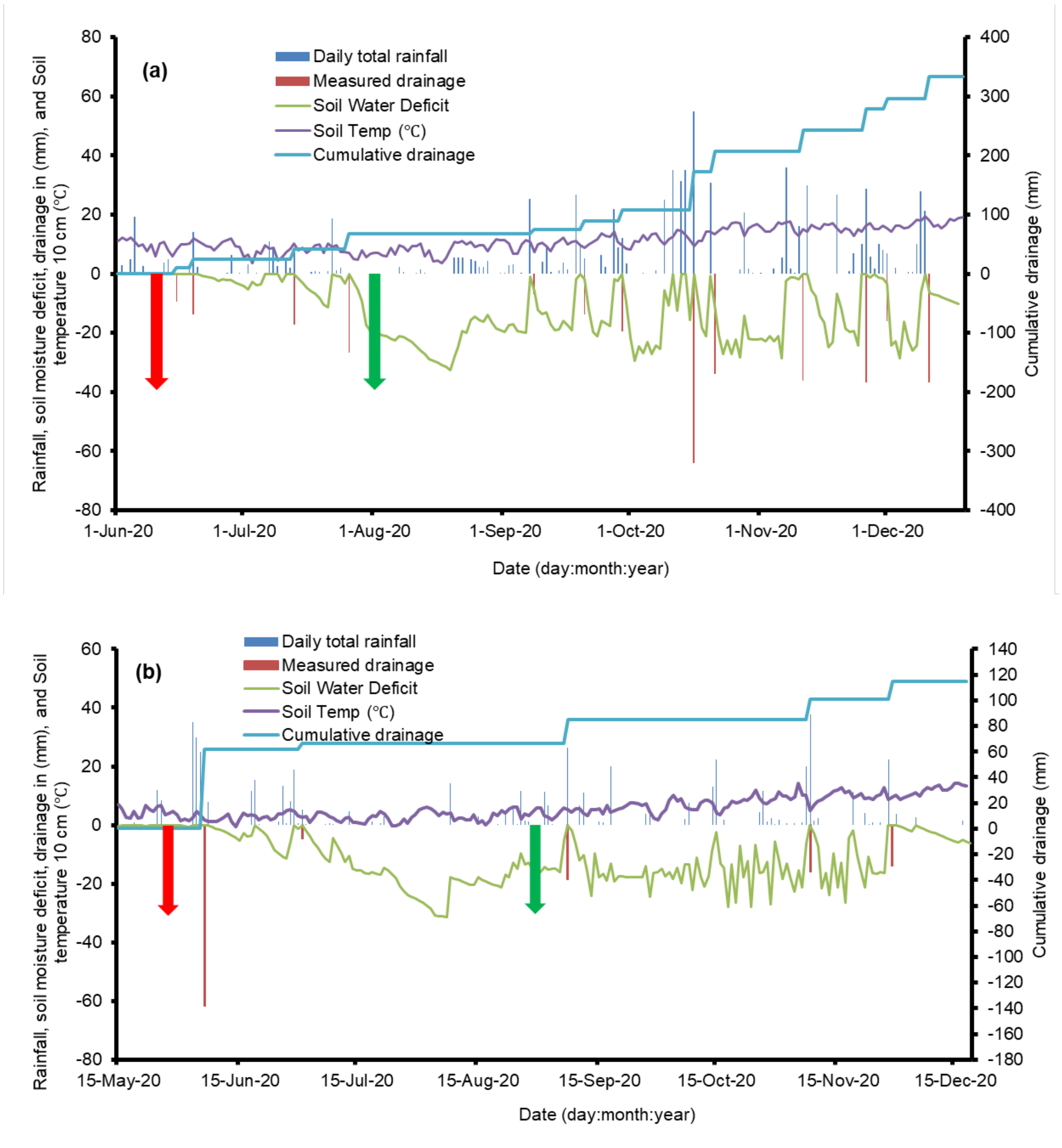


Figure 5.1 Daily total rainfall, soil water deficit, measured drainage, cumulative drainage, and average soil temperature (10 cm) at the (a) Manawatu and (b) Canterbury site during the experimental period of May 2020 to December 2020. The red arrow shows the late-autumn treatment application, while the green arrow shows the mid-winter treatment application.

5.3.2 Late-autumn treatments application

5.3.2.1 Mineral N leaching losses

In the Pumice soil lysimeters, twelve late-autumn leaching events were recorded resulting in cumulative drainage of 333 mm (Appendix 2 Figure A2.2a and A2.2c). Maximum leaching occurred at 172.1 mm of cumulative drainage with leached NO_3^- and NH_4^+ rates of 21 to 52 kg NO_3^- -N ha^{-1} and from 0.6 to 1.4 kg NH_4^+ -N ha^{-1} (Appendix 2 Figure A2.2a and A2.2c). The cumulative leached NO_3^- -N and NH_4^+ -N for the late-autumn urine treatments in the Pumice soil lysimeters ranged from 51.8 to 90.7 kg NO_3^- -N ha^{-1} and from 1.4 to 2.1 kg NH_4^+ -N ha^{-1} (Table 5.4). The applied treatments induced significant differences in total mineral N leaching. The DCD, 2LS and GA + LS treatments reduced the total mass of mineral N leaching in the Pumice soil by 8, 16, and 35%, respectively, compared to the application of the urine-only treatment (Table 5.4). Whereas, the application of PA-MA and LS had no significant effect on the total mineral N leaching relative to the application of the urine-only treatment.

In the Pallic soil lysimeters, five late-autumn leaching events were recorded resulting in cumulative drainage of 114.9 mm (Appendix 2 Figure. A2.2b and A2.2d). Maximum leaching occurred during the first drainage event (at 61.8 mm of cumulative drainage) at rates of 32 to 69 kg NO_3^- -N ha^{-1} and from 36 to 83 kg NH_4^+ -N ha^{-1} (Appendix 2 Figure. A2.1b and A2.1d). The cumulative NO_3^- -N and NH_4^+ -N leaching for late-autumn urine treatments ranged from 39.7 to 81.1 kg NO_3^- -N ha^{-1} and from 36.2 to 83.4 kg NH_4^+ -N ha^{-1} (Table 5.4). Reductions in total mineral N leaching from the Pallic soil were 34, 11, and 35% for the DCD, 2LS, and GA + LS treatments, respectively ($P < 0.05$), relative to the application of the urine-only treatment (Table 5.4). The applications of PA-MA and LS treatments had no significant effect on total mineral N leaching, relative to the application of the urine-only treatment.

Table 5.4 Cumulative NO_3^- -N and NH_4^+ -N leaching, and total mineral N following late-autumn treatment application in the Pumice soil for the period 09 June 2020 to 15 December 2020 and Pallic soil for the period 27 May to 16 December 2020.

	Pumice soil		
Treatments	**Cumulative nitrate leaching kg NO_3^- -N ha^{-1}**	**Cumulative ammonia leaching kg NH_4^+ -N ha^{-1}**	**Total mineral N leaching (kg N ha^{-1})**
Control	6.9 ± 0.3 e	1.8 ± 0.1 ab	8.7 ± 0.5 d
Urine-only	84.3 ± 2.8 ab	1.4 ± 0.1 b	85.7 ± 3.0 a
Urine + DCD	76.6 ± 2.6 bc	1.9 ± 0.11 ab	78.5 ± 4.6 ab
Urine + PA-MA	90.7 ± 2.0 a	1.4 ± 0.90 b	92.1 ± 3.1 a
Urine + LS	87.4 ± 3.1 ab	1.7 ± 0.3 ab	89.1 ± 2.2 a
Urine + 2LS	70.4 ± 1.5 c	1.7 ± 0.2 ab	72.1 ± 2.7 b
Urine + GA + LS	51.8 ± 3.1 d	2.1 ± 0.1 a	53.9 ± 3.6 c
	Pallic soil		
Treatments	**Cumulative nitrate leaching kg NO_3^- -N ha^{-1}**	**Cumulative ammonia leaching kg NH_4^+ -N ha^{-1}**	**Total mineral N leaching (kg N ha^{-1})**
Control	10.5 ± 1.2 d	3.3 ± 0.2 d	13.7 ± 0.7 d
Urine only	62.9 ± 4.7 b	83.4 ± 2.7 a	146.3 ± 5.7 ab
Urine + DCD	39.7 ± 3.4 c	57.7 ± 1.3 bc	97.3 ± 9.8 bc
Urine + PA-MA	81.1 ± 0.7 a	68.4 ± 1.5 ab	149.5 ± 8.2 a
Urine + LS	76.5 ± 1.8 a	75.0 ± 2.7 ab	151.5 ± 5.8 a
Urine + 2LS	58.4 ± 3.0 b	71.4 ± 1.7 ab	130.1 ± 8.3 abc
Urine + GA + LS	50.4 ± 3.3 bc	44.0 ± 2.3 c	94.5 ± 1.1 c

Note: Numbers after ± represent standard error of mean. Different small letters in each column of each soil indicate a significant difference at $P < 0.05$.

5.3.2.2 Cumulative N uptake and cumulative DW

Herbage N uptake and DM yield varied among treatments. Application of DCD, 2LS, and GA + LS treatments to the Pumice soil lysimeters induced a nominal but non-significant increase in cumulative N uptake, and cumulative DM yield (Table 5.5) relative to the application of the urine-only treatment.

Similarly, for the Pallic soil, there was no significant ($P > 0.05$) increase in cumulative N uptake and cumulative DM yield following the application of inhibitors (Table 5.5) compared to the application of the urine-only treatment.

Table 5.5 Cumulative pasture N uptake (kg N ha^{-1}), and cumulative DM yield (kg DM ha^{-1}) following late-autumn treatment application in the Pumice soil for the period 09 June 2020 to 15 December 2020 and Pallic soil for the period 27 May to 16 December 2020.

Treatments	Pumice soil	
	Cumulative N uptake (kg N ha^{-1})	**Cumulative DM yield (kg DM ha^{-1})**
Control	48.2 ± 1.2 d	2783 ± 176 b
Urine-only	232.5 ± 2.6 ab	9568 ± 156 a
Urine + DCD	254.8 ± 15.7 a	10276 ± 669 a
Urine + PA-MA	204.0 ± 7.3 c	9941 ± 596 a
Urine + LS	213.2 ± 11.4 bc	9474 ± 562 a
Urine + 2LS	258.1 ±16.0 a	10301 ± 719 a
Urine + GA + LS	261.1 ± 7.02 a	9583 ± 885 a
Treatments	**Pallic soil**	
	Cumulative N uptake (kg N ha^{-1})	**Cumulative DM yield (kg DM ha^{-1})**
Control	93.2 ± 9.5 b	4421 ± 300 b
Urine-only	280.9 ± 14.0 a	10106 ± 421 a
Urine + DCD	327.3 ± 21.5 a	10971 ± 743 a
Urine + PA-MA	286.4 ± 11.9 a	10223 ± 343 a
Urine + LS	308.1 ± 20.1 a	10596 ± 842 a
Urine + 2LS	312.5 ± 21.4 a	10892 ± 1080 a
Urine + GA + LS	314.4 ± 19.2 a	11286 ± 606 a

Note: Numbers after ± represent standard error of mean. Different small letters in each column of each soil indicate a significant difference at $P < 0.05$.

5.3.2.3 Herbage total Cu and macronutrient concentration

Late-autumn application of compounds (PA-MA, LS, 2LS, and GA + LS) did not induce significant ($P > 0.05$) changes in herbage Cu concentration at any harvest for either the Pumice or Pallic soil when compared to the urine-only lysimeters (Figure 5.2 a and b). Similarly, there were no signifcant changes in macronutrient uptake between applied inhibitors and urine-only treatment in either Pumice or Pallic soil (Appendix 2 Table A2.4).

5.3.2.4 Soil mineral N

There were no significant changes in residual soil mineral N between the applied inhibitors and the application of the urine-only treatment in either the Pumice or Pallic soils (Table 5.6).

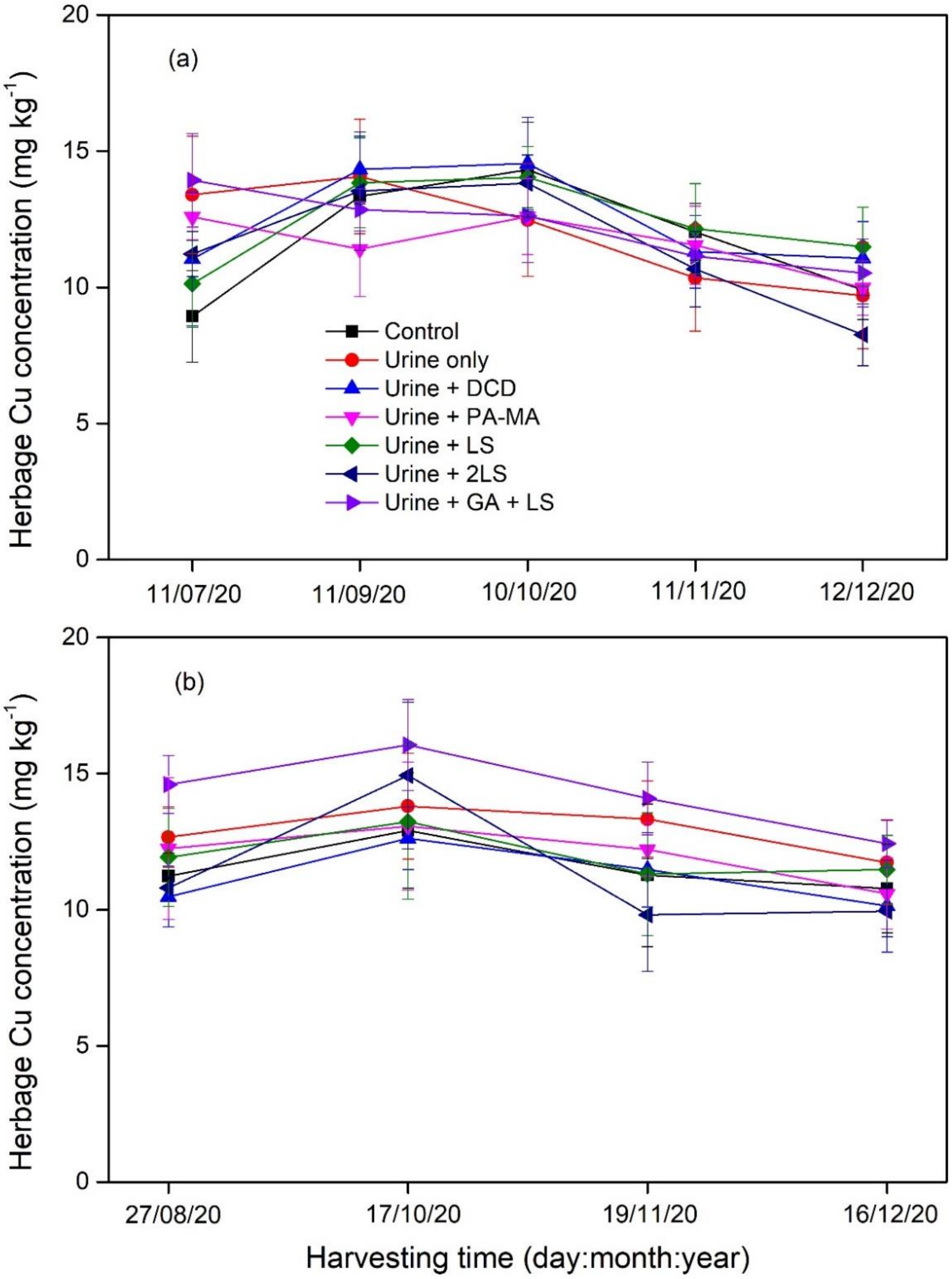


Figure 5.2 Late-autumn treatment application herbage Cu concentration in the Pumice (a) and Pallic soils (b).

Table 5.6 Soil NO_3^- -N, NH_4^+ -N, and soil total mineral N analysed at the end of the experiment following late-autumn treatment application in the Pumice and Pallic soils.

Treatments	Pumice soil		
	Soil nitrate ($kg\ NO_3^-$ -N ha^{-1})	**Soil ammonia ($kg\ NH_4^+$ -N ha^{-1})**	**Soil total mineral N ($kg\ N\ ha^{-1}$)**
Control	15.8 ± 0.8 a	0.13 ± 0.01b	16.0 ± 0.8 a
Urine only	16.2 ± 1.4 a	0.13 ± 0.02 ab	16.3 ± 1.4 a
Urine + DCD	18.6 ± 2.0 a	0.18 ± 0.01 a	18.7 ± 2.0 a
Urine + PA-MA	16.9 ± 1.7 a	0.14 ± 0.01 ab	17.1 ± 1.7 a
Urine + LS	14.3 ± 1.0 a	0.15 ± 0.01 ab	14.4 ± 1.0 a
Urine + 2LS	12.7 ± 0.5 a	0.18 ± 0.01 a	12.9 ± 0.5 a
Urine + GA + LS	16.6 ± 1.5 a	0.13 ± 0.02 ab	16.7 ± 1.5 a
Treatments	**Pallic soil**		
	Soil nitrate ($kg\ NO_3^-$ -N ha^{-1})	**Soil ammonia ($kg\ NH_4^+$ -N ha^{-1})**	**Soil total mineral N ($kg\ N\ ha^{-1}$)**
Control	17.4 ± 1.9 b	11.4 ± 0.9 a	28.8 ± 2.2 b
Urine only	31.7 ± 2.9 ab	3.8 ± 0.4 bc	35.5 ± 3.2 ab
Urine + DCD	29.1 ± 1.9 ab	3.1 ± 0.3 c	32.2 ± 1.3 b
Urine + PA-MA	35.3 ± 2.4 a	5.4 ± 0.6 b	40.7 ± 1.0 ab
Urine + LS	31.9 ± 2.7 ab	9.6 ± 0.2 c	34.6 ± 3.1 b
Urine + 2LS	40.6 ± 5.2 a	9.6 ± 0.5 a	50.2 ± 5.3 a
Urine + GA + LS	36.3 ± 4.5 a	2.8 ± 0.5 c	39.2 ± 4.4 ab

Note: Numbers after ± represent standard error of mean. Different small letters in each column of each soil indicate significant difference at $P < 0.05$.

5.3.3 Mid-winter treatment application

5.3.3.1 Mineral N leaching losses

Ten mid-winter leaching events were recorded for the Pumice soil resulting in cumulative drainage of 282.6 mm (Appendix 2 Figure A2.3a and A2.3c). Nitrate-N was the dominate form of N leached. Maximum leaching occurred from the urine treated lysimeters for a cumulative drainage of 137.3 mm with rates of 46-58 kg NO_3^- -N ha^{-1} and 0.5-1 kg NH_4^+ -N ha^{-1} (Appendix 2 Figure A2.3a and A2.c). Overall, the GA-only and GA + LS treatments significantly ($P <$ 0.05) reduced the total amount of mineral N leaching from the Pumice soil by 23 and 20%, respectively, relative to the application of the urine-only treatment (Table 5.7).

Three leaching events were recorded for the Pallic soil with a cumulative drainage of 48.3 mm (Appendix 2 Figure A2.3b and A2.3d). This low drainage resulted in low NO_3^- -N leaching for the different treatments. Maximum leaching from the urine-treated lysimeters was recorded for a cumulative drainage of 34.3 mm with rates of 1.8 to 28.3 kg NO_3^- -N ha^{-1} and 0.1-0.6 kg NH_4^+ -N ha^{-1} (Appendix 2 Figure A2.3b and A2.3d). Overall, lysimeters treated with GA alone showed a significant (P < 0.05) increase in mass of N leaching compared to the application of the urine-only treatment. However, there was no significant difference in total mineral N leaching between the urine-only and GA + LS treatment (Table 5.7).

Table 5.7 Cumulative NO_3^- -N leaching, cumulative NH_4^+ -N leaching, and total mineral N following mid-winter treatment application in the Pumice soil for the period 29 July 2020 to 15 December 2020 and Pallic soil for the period 26 August 2020 to 16 December 2020.

Treatments	**Pumice soil**		
	Cumulative nitrate leaching (kg NO_3^- -N ha^{-1})	**Cumulative ammonia leaching (kg NH_4^+ -N ha^{-1})**	**Total mineral N leaching (kg N ha^{-1})**
Control	2.5 ± 0.2 c	1.4 ± 0.2 a	3.9 ± 0.01 c
Urine only	136.7 ± 3.0 a	0.9 ± 0.1 a	137.8 ± 3.0 a
Urine + GA	104.8 ± 2.7 b	0.9 ± 0.2 a	105.6 ± 2.6 b
Urine + GA + LS	109.0 ± 4.4 b	1.1 ± 0.3 a	110.1 ± 3.0 b
Treatments	**Pallic soil**		
	Cumulative nitrate leaching (kg NO_3^- -N ha^{-1})	**Cumulative ammonia leaching (kg NH_4^+ -N ha^{-1})**	**Total mineral N leaching (kg N ha^{-1})**
Control	8.3 ± 0.5 c	0.2 ± 0.1 bc	8.5 ± 0.4 c
Urine only	53.1 ± 0.8 b	0.7 ± 0.1 a	53.8 ± 0.8 b
Urine + GA	57.1 ± 0.9 a	0.2 ± 0.01 c	57.3 ± 0.9 a
Urine + GA + LS	50.5 ± 0.9 b	0.4 ± 0.1 ab	50.9 ± 0.8 b

Note: Numbers after ± represent standard error of mean. Different small letters in each column of each soil indicate a significant difference at P < 0.05.

5.3.3.2 Cumulative N uptake and cumulative DM yield

Application of GA-only and GA + LS treatments to the Pumice soil lysimeters showed a significant (P < 0.05) increase in cumulative N uptake (22% for GA only and 13% for GA + LS) and cumulative DM yield (18% for GA only and 15% for GA + LS) relative to the application of the urine-only treatment (Table 5.8). The treatment effect on cumulative N uptake and cumulative yield for the Pallic soil was also significant (*P* < 0.05), with corresponding values of 19 and 12% for GA only, and 24 and 19% for GA + LS, respectively (Table 5.8).

Table 5.8 Cumulative N uptake (kg N ha^{-1}), and cumulative DM yield (kg DM ha^{-1}) following mid-winter treatment application in the Pumice soil for the period 29 July 2020 to 15 December 2020 and Pallic soil for the period 26 August 2020 to 16 December 2020.

Treatments	Pumice soil	
	Cumulative N uptake (kg N ha^{-1})	**Cumulative DM yield (kg DM ha^{-1})**
Control	45.5 ± 0.9 c	2692 ± 166 c
Urine-only	201.0 ± 5.2 b	8061 ± 380 b
Urine + GA	245.8 ± 9.2 a	9519 ± 141 a
Urine + GA + LS	227.3 ± 15.6 a	9270 ± 526 a
Treatments	**Pallic soil**	
	Cumulative N uptake (kg N ha^{-1})	**Cumulative DM yield (kg DM ha^{-1})**
Control	81.8 ± 8.8 d	3992 ± 425 d
Urine-only	271.5 ± 18.3 c	9405 ± 719 c
Urine + GA	321.9 ± 11.0 b	10506 ± 328 b
Urine + GA + LS	336.0 ± 17.7 a	11148 ± 755 a

Note: Numbers after ± represent standard error of mean. Different small letters in each column of each soil indicate a significant difference at *P* < 0.05.

5.3.3.3 Soil mineral N

In the Pumice soil, the applied treatments resulted in significant differences in residual soil mineral N in the lysimeters. The soil mineral N was significantly (*P* < 0.05) higher in the GA + LS treatment compared to the application of the urine-only treatments (Table 5.9). However, there were no significant changes between urine-only and GA treatments.

In the Pallic soil, there were no significant differences in residual mineral N observed between urine treated lysimeters (Table 5.9).

Table 5.9 Soil NO_3^- -N, NH_4^+ -N, and soil total mineral N analysed at the end of the experiment following mid-winter treatment application in the Pumice and Pallic soils lysimeters.

	Pumice soil		
Treatments	**Soil nitrate (kg NO_3^- -N ha^{-1})**	**Soil ammonia (kg NH_4^+ -N ha^{-1})**	**Soil total mineral N (kg N ha^{-1})**
Control	15.8 ± 0.8 b	1.1 ± 0.01 a	16.9 ± 0.8 b
Urine only	14.0 ± 1.5 b	0.7 ± 0.01 b	14.8 ± 1.5 b
Urine + GA	18.2 ± 1.3 ab	1.1 ± 0.1 a	19.3 ± 1.3 ab
Urine + GA + LS	22.7 ± 2.0 a	1.3 ± 0.01 a	24.0 ± 2.0 a
	Pallic soil		
Treatments	**Soil nitrate (kg NO_3^- -N ha^{-1})**	**Soil ammonia (kg NH_4^+ -N ha^{-1})**	**Soil total mineral N (kg N ha^{-1})**
Control	17.4 ± 1.6 b	11.4 ± 0.9 a	28.8 ± 2.2 a
Urine only	58.0 ± 2.3 a	4.9 ± 0.6 b	62.9 ± 2.8 b
Urine + GA	49.1 ± 4.0 a	4.3 ± 0.6 b	53.4 ± 4.5 b
Urine + GA + LS	54.1 ± 3.1 a	5.9 ± 0.4 b	60.0 ± 3.5 b

Note: Numbers after ± represent standard error of mean. Different small letters in each column of each soil indicate significant difference at $P < 0.05$.

5.4 Discussion

5.4.1 Leachate mineral N

Lysimeter leachate analysis before treatment application (Appendix 2 Table A2.1 and A2.2) showed that there was extremely low background mineral N in leachate. Results from this study show that NO_3^- -N was the major form of N leaching from the Pumice soil for both late-autumn and mid-winter urine applications (Table 5.4 and Table 5.7). This aligns with the general expectation that NO_3^- -N is the predominant form of mineral N in drainage water. However, large quantities of NH_4^+ -N were leached from the Pallic soil lysimeters in late-autumn, and this was associated with the first collected drainage (Appendix 2 Figure A2.2d). High NH_4^+ -N leachate losses have been previously reported for Pallic soil in Canterbury (Talbot et al. 2021), where late-autumn (May) urine application to stony Pallic orthic brown soil in Canterbury resulted in NH_4^+ -N leaching ranging from 33.0 to 58.7 kg NH_4^+ -N ha^{-1}, due to urine flowing via macro-pore into the lower gravel layers of the lysimeters. In this Chapter, an average of 60.8 kg NH_4^+ -N ha^{-1} was leached during the first cumulative drainage of 61.8 mm.

The high rainfall event and combination of the free-draining shallow stony soil, limited CEC, and low water holding capacity (40-60 mm) allowed the leaching of NH_4^+ -N. In contrast, the Pumice soil could hold between 150-200 mm of water with a higher CEC. In addition to the differences in water holding capacity and CEC between the soils, the stony nature of the Pallic A horizon (0-30 cm, 50-60% stones) allows macro pore flow of urine into the predominantly stone and sand Ap horizon (30-50 cm, 71-75% stones) (Carrick et al. 2017).

The late-autumn application of 2LS, and GA + LS, significantly ($P < 0.05$) reduced the total amount of mineral N leaching from the Pumice soil lysimeters relative to the application of urine-only, while only lysimeters treated with GA + LS showed a significant ($P < 0.05$) reduction in the total mineral N concentration in leachate from the Pallic soil lysimeters (Table 5.4). Application of 2LS proved to be more effective in reducing total mineral N leaching than a single application of LS for the Pumice soil. Therefore, the application of a second dose might have helped to prolong the effectiveness of these compounds in reducing total mineral N losses. However, in the Pallic soil lysimeters, application of a second dose did not yield any reduction in total mineral N leaching. This can be attributable to the fact that a higher proportion of the applied N was leached in the first cumulative drainage event before the second dose application. Further, application of PA-MA and LS treatments resulted in non-significant changes in total mineral N leaching in either Pumice or Pallic soil lysimeters relative to the urine-only treatment. The higher CEC of the Pumice soil might support the adsorption of inhibitors to soil organic matter (Zhang et al. 2020). On the other hand the low CEC and low water holding capacity of the Pallic soil might have exacerbated the possibilities of leaching these inhibitors during drainage (Martikainen 2022). These factors might have contributed to the reduction in inhibitor’s effectiveness.

The combination of GA + LS treatment reduced total mineral N leaching in both the Pumice and Pallic soils. In this study, GA was applied to improve N uptake and plant growth as a complimentary mechanism to the effect of LS. First herbage cut N uptake data from both sites suggests that this combination might have reduced total N losses through increasing N uptake when compared to the other treatments (Appendix 2 Table A2.3). This increase in herbage N uptake may have resulted in less soil mineral N available for leaching during drainage events. However, further studies are needed to provide clear evidence on the mode of action of this treatment. In a similar study (Bishop and Jeyakumar 2021), late-autumn GA + LS application significantly reduced NO_3^- -N leaching in Pumice and Pallic soils by 15 and 22%, respectively.

Mid-winter application of GA alone and GA + LS significantly ($P < 0.05$) reduced mineral N leaching loss from the Pumice soil (Italian ryegrass); however, the same result was not observed for the Pallic soil (perennial ryegrass/clover mixture) where GA alone increased the total mineral N leaching, and GA + LS had no significant effect when compared to urine-only (Table 5.7). The increase in N leaching in the Pallic soil associated with GA alone might be attributed to the interaction between the GA and white clover. Several studies have provided evidence that the application of GA increases nodule formation in legumes (Ferguson et al. 2011; Rafique et al. 2021), and high nodulation in legumes can increase biological nitrogen fixation (BNF). Rafique et al. (2021) reported that the application of GA_3 (10^{-5} M) as foliar spray to *Rhizobium* inoculated chickpea plants significantly increased nodules per plant by 55% relative to the control. Increased BNF by *Rhizobium* bacteria associated with clover nodules might have reduced the utilisation of urine applied N, thus making it susceptible to leaching. Further, the increase in N fixation might lead to an increase in the total N input, and eventually an increase in the NO_3^- -N leaching potential. Reduced N leaching by GA + LS was a combination effect of LS, and the complementary effect of GA. Evidence of this theory is illustrated through the significantly ($P < 0.05$) higher cumulative N uptake due to the GA + LS treatment when compared to GA alone.

5.4.2 Pasture N uptake and dry matter yield

The application of late-autumn inhibitors did not lead to any significant increase in cumulative herbage N uptake and cumulative DM yield for either of the lysimeter soils (Table 5.5) when compared to the urine-only lysimeters. The non-significant increase in cumulative N uptake and cumulative yield associated with the applied treatments was influenced by the form of N present in both soils. Complete nitrification in soil occurs within 2-4 weeks when conditions are favourable (Haynes and Williams 1992). Thereafter, most of the N in the soil is converted to the NO_3^- -N form. Pastures can only save energy for extra growth when they uptake N as either urea or the NH_4^+ ion. Therefore, pastures in this study were unlikely to realise a growth benefit due to the N speciation as NO_3^- -N. This effect explains the non-significant effect between applied treatments (Zaman and Blennerhassett 2010). Previous studies have also reported reduced NO_3^- -N leaching as a function of inhibition; however, these studies have not shown significant effect on cumulative N uptake and pasture DM (Cookson and Cornforth 2002; Zaman and Blennerhassett 2010), due to suppression of soil NO_3^- -N levels.

Although the applied treatments did not result in overall significant cumulative N uptake and variable yield between treatments, significant treatment effects were observed during the first harvest dates. For example, late-autumn application of DCD, 2LS, and GA + LS significantly ($P < 0.05$) increased herbage N uptake and DM yield for the first harvest in the Pumice soil compared to the urine-only treatment (Appendix 2 Tables A2.3). For the Pallic soil lysimeters this increase was not significant (Alexopoulos et al. 2007). The higher herbage N uptake in the first cut suggests that these treatments might have been effective in delaying the oxidation of

NH_4^+ -N during the period of rapid nitrification. However, their short effectiveness might be due to rapid degradation in the soil (Alexopoulos et al. 2007; Chibuike et al. 2022).

Mid-winter application of GA alone and GA + LS significantly ($P < 0.05$) increased both cumulative N uptake and DM yield in both the Pumice and Pallic soils relative to the urine-only treated lysimeters. The treatment effect in the Pumice soil lysimeters was due to the long period between the urine application and the first leaching event (Figure 5.1a). The longer period allowed high utilisation of applied N by lysimeter pasture thus giving significant differences between the applied treatments. The effectiveness of these treatments in the Pallic soil might have been accelerated by the low total mass of N leached from the Pallic soil lysimeters. As a result, a higher proportion of N was available for plant uptake.

5.4.3 Herbage Cu and macronutrients uptake

Results from Chapter 3 described how the amendments used in the current study (PA-MA, and LS) reduce nitrification rate through reducing the bioavailable Cu concentration in soil. Therefore, it was important to analyse the potential effect of these applied compounds on plant Cu uptake. The non-significant changes in herbage Cu suggests that any effect induced by these compounds might affect microbial processes in the soil while not affecting pasture Cu and macronutrients uptake. This is because soil microbial activity is more sensitive to small changes in the soil than plant growth (Shuaib et al. 2021). In Chapter 3, it was observed that only small changes (as low as 5 μg Cu kg^{-1}) in bioavailable Cu can induce significant changes in nitrification rate, which might not be significant for plant growth. These results suggests that these applied compounds had no unintended detrimental consequences on Cu and macronutrients uptake, thus providing a sustainable approach of reducing NO_3^- -N leaching in pastoral systems.

5.4.4 Soil mineral N and N recovered in the system

Soil mineral N results analysed at the end of the current study showed that there was no significant difference between urine treated and untreated lysimeters in both experimental soils with either late-autumn or mid-winter treatment application. This implies that all applied urine N was either utilised through pasture N uptake or lost through leaching or other pathways such as immobilisation or emission. In this study, the recovered N calculations show that an average of 0.01, 13.1, and 31.5% of the applied urine N in the Pumice soil (late-autumn treatments) was recovered through soil residual N, leached N, and plant N uptake, respectively (Table 5.10). While in the Pallic soil, soil residual N, leached N, and plant N uptake was 1.7, 19.1, and 35.3%, respectively of the applied urine N. In the Pumice and Pallic soils, the unaccounted N was 55.38 and 43.98%, respectively of the applied N. Further, mid-winter treatments in the Pumice soil lysimeters showed that an average of 0.4, 19.0, and 29.9% of applied urine N was recovered through soil residual N, leached N, and plant N uptake, respectively, while unaccounted N was 50.73%. Similarly, in the Pallic soil, average N recovered in soil, leaching, and herbage was 4.99, 7.57, and 49.44%, respectively. Unaccounted N corresponded to 49.44% in the Pallic soil (Table 5.10). The unaccounted N is mainly N lost through immobilisation in the soil microbial biomass and organic matter or through emission to the atmosphere. In this current Chapter, unaccounted N was nearly 50% and this percentage has been reported in previous studies. In the literature, an average of 26, 13, and 2% of applied urinary N has been reported to be lost through immobilisation, NH_3 volatilisation, and N_2O emissions (Selbie et al. 2015). In a field lysimeter study reported by Zaman and Blennerhassett (2010), the unaccounted N was 60.29 and 56.69% in autumn and spring, respectively. The values of urine applied N recovered through herbage N uptake in this study agree with other studies where similar trends were observed (Ball et al. 1979; Shepherd et al. 2010). For example, Ball et al. (1979) reported that in urine applied at 300 kg N ha^{-1}, the N recovered through plant N uptake was 37% of the applied urine-N. Overall, a higher percentage of unaccounted N was in the Pumice soil lysimeters. The differences in soil properties between the two sites might have played a major role in influencing this trend. The higher WHC of the Pumice soil, together with the frequent rainfall and higher temperatures (Figure 5.1a) between June to August, might have resulted in an increase in the population of denitrifying microorganisms. Denitrifying microbes might have released N from soil as N_2O leading to poor soil N utilisation by the pasture. Emissions can reach up to 28% of applied N due to the wet conditions which prevail between May and early July (Frase et al. 1994). However, emissions were not measured in the current study, and this is an area for future work.

Table 5.10 Percentage (%) of applied N recovered in soil, herbage, leachate, and unaccounted N in the late-autumn and mid-winter treatments urine application.

Treatments	Residual soil mineral N	Plant N uptake	Leached N	Unaccounted N
Late-autumn application				
Pumice soil	0.01	31.51	13.09	55.38
Pallic soil	1.66	35.29	19.07	43.98
Mid-winter application				
Pumice soil	0.41	29.87	19.00	50.73
Pallic soil	4.99	38.00	7.57	49.44

Note: All numbers are in percentages.

5.5 Conclusions

This study demonstrated that split application of LS significantly ($P < 0.05$) reduced the total mineral N leached only at the Manawatu site, whereas GA plus LS ($P < 0.05$) reduced mineral N leached from both Pumice and Pallic soils. These treatments provided valuable evidence on potential amendments that can be applied to urine patches to reduce mineral N leaching losses. The study shows that a split application of LS reduced mineral N leaching by means of increasing the LS reactive period in the soil, while the reduction associated with GA + LS is due to a combination of LS and GA effect. The timing of treatment was important, with the late-autumn application showing higher efficacy in reducing mineral N leaching from both soils than the mid-winter application. These results have demonstrated that for farmers to achieve the greatest reduction in N leaching during the period of high N losses and drainage, application of an inhibitor is necessary during the late-autumn period. These findings can potentially guide farm management practices with respect to the optimal timing of nitrification inhibitor application to grazed pastoral systems.

CHAPTER 6

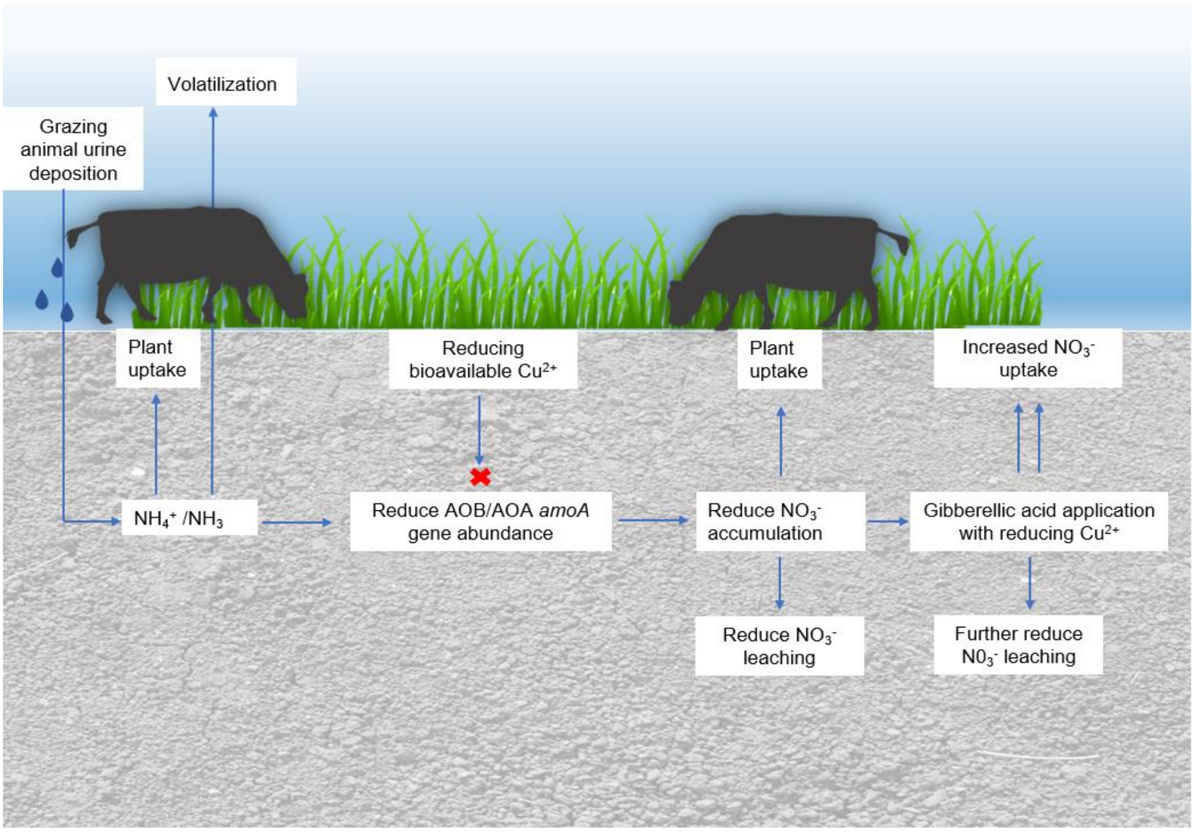
Nitrification rate in dairy cattle urine patches can be inhibited by changing soil bioavailable Cu concentration

*This chapter was published in the **Environmental Pollution Journal** in 2023. Citation:*

Matse, D. T., Jeyakumar, P., Bishop, P., & Anderson, C. W. N., (2023). Nitrification in dairy urine patches can be inhibited by changing soil bioavailable Cu concentration. Environmental Pollution. 320: 121107. doi: 10.1016/j.envpol.2023.121107

Results in Chapter 5 demonstrated that the organic compounds LS and PA-MA can potentially reduce NO_3^- -N leaching under field conditions. However, the mechanistic effect of LS and PA-MA on bioavailable Cu was not investigated. Therefore, Chapter 6 aimed to determine the effect of LS and PA-MA on bioavailable Cu, and their impact on nitrification rate and AOB/AOA functional genes. This Chapter also examines the co-treatment effect of LS and GA on reducing NO_3^- -N leaching under field conditions.

Graphical Abstract



Abstract

Ammonia oxidation to NH_2OH is catalyzed by the AMO enzyme and Cu is a key element for this process. We investigated the effect of soil bioavailable Cu changes induced through the application of Cu-complexing compounds on nitrification rate, AOB and AOA *amoA* gene abundance, and mineral N leaching in urine patches using the Manawatu Recent soil. Further, evaluated the combination of organic compounds LS with a growth stimulant GA. Treatments were applied in May 2021 as late-autumn treatments: control (no urine), urine-only at 600 kg N ha^{-1}, urine + DCD, urine + PA-MA, urine + LS, urine + split-application of LS (2LS), and urine + GA + LS. In addition, another four treatments were applied in July 2021 as mid-winter treatments: control, urine-only at 600 kg N ha^{-1}, urine + GA, and urine + GA + LS. Soil bioavailable Cu and mineral N leaching were examined during the experimental period. The AOB/AOA *amoA* genes were quantified using quantitative polymerase chain reaction. Changes in soil bioavailable Cu across treatments correlated with nitrification rate and AOB *amoA* abundance in late-autumn while the AOA *amoA* abundance did not change. The reduction in soil bioavailable Cu induced by the PA-MA and 2LS was linked to a significant ($P < 0.05$) reduction in mineral N leaching of 16 and 30%, respectively, relative to the urine-only treatment. The LS treatment did not induce a significant effect on either bioavailable Cu or mineral N leaching relative to urine-only. The GA + LS treatment reduced mineral N leaching by 10% relative to LS in late-autumn, however, there was no significant effect in mid-winter. This study demonstrates that reducing bioavailable Cu can be a potential strategy to reduce N leaching from urine patches.

Keywords: Bioavailable Cu; Nitrification rate; Ammonia-oxidizing bacteria; Ammonia-oxidizing archaea; Mineral N leaching.

6.1 Introduction

Urinary excretion by grazing livestock deposits a high N concentration (200-2000 kg N ha^{-1}) (Selbie et al. 2015) onto pastoral soils and this N becomes susceptible to leaching as NO_3^- -N during drainage events (Malcolm et al. 2022). Nitrate leaching to ground and shallow surface-water is a serious environmental concern (Sun et al. 2022) and high NO_3^- -N concentrations (>11.2 mg L^{-1} NO_3^- -N) in drinking water can pose a threat to both animal and human health (Menció et al. 2016; Richards et al. 2021). Mitigation of the environmental consequences of NO_3^- -N leaching in agriculture systems is dependent on scientific knowledge of the mechanisms behind nitrification.

Nitrification is the oxidation of NH_4^+ -N to NH_2OH, followed by the oxidation of NH_2OH to NO_2, and eventually from NO_2^- to the mobile NO_3^- -N (Muller et al. 2022; Yi et al. 2023). The ammonia oxidizing process is carried out by the [3]AOB or [4]AOA (Lourenço et al. 2022; Lu et al. 2022; Soliman and Eldyasti 2018). These two groups of ammonia oxidizers possess the AMO enzyme encoded by the *amoA* gene that catalyses the first step of nitrification (Bozal-Leorri et al. 2022; Hayatsu et al. 2021). Nitrification in soil is therefore dependent on AMO activity. However, several studies have reported that the functioning of AMO is influenced by several factors: including soil pH, N availability, and Cu bioavailability (Matse et al. 2022a; Norton and Ouyang 2019; Ramotowski and Shi 2022).

Many studies have shown the importance of Cu in the oxidation of ammonia in drinking and wastewater treatment plants, pure cell culture, soil incubations, and glasshouse pot experiments (Corrochano-Monsalve et al. 2021; Gwak et al. 2020; Wagner et al. 2016). For example, Gwak et al. (2020) reported that ammonia oxidizers were inhibited in the presence of organic

[3] Ammonia-oxidizing bacteria
[4] Ammonia-oxidizing archaea

amendments that can complex with Cu^{2+}. They also demonstrated that Cu addition significantly increased AOA growth in a municipal nitrifying activated sludge. Chapter 3 demonstrates that increasing Cu concentration (0.1-3 kg Cu kg^{-1}) significantly increased nitrification rate in three pastoral soils. It further reported that reducing bioavailable Cu through the application of LS and PA-MA (organic compounds) reduced nitrification rate.

However, the use of organic compounds such as LS and PA-MA to reduce nitrification through reducing bioavailable Cu have only been examined in a controlled environment. As a result, their efficiency under field conditions is unknown. Studies that have evaluated the efficiency of nitrification inhibitors under environmental conditions have shown the effect of inhibition is reduced under field conditions relative to the potential reported in controlled studies (Guardia et al. 2018; Kim et al. 2012) and a potential causative factor is poor plant growth. One strategy to overcome this challenge is to improve plant growth through the application of an external plant growth stimulant such as GA (Parsons et al. 2013). Gibberellic acid is responsible for various aspects of plant growth and elongation (Swain and Singh 2005). Several studies have reported increased herbage N concentration and dry matter following GA application (Matthew et al. 2009; Zaman et al. 2014). The extra boost in growth induced through GA could potentially increase N uptake in soil during periods of low N uptake, thus complementing the Cu reducing mechanism of inhibitors.

The study in Chapter 6 was therefore conducted to determine the effect of Cu-complexing organic compounds on the concentration of bioavailable Cu in soil under field conditions, and the impact of such treatment on nitrification rate, AOB/AOA *amoA* gene abundance, and mineral N leaching. A second objective was to evaluate the effect of a combination of organic compounds with GA as a strategy to reduce mineral N leaching. Understanding the mechanism of Cu's effect on nitrification could support the development of alternative methods that may reduce nitrification rate in pastoral soils.

6.2 Materials and Methods

6.2.1 Site and soil type

This study was conducted in a lysimeter facility at the Dairy 1 farm (40°23'0.95"S175°36'36.16"E), Massey University, Palmerston North, NZ. The annual rainfall and daily temperature in Palmerston North is around 900 mm and 9.1 ℃, respectively (NIWA 2016). The soil used was the Manawatu Recent soil, a fine sandy loam described as dystric Fluventic Eutrudept according to US Soil Taxonomy Classification (Hewitt 2010). This is a well-drained soil with a moderately developed structure. This soil was used in this current study because it has low organic carbon (C) content compared to other NZ pastoral soils (Matse et al. 2022a; McNally et al. 2017). Low C can eliminate the C effect on influencing the performance of ammonia oxidizing microorganisms in the soil.

6.2.2 Soil column preparation

The field experiment was conducted using repacked lysimeter soil columns made from polyvinyl chloride (PVC) tubes, each measuring 500 mm internal diameter and 600 mm length. These lysimeters were installed following the protocol of Cameron et al. (1992). Each lysimeter was fitted with glass fabric at the bottom to prevent loss of soil and was connected to a drainage collection PVC pipe (10 mm) that percolated into a 20 L container. Soil was collected at 20 cm depth from a site that had been fenced for approximately 2 years without any grazing; soil collection was designed to prevent the inclusion of any residual N from grazing animals with soil packed into lysimeters. The general soil properties (0-20 cm) from the soil collection site were measured at the time of soil collection and are presented in Table 6.1. Soil was homogenised by sieving through a 25 mm sieve to remove any plant debris. Soil was repacked into the lysimeters, then sulphur was applied to all lysimeters at a rate of 20 kg ha^{-1} as K_2SO_4 to correct a recorded deficiency in Sulphur (Table 6.1). Italian ryegrass was sown at a rate of 25 kg ha^{-1} after four weeks. All lysimeters received Calcium Ammonium Nitrate (CAN) applied at a rate of 50 kg N ha^{-1} as a maintenance N fertiliser four weeks after ryegrass germination. Ryegrass was allowed to establish for two months before treatment application.

Table 6.1 Summary of determined soil properties

Soil property	Value
pH	5.5
Olsen P (mg L^{-1})	14
Sulphate S (mg kg^{-1})	2
Bioavailable Cu (mg kg^{-1})	0.19
Cation Exchange Capacity (CEC, cmol$_c$ kg^{-1})	11
Exchangeable cations (cmol$_c$ kg^{-1})	
Exchangeable K	0.44
Exchangeable Ca	4.50
Exchangeable Mg	1.15
Exchangeable Na	0.11

6.2.3 Treatments and application

Synthetic urine was prepared using chemical components as explained in Chapter 4, section 4.2.3 and was applied to each urine-treated lysimeter at an equivalent application rate of 600 kg N ha^{-1}. The control treatments received the same amount of water. Two sets of treatments were applied as late-autumn (first treatments) and mid-winter (second treatments) applications of urine (Table 6.2). Pasture in all lysimeters was cut to a uniform standing height of 50 mm above the soil surface before treatment application. The combination of LS and GA was examined for both the late-autumn and mid-winter applications because literature evidence shows that GA is more effective when applied during winter temperatures (Williams and Arnold 1964). The nitrification inhibitor DCD was used as a reference chemical in this study.

All treatments were applied in a completely randomised block design and replicated four times. The synthetic urine was applied in a single application in both late-autumn and mid-winter. Other treatments (DCD, LS, PA-MA, and GA) were applied as a uniform spray application immediately after 4 h of urine application to the lysimeter surface for both application periods.

Table 6.2 Details of treatments applied in late-autumn (applied 25 May 2021 to 16 November 202) and mid-winter (applied 25 July 2021 to 16 November 2021). DCD, LS, and PA-MA were used in this study as nitrification inhibitors.

Number	Treatments	Urine N rate (kg N ha^{-1})
	Late-autumn treatments	
1	Control (water only)	No urine
2	Urine only	600
3	Urine + DCD at 10 kg ha^{-1}	600
4	Urine + LS at 120 kg ha^{-1}	600
5	Urine + PA-MA at 10 kg ha^{-1}	600
6	Urine + split application of LS (2LS) at same rate, initial and after a month of first application	600
7	Urine + ProGibb SG at 80 g ha^{-1} (GA) + LS	600
	Mid-winter treatments	
1	Control (water only)	No urine
2	Urine only	600
3	Urine + ProGibb SG at 80 g ha^{-1} (GA)	600
4	Urine + ProGibb SG at 80 g ha^{-1} (GA) + LS	600

Note: Water was applied at same volume as the urine.

6.2.4 Soil sampling

Soil was sampled from each lysimeter at days 1, 7, 14, 28, 37, and 64 after urine application using a stainless-steel corer with dimensions of 150 mm depth x 30 mm diameter. However, for the mid-winter treatments soil sampling was only possible at days 1, 7, 14, and 64 due to COVID-19 lockdown in NZ during this period. Two soil cores were sampled per lysimeter and cores from the same treatments were composited to form a bulk sample. Sample holes were immediately backfilled using bulk soil and white plastic markers were inserted to identify sampled areas. Fresh soil samples were immediately divided into two parts; (a) one was put in a cooler (on ice) in the field and transferred to -20 °C freezer for ammonia oxidizers analysis, and (b) the second part was transferred to <4 °C and stored until analysis.

6.2.5 Soil chemical analysis

Soil was mixed manually and sieved through a 2 mm stainless steel sieve prior to analysis. Four replicate samples were analysed from each treatment. Soil moisture content was determined using the method outlined in Chapter 3, section 3.2.1.1 and soil mineral N (NH_4^+ -N/NO_3^- -N), and soil bioavailable Cu were analysed according to the methods described in Chapter 4, section 4.2.5. Soil mineral N and bioavailable Cu were calculated on a soil dry weight basis.

6.2.6 Soil DNA extraction and real-time quantitative PCR

The soil DNA was extracted from 0.25 g of soil sampled at days 1, 37, and 64 for late-autumn treatment and at days 1, 14, and 64 for mid-winter treatment using the PowerSoil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. Soil DNA quality and quantity and AOB/AOA amoA gene quantification was made using the method described in Chapter 4, section 4.2.7. Briefly, AOB/AOA amoA gene quantitative PCR (qPCR) was performed in a LightCycler® 480III (Roche, software release 1.2.1.62 SP 3). The primers, primer sequence and reaction conditions are as outlined in Table 4.2 and Table 4.3. Each qPCR (10 µL mixture) contained 1 µL 10-fold diluted DNA samples, 5 µL SsoFast™ EvaGreen® Supermix, 0.52 µL of each forward and reverse primer, and 2.96 µL RNA/DNA free water. A 10-fold diluted DNA samples of known concentration was used as a standard. PCR efficiency for AOB/AOA were, 97 and 104%, respectively.

6.2.7 Drainage water collection and analysis

Drainage water was collected after every rainfall event except when NZ was under lock down (between 17 August 2021 to 07 September 2021). Total drainage water was collected and analysed as outlined in section 5.2.5. Leached NO_3^- -N and NH_4^+ -N were calculated from the NO_3^- -N/NH_4^+ -N concentration in drainage water and the total drainage volume. Total mineral N leaching was the sum of NH_4^+ -N and NO_3^- -N leaching.

6.2.8 Herbage sampling and analysis

Lysimeter herbage was cut at approximately 50 mm height using an electric cutter to simulate typical residual pasture height after grazing (Di and Cameron 2002b). Fresh herbage samples were oven dried at 65 °C for a week and weighed to record pasture DW. Oven dried pasture samples were prepared for N concentration analysis as outlined in Chapter 5, section 5.2.7.

6.2.9 Climatic data

Climate data was sourced from NIWA's (National Institute of Water and Atmosphere) climatic data site (https://cliflo.niwa.co.nz). Additionally, soil temperature sensors (HortPlus Microl Loggers, Model Z) were placed at 10 cm soil depth and a manual rain gauge was installed onsite.

6.2.10 Data analysis and net nitrification rate calculation

The collected data was tested for normality and analysed using Minitab (Version 19. Minitab Inc., USA). Significant ($P < 0.05$) differences between means were assessed using Turkey post hoc tests. Graphs were drawn using Origin Pro 9.

The net nitrification rate was calculated using equation 3.1 as outlined in Chapter 3, section 3.2.3.4 of this thesis.

6.3 Results

6.3.1 Climatic data and drainage

Total rainfall during the experimental period (May to December) was 583.8 mm with 183.8 mm collected as drainage (Figure 6.1). The soil water deficit was low between May and June and extremely high between September to November. The average daily soil temperature was below 10 °C between June and July, increasing to an average of above 15 °C in November (Figure 6.1).

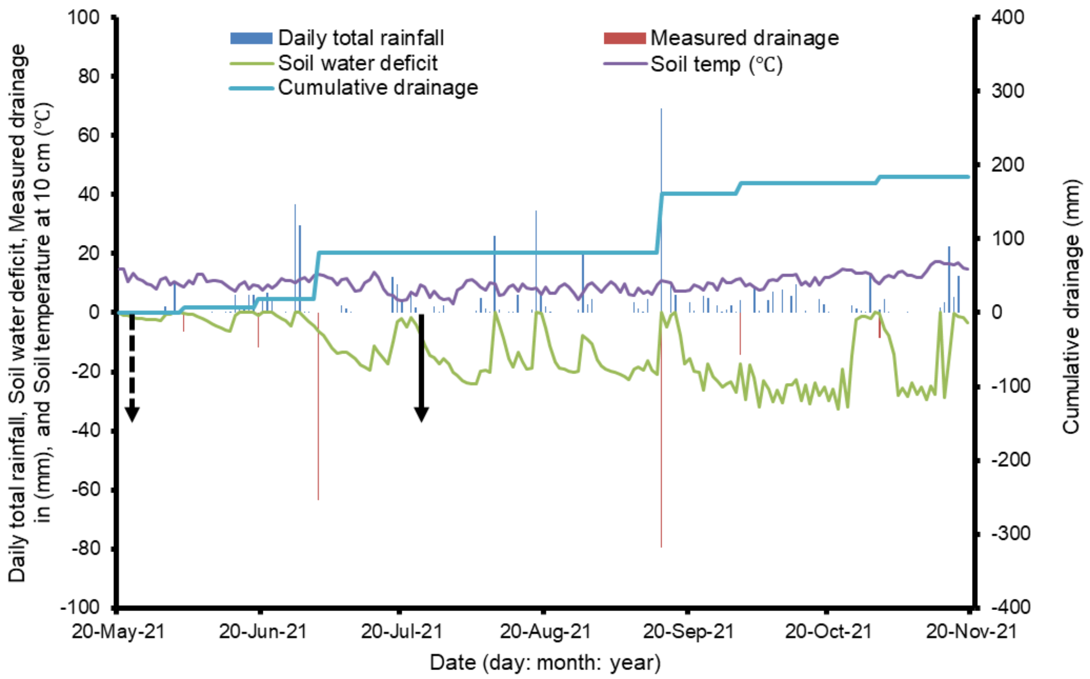


Figure 6.1 Daily total rainfall, soil water deficit, measured drainage, cumulative drainage, and average soil temperature at 10 cm depth during the experimental period. The black-broken line arrow shows the late-autumn treatment application, while the black-solid line arrow shows the mid-winter treatment application.

6.3.2 Soil bioavailable Cu

The soil bioavailable Cu concentration for the late-autumn application was significantly influenced by the application of nitrification inhibitors relative to the urine-only treatment (Figure 6.2A). The PA-MA treatment significantly ($P < 0.05$) reduced bioavailable Cu by an average of 36% for all samplings, except for Day 1. There was a significant ($P < 0.05$) reduction in bioavailable Cu associated with the application of DCD on Days 1 and 7. Both LS and GA + LS induced a nominal reduction in bioavailable Cu over the duration of the experiment, with the exception of Day 7. Generally, the application of 2LS reduced the bioavailable Cu concentration relative to the urine-only treatment, however, this effect was only significant on Days 7 and 37.

For the mid-winter treatments, there was no significant change in bioavailable Cu concentration associated with the application of the GA-only treatment relative to urine-only across the different sampling times (Figure 6.2B). Generally, there was a reduction in bioavailable Cu concentration induced by GA + LS, however this reduction was only significant (26% reduction) relative to the urine-only treatment at Day 1.

6.3.3 Late-autumn NH_4^+ -N and NO_3^- -N concentration

The soil NH_4^+ -N concentration for the late-autumn urine-only treatment decreased from 782.2 to 5.9 NH_4^+ -N kg ha^{-1} after 64 days of urine application (Figure 6.3A). Significantly ($P < 0.05$) higher NH_4^+ -N concentrations in the DCD and PA-MA treatment lysimeters were observed throughout the sampling period (except for Day 1) relative to the urine-only treatment. The NH_4^+ -N concentration in soil treated with DCD and PA-MA at Day 64 was 374 and 193 kg NH_4^+ -N ha^{-1}, respectively, relative to 5.86 kg NH_4^+ -N ha^{-1} for the urine-only treatment at this time. Generally, there were no significant changes in NH_4^+ -N concentration induced by the application of LS and GA + LS treatments relative to the urine-only treatment at any sampling time except Day 1 where there was a significantly ($P < 0.05$) lower NH_4^+ -N concentration. The 2LS treatment showed a significantly ($P < 0.05$) lower NH_4^+ -N concentration at Day 1 (relative to the urine-only treatment) but significantly higher NH_4^+ -N concentration at Day 37 which was after application of the second dose of LS.

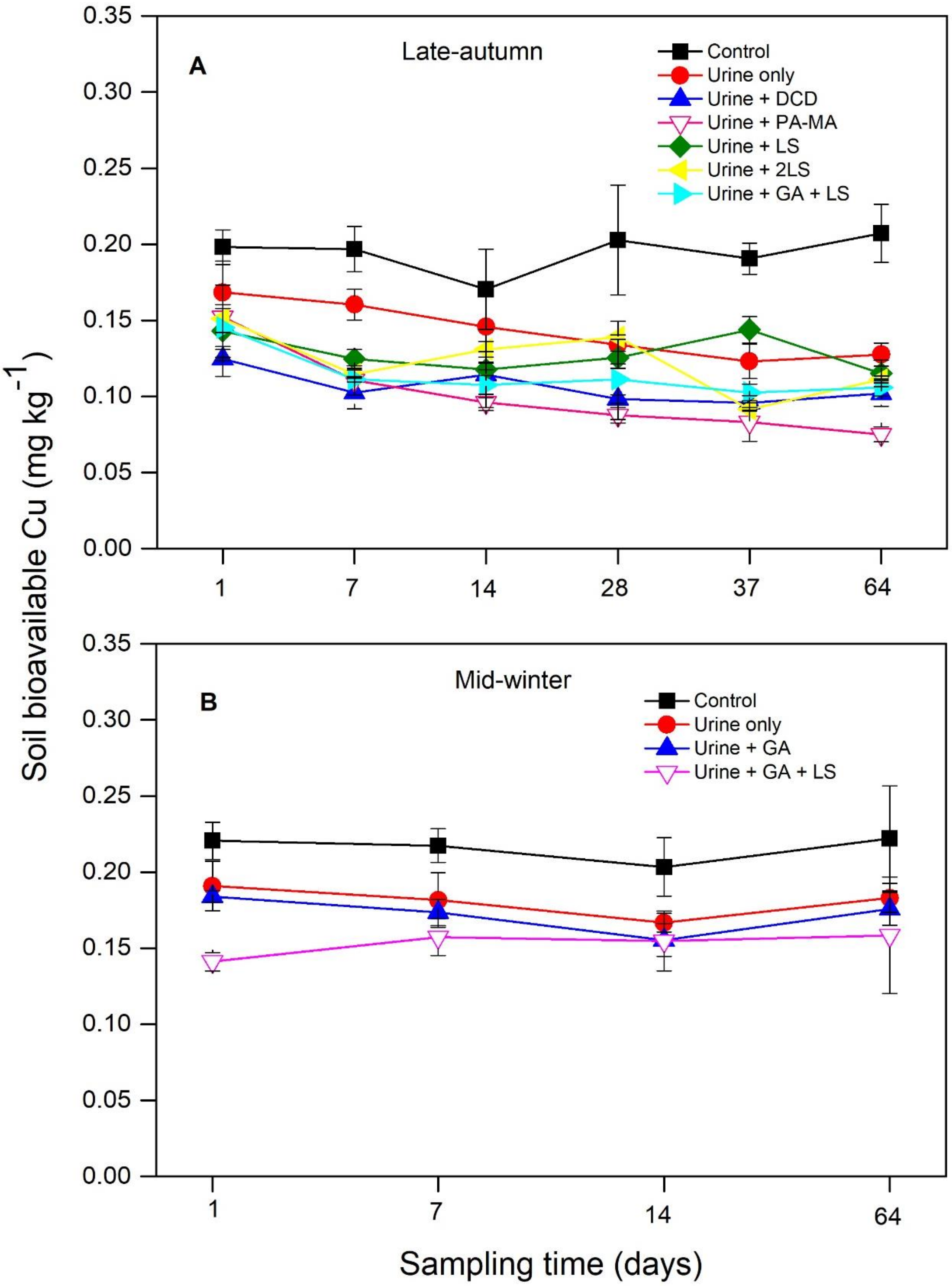


Figure 6.2 Bioavailable Cu after late-autumn (A) and mid-winter (B) treatment application as a function of treatment and sampling time. Vertical error bars indicate standard deviation (*n = 4*).

There was significant (P < 0.05) reduction of soil NO_3^- -N concentration associated with the late-autumn application of DCD and PA-MA by average values of 72 and 33%, respectively, in the first 37 Days (Figure 6.3B). However, both treatments showed significantly (P < 0.05) higher NO_3^- -N concentrations at Day 64, relative to the urine-only treatment. The LS, 2LS, and GA + LS treatments significantly (P < 0.05) reduced the soil NO_3^- -N concentration by an average of 38, 34, and 46%, respectively over the first 14 Days of sampling compared with the urine-only treatment (Figure 6.3B). After Day 14 the results greatly varied.

6.3.4 Mid-winter NH_4^+ -N and NO_3^- -N concentration

The soil NH_4^+ -N concentration for the mid-winter urine-only treatment decreased from 500.8 to 33.6 kg NH_4^+ -N ha^{-1} after 64 Days (Figure 6.3C). Application of the GA-only treatment significantly (P < 0.05) reduced the NH_4^+ -N concentration (relative to urine-only treatment) at all sampling times with the exception of Day 64. The reduction was by values of 17, 30, and 25% at Days 1, 7, and 14, respectively. The GA + LS treatment significantly (P < 0.05) reduced the NH_4^+ -N concentration by values of 19 and 11% at Days 1 and 7, respectively, relative to the urine-only treatment. However, at Days 14 and 64 there was an increase in NH_4^+ -N concentration by 13 and 17%, respectively.

Mid-winter application of GA-only and GA + LS significantly (P < 0.05) reduced the soil NO_3^- -N concentration by an average of 24 and 19% relative to the urine-only treatment at all sampling times, with the exception of Day 64 (Figure 6.3D).

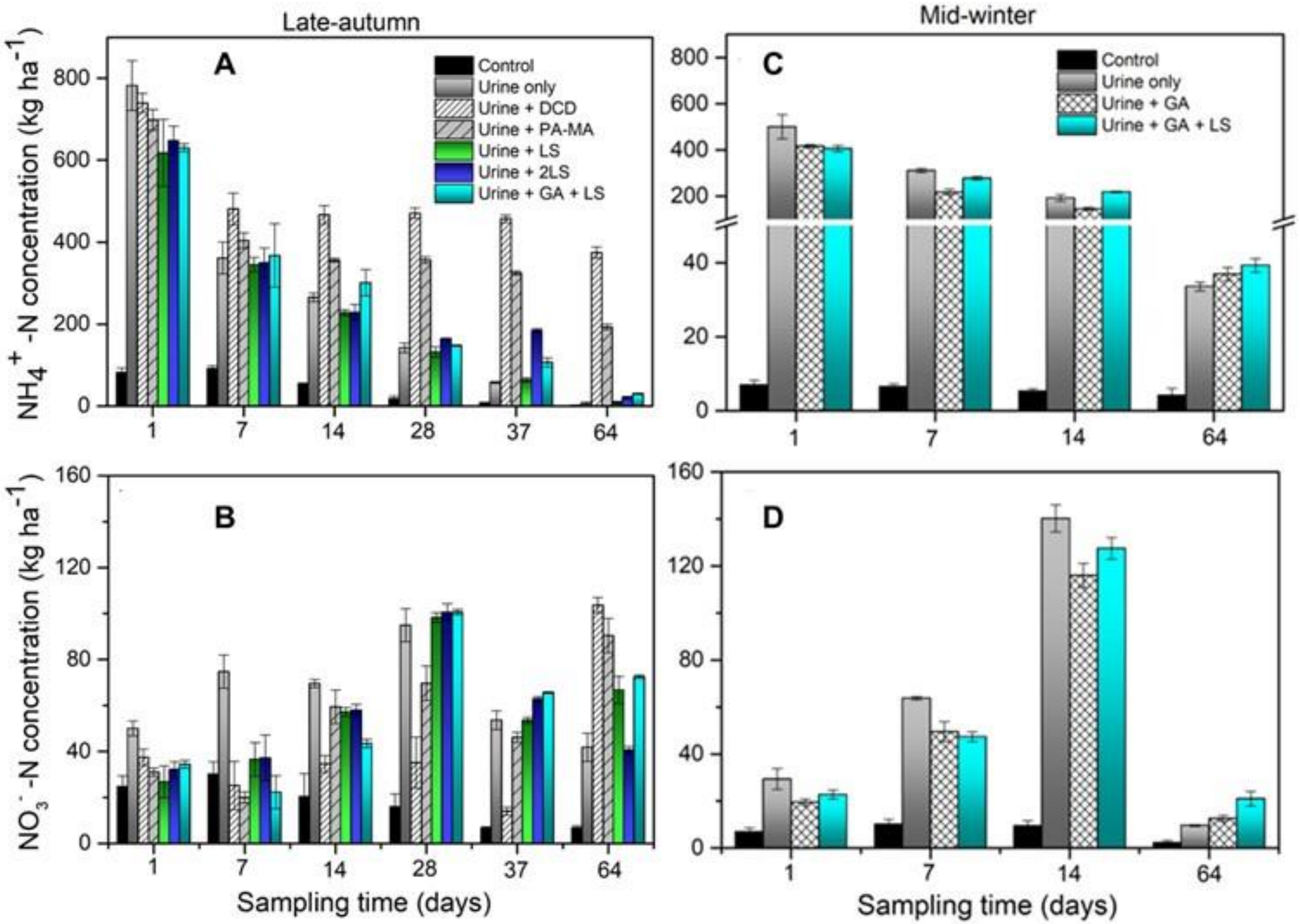


Figure 6.3 NH_4^+ -N concentration (A) and NO_3^- -N concentration (B) following late-autumn treatment application; and NH_4^+ -N concentration (C) and NO_3^- -N concentration (D) following mid-winter treatment application. Vertical error bars indicate standard deviation of mean (*n = 4*).

6.3.5 Net nitrification rate

To further analyse the effect of the applied nitrification inhibitors on nitrification rate, net nitrification rate was calculated (Figure 6.4). The net nitrification rate for all treatments following late-autumn application indicated that nitrification was higher in the first 7 Days after urine application. Both DCD and PA-MA treatments significantly ($P < 0.05$) reduced net nitrification rate (relative to urine-only) by an average factor of 70 and 55%, respectively, in the first 37 Days (Figure 6.4A). Application of LS, and GA + LS induced significant ($P < 0.05$) changes in net nitrification rate in the first 7 Days after urine application, while the 2LS treatment significantly ($P < 0.05$) reduced the net nitrification rate by values of 32, 60, and 90% at Days 7, 28, and 37 compared with the urine-only treatment. There was a significant and positive correlation ($R^2 = 0.385$, $P < 0.001$) between changes in bioavailable Cu and net nitrification rate across the different sampling periods and treatments (Figure 6.4C).

The mid-winter results demonstrated that the GA-only treatment did not induce significant changes in net nitrification rate relative to the urine-only treatment at any sampling time (Figure 6.4B). In contrast, the combined GA + LS treatment significantly ($P < 0.05$) reduced net nitrification by an average of 41% in the first 14 Days relative to the urine-only treatment. There was no relationship between changes in bioavailable Cu and net nitrification rate across the mid-winter treatments (Figure 6.4D).

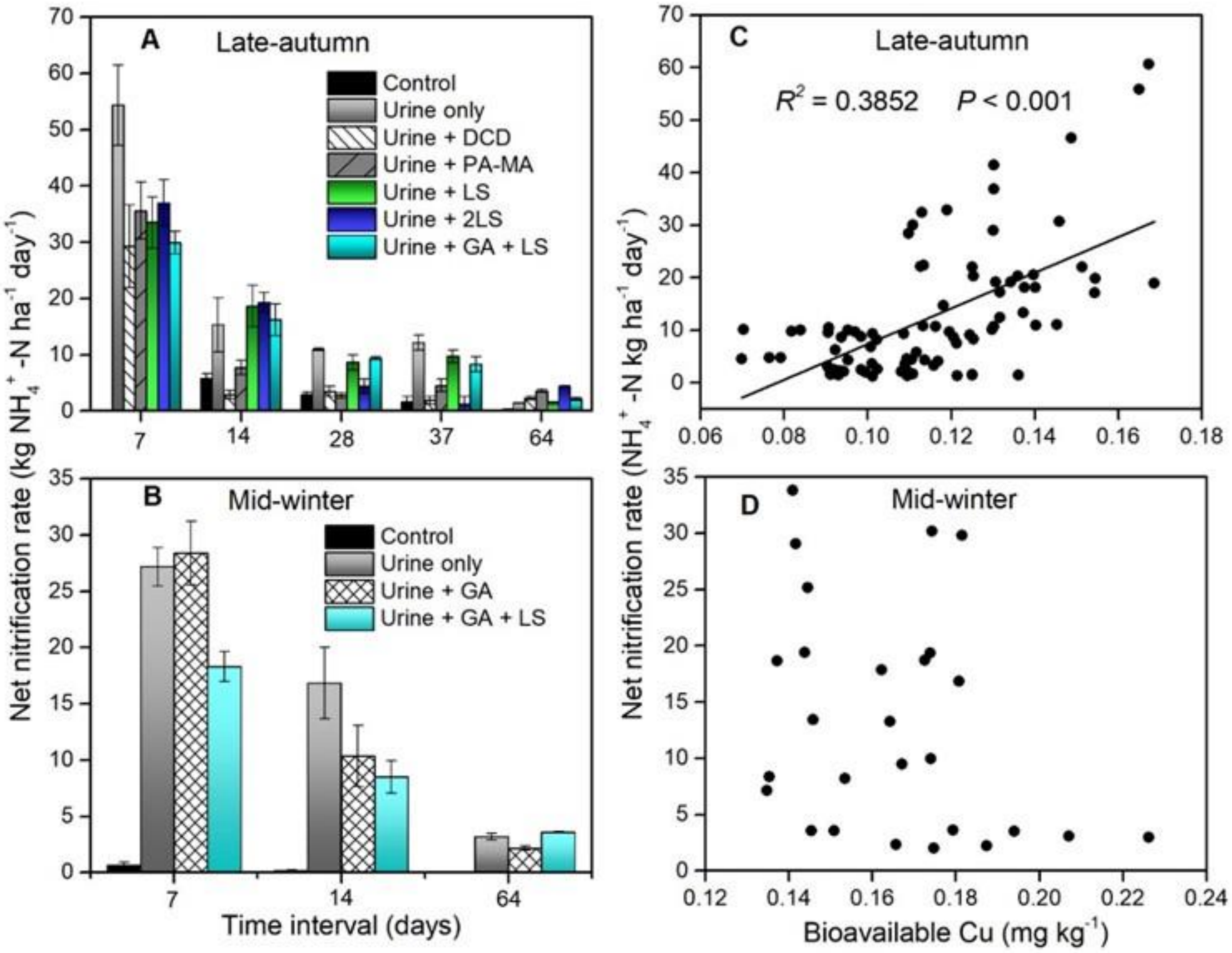


Figure 6.4 Net nitrification rate at the different sampling intervals as influenced by the application of late-autumn (A) and mid-winter (B) treatments. Correlation between bioavailable Cu and net nitrification rate during the experimental period in late-autumn (C) and mid-winter (D).

6.3.6 Abundance of AOB and AOA amoA gene

6.3.6.1 Late-autumn treatment application

During late-autumn, the DCD and PA-MA treatments significantly (P < 0.05) reduced AOB *amoA* gene abundance by average factors of 1.7 and 1.6, respectively, relative to the urine-only treatment at all sampling times (Figure 6.5A). The LS treatment significantly (P < 0.05) reduced AOB *amoA* gene abundance only at Day 1 by a factor of 2.2 when compared to lysimeters treated with urine-only. AOB *amoA* gene abundance was significantly (P < 0.05) reduced by factors of 1.9 and 1.6 in the 2LS treatment, and by 2.1 and 1.4 in the GA + LS treatment on Days 1 and 37, respectively, relative to the urine-only treatment. No significant changes in AOB *amoA* gene abundance were observed between the urine-treated lysimeters on Day 64. Correlation analysis showed that abundances of AOB *amoA* genes in the different urine treated lysimeters was influenced by changes in bioavailable Cu within the sampling periods (R^2 = 0.6129, P < 0.001) (Figure 6.5C).

There were no significant changes in AOA *amoA* gene abundance between all urine treated lysimeters across the late-autumn sampling times (Figure 6.5B). Recorded changes in AOA *amoA* gene abundance did not show a significant corelation (R^2 = 0.1865, P > 0.001) with changes in bioavailable Cu for the different sampling times and treatments (Figure 6.5D).

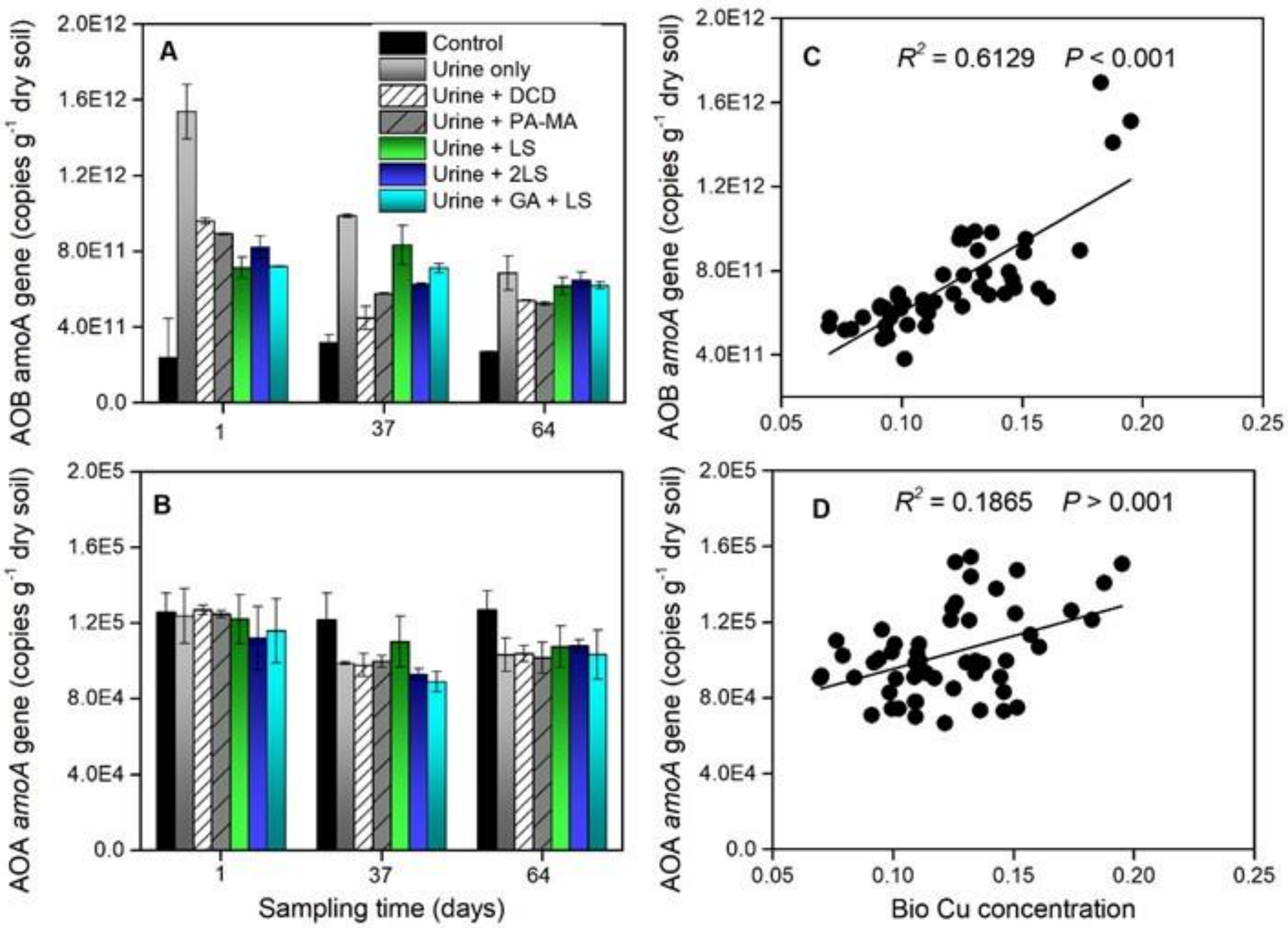


Figure 6.5 Late-autumn treatment application on AOB *amoA* gene abundance (A) and AOA amoA gene abundance (B) as a function of treatments and sampling time. Vertical error bars represent standard deviation of mean (*n = 4*). Correlation between AOB *amoA* gene abundance and bioavailable Cu (C), and AOA *amoA* gene abundance and bioavailable Cu (D)in late-autumn treatment.

6.3.6.2 Mid-winter treatment application

There was no significant reduction in AOB *amoA* gene abundance as a function of GA treatment relative to the urine-only treatment for any sampling time (Figure 6.6A). However, the combination of GA + LS treatment significantly ($P < 0.05$) reduced AOB *amoA* gene abundance (relative to urine-only) by factors of 2.5 and 1.4 on Days 1 and 14, respectively. The AOB *amoA* gene copy number across the samplings was correlated ($R^2 = 0.5009$, $P < 0.001$) to the bioavailable Cu within each sampling time (Figure 6.6C).

There were no significant differences in AOA *amoA* gene abundance between treatments for all mid-winter samplings (Figure 6.6B). The change in AOA *amoA* gene copy numbers for the different treatments did not correlate with changes in bioavailable Cu concentration at each sampling (Figure 6.6D).

6.3.7 Mineral N leaching (NO_3^- -N and NH_4^+ -N)

In late-autumn, rainfall generated six leaching events resulting in cumulative drainage of 183.8 mm (Appendix 3 Figure A3.1A-B). Nitrate-N and NH_4^+ -N leaching across the drainage events was summed to give the cumulative NH_4^+ -N and NO_3^- -N leaching (mineral N leaching) during the experimental period. Cumulative NO_3^- -N leaching was the main source of mineral N leaching with values ranging from 281 to 383 kg ha^{-1} for the urine treated lysimeters (Table 6.3). In comparison, cumulative NH_4^+ -N leaching was negligible ranging from 9 to 11 kg ha^{-1}. Application of DCD, PA-MA, 2LS, GA + LS treatments significantly ($P < 0.05$) reduced total mineral N leaching by 39, 16, 30, and 19%, respectively, relative to the urine-only treatment (Table 6.3). However, there was no significant difference in total mineral N leaching between the LS and urine-only treatment.

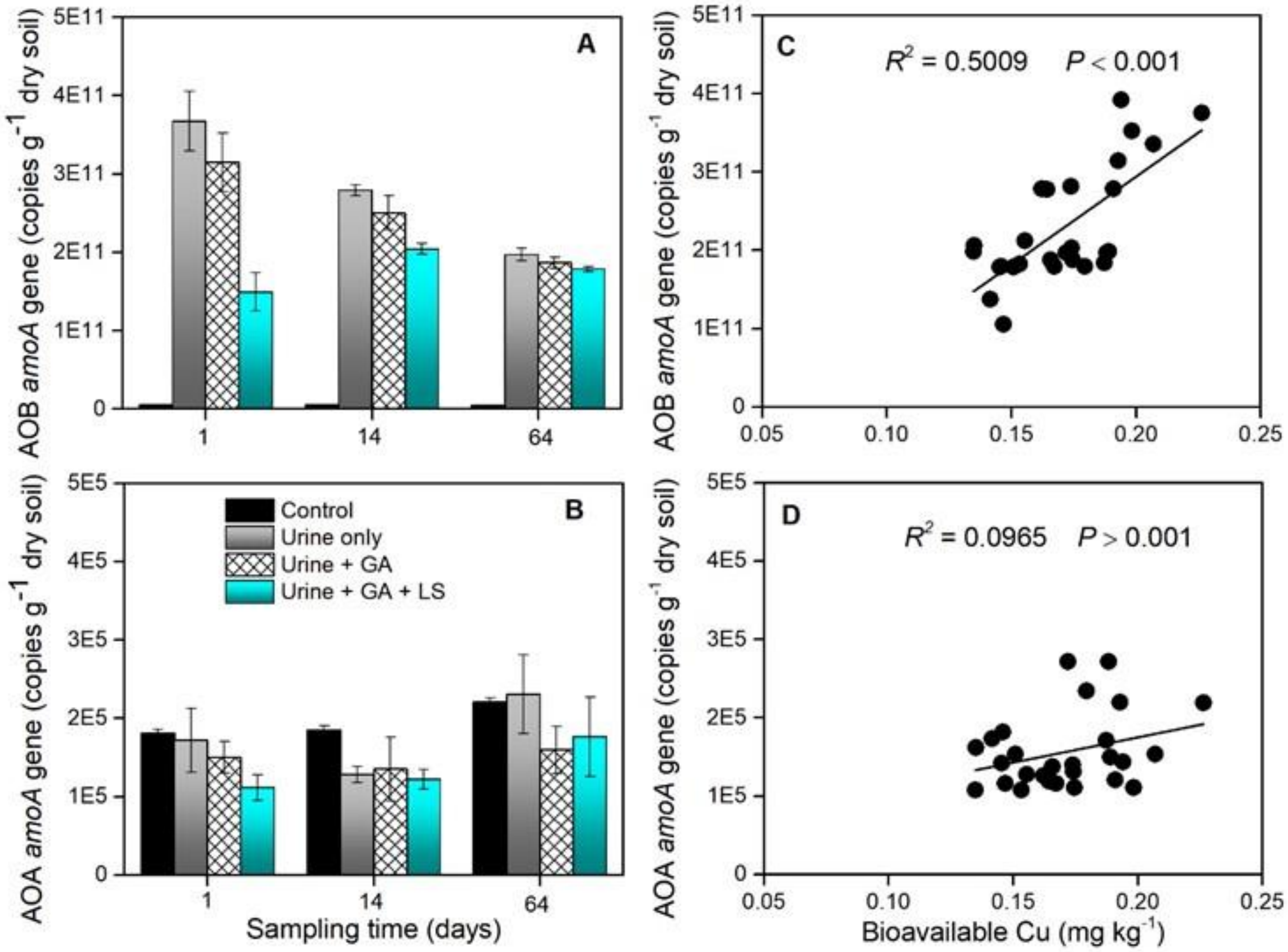


Figure 6.6 Mid-winter treatment application on AOB *amoA* gene abundance (A) and AOA *amoA* gene abundance (B) as a function of treatments and sampling time. Vertical error bars represent standard deviation of mean ($n = 4$). Correlation between AOB *amoA* gene abundance and bioavailable Cu (C), and AOA *amoA* gene abundance and bioavailable Cu (D) in mid-winter treatment.

In mid-winter, rainfall resulted in three leaching events for 103.3 mm cumulative drainage (Appendix 3 Figure A3.1C-D). Cumulative mineral N leaching during the experimental period ranged from 186 to 225 kg NO_3^- -N ha^{-1} and 8 to 10 kg NH_4^+ -N ha^{-1} in the urine treated lysimeters (Table 6.3). The GA-only treatment significantly ($P < 0.05$) increased total mineral N leaching by 24.4% relative to the urine-only treatment. The GA + LS treatment nominally increased total mineral N leaching by 12.4% compared with the urine-only treatment.

Table 6.3 Late-autumn and mid-winter treatment application cumulative NO_3^- -N leaching, cumulative NH_4^+ -N leaching, and total mineral N leaching (kg N ha^{-1}).

Treatments	Cumulative nitrate leaching (kg NO_3^- -N ha^{-1})	Cumulative ammonia leaching (kg NH_4^+ -N ha^{-1})	Total mineral N leaching (kg N ha^{-1})
Late-autumn application			
Control	117.9 ± 1.4 e	10.9 ± 0.5 a	-
Urine only	382.7 ± 5.3 a	9.7 ± 1.0 a	263.2 ± 6.2 a
Urine + DCD	281.3 ± 5.8 d	9.2 ± 0.4 a	161.9 ± 5.5 d
Urine + PA-MA	341.0 ± 4.6 b	8.5 ± 0.8 a	220.2 ± 5.4 b
Urine + LS	378.8 ± 8.8 a	10.7 ± 0.6 a	260.4 ± 9.7 a
Urine + 2LS	303.3 ± 5.1 cd	9.4 ± 1.1 a	183.9 ± 5.5 cd
Urine + GA + LS	332.6 ±1 0.1 bc	10.5 ± 0.3 a	212.8 ± 10.4 bc
Mid-winter application			
Control	17.6 ± 1.7 c	8.2 ± 0.5 a	-
Urine only	186.0 ± 5.3 b	7.7 ± 0. 4a	167.8 ± 5.5 b
Urine + GA	224.7 ± 3.8 a	10.1 ± 0.7 a	208.8 ± 3.7 a
Urine + GA + LS	205.6 ± 9.7 ab	8.9 ± 0.5 a	188.6 ± 9.8 ab

Note: Numbers after ± represent standard error of mean. Different small letters between variables in a column represent significant difference, $P < 0.05$.

6.3.8 Cumulative N uptake and cumulative DM yield

Cumulative N uptake and yield refers to the sum of the individual harvests (four cuts for late-autumn [Appendix 3 Figure A3.2] and three cuts for mid-winter [Appendix 3 Figure A3.2]) over the two application periods. Following late-autumn treatment, cumulative N uptake for the PA-MA and GA + LS treatments significantly ($P < 0.05$) increased by 25 and 24%, respectively, relative to the urine-only treatment (Table 6.4). However, application of the DCD, LS, and 2LS treatments only induced a nominal increase in cumulative N uptake relative to the urine-only treatment (Table 6.4). In terms of late-autumn cumulative DM yield, all inhibitor treated lysimeters (except for LS) showed increased cumulative DM yield relative to the urine-only treatment, however the increase was not significant (Table 6.4). In contrast, cumulative DM yield associated with LS treatment showed a nominal 7.9% reduction relative to urine-only treatment.

For the mid-winter treatments there was no significant change in either cumulative N uptake or DM yield induced by the application of GA-only relative to the urine-only treatment. In contrast, the combination of GA + LS significantly ($P < 0.05$) increased cumulative N uptake by 18% and the increase in N uptake resulted in a 16% increase in cumulative DM yield relative to the urine-only treatment (Table 6.4).

Table 6.4 Late-autumn and mid-winter treatment application cumulative N uptake, % N uptake changes relative to urine-only, cumulative yield, % yield changes relative to urine-only.

Treatments	**Cumulative N uptake (kg N ha^{-1})**	**% N uptake relative to urine-only**	**Cumulative DM yield (kg DM ha^{-1})**	**% Yield relative to urine-only**
Late-autumn application				
Control	148.2 ± 15.3 c	-	7753 ± 448 b	-
Urine only	387.7 ± 23.6 b	-	12997 ± 762 a	-
Urine + DCD	462.8 ± 23.0 ab	+19. 4	13554 ± 684 a	+4.3
Urine + PA-MA	484.6 ± 21.1 a	+25.0	14435 ± 1267 a	+11.1
Urine + LS	389.2 ± 19.0 b	+0.4	11966 ± 764 a	-7.9
Urine + 2LS	418.5 ± 25.0 ab	+7.8	13980 ± 653 a	+7.6
Urine + GA + LS	494.1 ± 14.3 a	+24.4	14488 ± 455 a	+11.5
Mid-winter application				
Control	91.8 ± 15.0 c	-	6539 ± 435 b	-
Urine only	353.0 ± 12.1 b	-	11642 ± 326 a	-
Urine + GA	346.0 ± 9.5 b	-2.0	12057 ± 594 a	+3.6
Urine + GA + LS	417.9 ± 16. 6a	+18.4	13487 ± 592 a	+15.8

Note: Numbers after ± represent standard error of mean. Different small letters between variables in a column represent significant difference, $P < 0.05$. (+) represents increase; (-) represents decrease.

6.4 Discussion

6.4.1 Application of organic inhibitors

6.4.1.1 Effect on bioavailable Cu, nitrification rate, and ammonia oxidizers

The application of the PA-MA treatment to soil significantly (P < 0.05) reduced the concentration of bioavailable Cu in soil from Day 1 at all sampling times during the late-autumn season, relative to the urine-only treatments (Figure 6.2A). This significant reduction may be due to the complexation of Cu by carboxylic and hydroxy functional groups of the PA-MA inhibitor which effectively reduced the concentration of bioavailable Cu in soil solution (Li et al. 2021; Qiu and Mao 2013; Zhou et al. 2020). This is consistent with the previous report of reduced bioavailable Cu in the presence of the PA-MA in Chapter 3. However, application of treatment LS induced only a nominal reduction in bioavailable Cu concentration. This result is not consistent with the incubation results reported in Chapter 3, where LS application significantly reduced the bioavailable Cu concentration in soil. The reduced effectiveness of LS relative to PA-MA may have been influenced by the ambient environmental conditions under which the study was completed; Byrne et al. (2020) reported that soil moisture and soil temperature might reduce inhibitor efficiency. These data suggest that to realise a significant reduction in bioavailable Cu through LS, another dose should be applied one month after the first (Day 37 in the current study). The reference nitrification treatment in this study (DCD) also significantly reduced the concentration of soil bioavailable Cu.

The changes in bioavailable Cu induced by the inhibitors had a significant influence on nitrification. This is evidenced by the significant and positive correlation (R^2 = 0.3850, P < 0.001) between bioavailable Cu and net nitrification rate over the late-autumn experimental period (Figure 6.4C). The PA-MA treatment was more effective in reducing net nitrification rate during the first 37 Days than all other treatments except DCD for which the reduction in nitrification rate was the same. The greater reduction in net nitrification rate as a function of PA-MA can be associated with the increased reduction in bioavailable Cu presented in Figure 6.2A. Literature evidence has demonstrated that Cu deficiency can limit the functioning of nitrifiers (Wagner et al. 2016), thus reducing nitrification rate. The 2LS treatment significantly ($P < 0.05$) reduced net nitrification rate by 90% at Day 37 which was 5 days after application of the second dose of LS. These results suggest that the application of the second dose of LS enhanced the reduction in bioavailable Cu concentration, and this is linked to the observed reduction in net nitrification rate relative to one dose application of LS. The application of LS alone significantly ($P < 0.05$) reduced net nitrification only in the first 7 Days after urine application, and these results suggest that LS may have been effective but only for a short period in the soil. Several factors can influence the efficiency of LS in the soil, including the adsorption of LS to soil organic matter and clay or microbial degradation (Martikainen 2022). Zhang et al. (2020) demonstrated that inhibitor effectiveness in the soil can be influenced by both decomposition and adsorption to soil organic matter. In addition, the increase in microbial activity after urine application can degrade the lignin compound in the LS (Khatami et al. 2019), thus making it less effective in reducing bioavailable Cu in the soil. The reference material DCD showed more effective reduction in net nitrification rate relative to the other treatments.

In the present study, the positive and significant correlation ($R^2 = 0.6129$ $P < 0.001$) between bioavailable Cu concentration in soil with the abundance of AOB *amoA* gene (Figure 6.5C) provides strong evidence for the role of Cu as the co-factor in the functioning of the AMO enzyme (Ayub et al. 2022). A reduction in bioavailable Cu in soil will therefore reduce AOB activity and inhibit the conversion of NH_4^+ -N to NO_3^- -N. In contrast, a scenario of higher bioavailable Cu concentration will increase AOB activity. However, there was no effect of bioavailable Cu reduction on AOA *amoA* gene abundance. This indicates that the contribution of AOA to nitrification under high N conditions might be less than AOB. These results support the findings presented in Chapter 4 that nitrification rate under high N content conditions is more influenced by AOB population growth than AOA population (Chapter 4): AOA populations may be better adapted to low N conditions (Shen et al. 2008). Further support for the data interpretation is provided by Di et al. (2009) who demonstrated a significant correlation (R^2 = 0.51, P = 0.01) between AOB *amoA* gene and nitrification rate in urine patches but no correlation for the AOA *amoA* gene.

6.4.1.2 Effect on mineral N leaching, pasture N uptake and DM yield

Results in this Chapter result show that there was NO_3^- -N leaching in the control treatment, and this may be associated with the high organic N mineralisation. The soil loss during repacking of lysimeters may have increased aeration, which has a positive influence on microbial activity in the soil thus contributing to high N mineralisation as reported by Szostek et al. (2022). An increase in N mineralisation increases the NO_3^- -N production within the soil system, resulting in a higher proportion of the produced NO_3^- -N being susceptible to leaching during drainage events. The investigation of N leaching using treatments PA-MA and 2LS over the late-autumn season showed a significant (P < 0.05) reduction in mineral N leaching, however lysimeters treated with LS alone showed no significant reduction in mineral N leaching relative to the urine-only control. The effective reduction of mineral N leaching by PA-MA and 2LS in this study is linked to the significant reduction in bioavailable Cu concentration which reduced the net nitrification rate and AOB *amoA* gene abundance in the soil. This effect of PA-MA and 2LS reduced mineral N leaching by values of 16 and 30% relative to the urine-only treatment. The reduction in mineral N leaching induced by PA-MA and 2LS increased N uptake by 25 and 7.8% and herbage DM yield by factors of 11 and 8%, respectively, relative to the urine-only control. These results suggest that PA-MA and 2LS were effective in delaying the oxidation of NH_4^+ -N during the period of rapid nitrification, which eventually benefited pasture growth. The delay of NH_4^+ -N oxidation helps to keep N in a stable form (NH_4^+) for plant utilisation and in this study, even after 64 Days of urine application, the PA-MA treatment recorded a higher NH_4^+ -N relative to the urine-only and LS treatments. The higher NH_4^+ -N in the PA-MA treatment is further confirmed by the higher herbage N uptake in all individual herbage cut results (Appendix 3 Figure A3.2) relative to both urine-only and LS, which provides strong evidence that PA-MA reduced nitrification. The application of the single dose of LS did not lead to a significant ($P > 0.05$) change in mineral N leaching. This lack of effectiveness can be attributed to the nominal reduction in bioavailable Cu concentration induced by LS which only reduced net nitrification and AOB *amoA* gene abundance during the first 7 Days after urine application. As a consequence, it is inferred that nitrification rate increased after 7 Days, increasing the proportion of mineral N that was in the form of NO_3^- -N (Figure 6.3B) and susceptible to leaching. The reference material (DCD) also showed a significant reduction in mineral N leaching by 39% relative to urine-only. Several studies have demonstrated the efficacy of DCD on reducing mineral N leaching from pasture field studies is within the range of 20-69% depending on soil type and other factors (Cameron et al. 2014; Kim et al. 2014; Ledgard et al. 2014). In this Chapter, the effectiveness of DCD is associated with the significant Cu reduction which may have inhibited the enzyme AMO functioning in the ammonia-oxidizing microorganisms.

6.4.2 Effect of a growth hormone on mineral N leaching

The inclusion of GA with the inhibitor treatments improved the effectiveness of LS in reducing mineral N leaching during the late-autumn application period. The combined application of GA + LS reduced mineral N leaching by 10% relative to LS treatment alone and this was associated with increased N uptake and DM yield by 24 and 21%, respectively, relative to LS alone. The effectiveness of GA + LS treatment to reduce mineral N leaching can be associated with an increase in N uptake induced by the growth hormone. The observed effect of GA to reduce mineral N leaching and increase N uptake is consistent with other studies that have observed a similar effect (Bishop and Jeyakumar 2021; Matse et al. 2022b). For example, in Chapter 5, it is reported that a late-autumn application of GA + LS to Pumice and Pallic soils significantly ($P < 0.05$) reduced mineral N leaching by 40 and 38%, respectively, relative to treatment with LS alone. This suggests that LS alone is not effective in reducing mineral N leaching.

Mid-winter application of GA-only and GA + LS did not significantly reduce mineral N leaching when compared to the urine-only control. However, the combination of GA + LS increased N uptake and DM yield by 21 and 12% relative to GA-only (Table 3). This lack of a significant treatment effect of GA on N leaching for the mid-winter application may have been influenced by weather conditions over the treatment period. For example, Figure 6.1 shows that after the application of mid-winter treatments, there was high water deficit. This may have reduced the effect of GA's to enhance plant growth.

In summary, the results from this study provide fundamental information that can underpin ongoing research to design a sustainable mechanism for reducing N losses in grazed pasture systems. In this current study, DCD proved to be a highly effective inhibitor in reducing mineral N. However, the sale of DCD has been voluntarily withdrawn from the market due to the detection of traces of DCD found in milk powder (Pal et al. 2016). The alternative and novel inhibitors PA-MA, 2LS, and the combination GA + LS demonstrated great potential under late-autumn conditions explored in the current study. These inhibitors showed a similar effect to DCD in terms of reducing mineral N leaching during the study period. In addition, the application of PA-MA and GA + LS increased N uptake by 6 and 5% and DM yield by values of 6.8 and 7.2%, respectively, relative to DCD. This provides strong evidence that application of these inhibitors (PA-MA and GA + LS) can be helpful to farmers to minimise environmental contamination associated with N losses, while also improving N use efficiency.

6.5 Conclusions

The results discussed in this Chapter have demonstrated that the application of Cu-complexing compounds to soil will induce a reduction in soil bioavailable Cu concentration and this has a significant influence on soil nitrification rate. This was evidenced by the strong and positive correlation between bioavailable Cu and net nitrification rate and AOB *amoA* gene abundance during the late-autumn treatment period. However, the changes in soil bioavailable Cu concentration did not influence the AOA *amoA* gene abundance for both late-autumn and mid-winter applications. The PA-MA and 2LS treatments showed effective reduction of mineral N leaching which increased pasture N uptake and DM yield. The inclusion of GA as a co-treatment with LS proved to be an effective strategy to improve the effectiveness of LS as an inhibitor in late-autumn. Future research should identify potential amendments or combinations that can improve the inhibition of nitrification rate described in this Chapter.

CHAPTER 7

Integrated discussion: Important findings, implications of the research and suggestions for future work

7.1 Introduction: Research summary

7.1.1 Why was this work conducted?

New Zealand produces about 3% of the world's milk and this means the dairy industry is a major contributor to the NZ economy (DairyNZ 2021). Dairy intensification with time has seen the dairy cow population in NZ increase by 2.81 million animals from 1975/76 to 2020/21(DairyNZ 2021). Global demand for dairy product to is expected to increase by 80% between 2000 and 2050 (Huang et al. 2010), and the NZ dairy industry is striving contribute to this forecast increase. The main challenge experienced by the dairy industry is the high contribution of the sector to environmental challenges such as NO_3^- -N leaching, which results in ground and surface water pollution (Coskun et al. 2017; Joy et al. 2022). Nitrate leaching is a function of dairy cows grazing N-rich pastures (with 18-20% crude protein). Dairy cows only utilise a small proportion (5-30%) of the ingested N with the remaining N content (70-95%) excreted in the urine. Urine is unevenly returned to fields, and this creates small areas of highly concentrated N, described as urine patches. A high percentage of the N in these urine patches become susceptible to leaching as NO_3^- -N during drainage events (Cameron et al. 2013; Di and Cameron 2016). Urine patches are, therefore, the main sources of NO_3^- -N leaching in grazed pastoral dairy systems. Since NO_3^- -N is a product of nitrification, broadening the understanding of the mechanism of nitrification is a vital step in the development of effective mitigation strategies.

7.1.2 What is already known?

The first step of nitrification is the oxidation of NH_4^+ -N to NO_2^- by AOB and AOA followed by the oxidation of NO_2^- to NO_3^- -N by nitrite oxidizing bacteria. The key enzyme responsible for ammonia oxidation to NH_2OH is the AMO enzyme, which is a Cu dependent enzyme. This suggests that copper is a co-factor in the functioning of the AMO enzyme. Therefore, changes in soil bioavailable Cu concentration induced through application of Cu-complexing compounds such as LS and PA-MA can potentially influence the oxidation of NH_4^+ -N to NO_3^- -N. Used in this way, the chemical compounds listed here can be considered as nitrification inhibitors. However, the influence of Cu bioavailability on nitrification rate in agricultural soils has not yet been fully defined. Several studies have highlighted that the effectiveness of inhibitors is reduced under field conditions, and the potential cause is poor N uptake and plant growth. To counter these issues, plant growth stimulants such as GA have been proposed to improve N uptake and plant growth as a complimentary mechanism to the Cu-complexing effect of organic inhibitors.

7.1.3 Research aim?

To address the recognised research gap, the work of this doctoral thesis was designed and implemented to investigate the influence of bioavailable Cu on nitrification rate, AOB/AOA *amoA* gene abundance, and the resulting mineral N leaching in different soils. The rationale for this work is to underpin new advances in the development of nitrification inhibitors and growth stimulants which could assist NZ dairy farmers to increase the productivity of agricultural land while reducing the environmental problems associated with dairy farming.

There are no published studies that characterise the relationship between bioavailable Cu, nitrification rate, and AOB/AOA *amoA* gene abundance and then link this science to mineral N leaching in pastoral soils. The literature search at the beginning of this doctoral study showed that Cu is an important trace element for the functioning of the AMO enzyme, and therefore, reducing bioavailable Cu in soil can potentially influence the functioning of the AMO enzyme. The influence of bioavailable Cu on nitrification rate, AOB/AOA *amoA* gene abundance, and the resulting mineral N leaching, was the key focal point of this thesis work.

7.1.4 How was it undertaken?

The work of this thesis consisted of an incubation study, a greenhouse-pot experiment, and field lysimeters experiments using different soils to understand the effect of Cu-complexing organic materials to reduce Cu bioavailability, and thus, to reduce nitrification in agricultural soils. The influence of bioavailable Cu on nitrification rate and AOB/AOA *amoA* gene abundance in soil was demonstrated in Chapters 3 and 4. In Chapter 3, LS and PA-MA were shown to significantly reduce Cu bioavailability which resulted in a significant inhibition of the nitrification rate, and which could potentially reduce NO_3^- -N leaching at field level. The application of LS and PA-MA at the field level was subsequently examined in field lysimeter studies conducted using Pumice soil under Manawatu climate conditions and Pallic soil under Canterbury climate conditions (Chapter 5). In addition, Chapter 5 examined the effect of combining a growth stimulant, GA with LS on reducing mineral N leaching. The mechanism of reducing bioavailable Cu through the application of organic compounds on nitrification rate, AOB/AOA *amoA* gene abundance and the resulting NO_3^- -N leaching was investigated in a field lysimeter trial using the Manawatu Recent soil (Chapter 6). The mechanism of combining GA and LS on influencing mineral N leaching in both autumn and winter applications was further investigated in Chapter 6

7.2 Key findings

1) ***Bioavailable Cu has significant influence on nitrification rate, but the effect depends on soil characteristics.***

A soil incubation (Chapter 3) and greenhouse experiment (Chapter 4) were established using three soils (Pumice, Pallic, and Recent soils) to examine the effect of different concentrations of Cu on nitrification rate and the abundance of ammonia oxidisers. Chapter 3 demonstrated that nitrification rate increased as a function of increasing levels of added Cu (0.1 to 3 mg kg^{-1}). Similarly, Chapter 4 demonstrated that bioavailable Cu ranging between 0.2 mg and 0.38 Cu kg^{-1} significantly ($P < 0.05$) increased nitrification rate in the Recent and Pallic soils (respectively) relative to a Cu0 (urine-only) treatment. This trend is confirmed by a significant correlation between bioavailable Cu concentration and NO_3^- -N concentration in the Pallic ($r = 0.748$, $P < 0.05$) and Recent ($r = 0.937$, $P < 0.01$) soils (Chapter 4). There was no effect of changing bioavailable Cu concentration on nitrification rate in the Pumice soil (Chapter 4). The importance of the element Cu in the nitrification process was further confirmed by the significant and positive relationship between bioavailable Cu and AOB *amoA* gene abundance in the Pallic ($r = 0.702$, $P < 0.05$) and Recent ($r = 0.940$, $P < 0.01$) soils (Chapter 4). Bioavailable Cu concentration above 6 mg Cu kg^{-1} significantly ($P < 0.05$) inhibited the growth of AOB *amoA* gene in both Recent and Pallic soils but not in the Pumice soil.

Results presented in Chapter 3 demonstrate that a reduction in bioavailable Cu concentration in soil induced through the application of Cu-complexing organic compounds (LS and PA-MA) had a negative effect on nitrification rate. This trend is confirmed by results presented in Chapter 6 which showed that reduction in bioavailable Cu was associated with a significant reduction of nitrification rate and AOB *amoA* gene abundance. The strong positive correlation between bioavailable Cu and nitrification rate ($R^2 = 0.3852$, $P < 0.001$) and AOB *amoA* gene abundance ($R^2 = 0.6129$, $P < 0.001$) provided strong evidence that Cu is a vital trace element in AMO functioning. However, based on the findings of the collective experiments in this thesis, soil characteristics (such as Al, Fe, CEC, and organic matter) also had a significant influence on Cu bioavailability, and this can account for the variable effect of the treatments used on nitrification rate and ammonia oxidiser activity in the different soils. Soil properties provided some level of buffering of the effect of bioavailable Cu on nitrification rate and AOB *amoA* gene abundance, and of the three soils studied, the order of effect was Recent > Pallic > Pumice soils where the Pumice soil showed the greatest ability to resist change (Chapter 4).

2) ***AOB amoA but not AOA amoA gene abundance changes as a function of Cu bioavailability: AOB amoA is therefore the dominant nitrifier in agricultural soil***

The reduction in soil bioavailable Cu that was associated with the application of Cu-complexing organic compounds significantly ($P < 0.05$) reduced AOB *amoA* gene abundance (Chapter 6). This trend was confirmed by the positive relationship between changes in bioavailable Cu and AOB *amoA* gene abundance in both late autumn ($R^2 = 0.6129$, $P < 0.001$) and mid-winter treatment application ($R^2 = 0.5009$, $P < 0.001$). However, changes in bioavailable Cu did not influence AOA *amoA* gene abundance for either the late-autumn or mid-winter treatments. Results reported in Chapter 4 confirmed that changes in AOB *amoA* gene abundance were associated with bioavailable Cu concentration, while there was no relationship between changes of AOA *amoA* gene abundance and bioavailable Cu concentration. The current research suggests that AOB *amoA* are the dominant nitrifiers in agricultural soils.

3) Application of the organic inhibitors has the potential to reduce NO_3^- -N leaching.

The results presented in Chapters 5 and 6 show that a significant reduction in mineral N leaching induced by LS can only be realised through application of a second dose applied one month after the first (2LS). Lysimeters treated with a double dose of LS (2LS) recorded significantly ($P < 0.05$) reduced mineral N leached by values of 16% (Pumice soil), 11% (Pallic soil), and 30% (Recent soil) relative to a urine-only control. The results from Chapter 6 suggest that application of the second dose prolonged the effectiveness of LS in the soil or increased the reactive time period of LS in the soil.

The current research also explored the effect of the Cu-complexing organic compound PA-MA on NO_3^- -N leaching. Application of PA-MA significantly reduced mineral N leaching by 16% in the Recent soil (Manawatu climate), but the same effect was not observed in the Pumice soil (Manawatu climate) and Pallic soil (Canterbury climate). These results suggest that the soil properties reduced the effectiveness of the tested inhibitors. When the different Cu-complexing organic compounds are compared, the double dose of LS treatment (2LS) proved to be the most effective treatment to reduce mineral N leaching in different soils across the range of tested climatic conditions.

4) Treatment with growth hormone improved the effectiveness of organic inhibitors in reducing mineral N leaching.

Late-autumn treatment combination of GA + LS reduced mineral N leaching by 40 and 38% in the Pumice and Pallic soils (respectively) relative to treatment with LS alone (Chapter 5). In Chapter 6, the late-autumn combination of GA + LS reduced mineral N leaching from the Recent soil by 9.6% relative to LS alone. These results provide strong evidence that the addition of GA effectively improved the reduction of mineral N leaching that was induced by LS through improving pasture growth. The reduction in mineral N leaching associated with GA +

LS treatment is therefore inferred to be a function of both the inhibition effect of LS and a hormonal effect of GA.

In Chapter 6 the effectiveness of the GA + LS treatment is shown to be associated with reduced conversion of NH_4^+ -N to NO_3^- -N. The mechanisms for this reduced nitrification rate are both a reduction in bioavailable Cu concentration (induced by LS) and increased plant uptake of N (induced by the GA) which may be in the form of NH_4^+ -N.

7.3 Importance of these key findings to primary production

7.3.1 For pastoral farming systems

Urine patches have been identified as N leaching hotspots which contribute to elevated NO_3^- -N concentrations in some water sources (McLay et al. 2001). This thesis presents evidence for a relationship between bioavailable Cu, AOB/AOA growth, and nitrification rate; the data show that an increase in bioavailable Cu results in an increase in nitrification rate and the abundance of AOB.

Understanding this relationship could support the development of novel Cu-complexing compounds that can reduce nitrification rate, and better correlate Cu discharge from animal remedies or dairy farm effluent with nitrification (Bolan et al. 2003). This is because dairy farms heavily supplement cows with Cu (López-Alonso and Miranda 2020; Silva et al. 2022) or apply farm effluent containing Cu (Panagos et al. 2018). Such practices could increase levels of bioavailable Cu in established dairy soils, and thereby increasing nitrification rate.

As a potential strategy to reduce soil nitrification rates, the results of this doctoral study demonstrate that application of Cu-complexing organic compounds (LS and PA-MA) can reduce nitrification rate through reducing the bioavailable Cu concentration in soil. New Zealand soils typically have low native Cu levels of about 20-30 μg g^{-1} (Morgan and Bowden

1993). However, even at these low levels, added organic compounds (LS and PA-MA) were able to induce significant reduction in bioavailable Cu concentration. Although the results showed a reduction in the bioavailable concentration of Cu in soil with the applied inhibitors, there was no significant difference in the herbage Cu and macronutrient concentration between the applied inhibitor and urine-only treatments (Figure 5.2). Any reduction in bioavailable Cu might affect microbial processes in the soil while not affecting pasture uptake due to the greater sensitivity of soil microbial activity than pasture to small changes of bioavailable Cu in the soil than pasture (Shuaib et al. 2021). This highlights that Cu levels can be manipulated in the soils and such a strategy could be applicable to NZ farming systems. There is minimal risk that manipulation of soil bioavailable Cu levels will impact on the Cu nutrient status of grazing animals. To further account for the applicability of this strategy, based on cost, the estimated cost of LS and PA-MA is US$ 36 ha^{-1} and US$ 11 ha^{-1}, respectively compared to DCD at US$ 16 ha^{-1}. However, another significant cost on the use of LS can be transport cost because LS is very bulky compared to both DCD and PA-MA.

The inclusion of a GA as a mechanism to increase N uptake and growth is an effective strategy for N management, especially during periods of low growth and when there is a high risk of leaching. The boost to plant growth associated with GA application complemented the effectiveness of inhibitors by increasing the uptake of soil available NH_4^+ -N.

7.3.2 For the horticulture industry

The findings of this doctoral thesis can also be potentially applied in the horticulture industry, where research has shown an increasing Cu concentration in orchard soils in both NZ and internationally due to the excessive use of fungicide and bacterial sprays containing Cu (Taylor et al. 2010; Victorino et al. 2021). For example, literature evidence shows that there is high N fertiliser use in the horticulture industry to meet plant demand but only approximately 30% of this applied N is utilised by plants (Gentile et al. 2022). This suggests that there is high conversion of NH_4^+ -N to NO_3^- -N and a high proportion of the NO_3^- -N becomes susceptible to leaching or lost through other pathways. The high nitrification rate in orchard soils could potential be linked with the increasing levels of Cu concentration in the soil due to accumulation of Cu residues from Cu sprays. For example, Jeyakumar et al. (2014) reported that Cu-based bactericide sprays are an effective strategy to reduce Psa in kiwifruit. However, continuous application of these Cu sprays can lead to significant Cu accumulation in soil. This thesis work demonstrates that increasing bioavailable Cu concentration in soil can potentially increase nitrification rate, leading to increased mineral N leaching.

7.3.3 Recommendations for future studies

To expand on the knowledge presented in this doctoral research, there are several ongoing knowledge gaps that justify further research, and these are summarised in this section.

i. Field experiments are recommended to examine the potential effect of the Cu complexing compounds in terms of causing plant Cu deficiency and complexing with other elements in diversifying soil types.

ii. The potential impact of using this Cu-complexing compounds may show effect on pasture tissue Cu, and to the grazing animal through food chain. Therefore, a detailed study is recommended to examine this issue.

iii. Investigate soil properties such as organic matter, Al, Fe, and soil pH that may influence the efficacy of the Cu-complexing compounds.

iv. Field experiments should be conducted to investigate the effect of LS and PA-MA on nitrous oxide emissions and the associated gene abundance in diverse soil types.

v. Experiments should be conducted on the potential combination of the plant growth hormone GA with other inhibitors to mitigate mineral N leaching in different soils and management practices.

vi. Research is needed to analyse the potential short-term and long-term effect of the applied organic compounds on non-target soil microbiota.

This doctoral research has provided critical knowledge on the relationship between the trace element Cu and AOB/AOA functional genes in pastoral soils. The findings of this research provide fundamental information that can underpin ongoing research to design sustainable mechanism that reduce N losses from grazed pasture. This will help both NZ and international agriculture systems to minimise environmental contamination associated with urine patches.

References

Aarons SR, Gourley CJP, Powell JM, Hannah MC (2017) Estimating nitrogen excretion and deposition by lactating cows in grazed dairy systems. Soil Res. 55(6): 489-499. https://doi.org/10.1071/SR17033

Adhikari KP, Chibuike G, Saggar S, Simon PL, Luo J, de Klein CAM (2021) Management and implications of using nitrification inhibitors to reduce nitrous oxide emissions from urine patches on grazed pasture soils – A review. Sci. Total Environ. 791: 148099. https://doi.org/10.1016/j.scitotenv.2021.148099

Alexander M (2000) Aging, bioavailability, and overestimation of risk from environmental pollutants. Environ. Sci. Technol. 34(20): 4259-4265. https://doi.org/10.1021/es001069+

Alexopoulos AA, Akoumianakis KA, Olympios CM, Passam HC (2007) The effect of the time and mode of application of gibberellic acid and inhibitors of gibberellin biosynthesis on the dormancy of potato tubers grown from true potato seed. J. Sci. Food Agric. 87(10): 1973-1979. https://doi.org/10.1002/jsfa.2954

Ali S, Bharwana SA, Rizwan M, Farid M, Kanwal S, Ali Q, Ibrahim M, Gill RA, Khan MD (2015) Fulvic acid mediates chromium (Cr) tolerance in wheat (Triticum aestivum L.) through lowering of Cr uptake and improved antioxidant defense system. Environ Sci Pollut Res 22(14): 10601-10609. 10.1007/s11356-015-4271-7

Allen HE, Chen P-H (1993) Remediation of metal contaminated soil by EDTA incorporating electrochemical recovery of metal and EDTA. Environ. Prog. 12(4): 284-293. https://doi.org/10.1002/ep.670120409

Anderson N, Strader R, Davidson C (2003) Airborne reduced nitrogen: ammonia emissions from agriculture and other sources. Environ. Int. 29(2): 277-286. https://doi.org/10.1016/S0160-4120(02)00186-1

Arp DJ, Sayavedra-Soto LA, Hommes NG (2002) Molecular biology and biochemistry of ammonia oxidation by Nitrosomonas europaea. Arch. Microbiol. 178(4): 250-255. https://doi.org/10.1007/s00203-002-0452-0

Ayub H, Kang M-J, Farooq A, Jung M-Y (2022) Ecological Aerobic Ammonia and Methane Oxidation Involved Key Metal Compounds, Fe and Cu. Life 12(11): 1806. https://doi.org/10.3390/life12111806

Ball R, Keeney DR, Thoebald PW, Nes P (1979) Nitrogen Balance in Urine-affected Areas of a New Zealand Pasture1. Agron. J. 71(2): 309-314. https://doi.org/10.2134/agronj1979.00021962007100020022x

Barth G, von Tucher S, Schmidhalter U (2001) Influence of soil parameters on the effect of 3,4-dimethylpyrazole-phosphate as a nitrification inhibitor. Biol. Fertility Soils 34(2): 98-102. https://doi.org/10.1007/s003740100382

Bédard C, Knowles R (1989) Physiology, biochemistry, and specific inhibitors of CH4, NH4+, and CO oxidation by methanotrophs and nitrifiers. Microbiol. Mol. Biol. Rev. 53(1): 68-84. https://doi.org/10.1128/mr.53.1.68-84.1989

Behrens S, Azizian Mohammad F, McMurdie Paul J, Sabalowsky A, Dolan Mark E, Semprini L, Spormann Alfred M (2008) Monitoring Abundance and Expression of "Dehalococcoides" Species Chloroethene-Reductive Dehalogenases in a Tetrachloroethene-Dechlorinating Flow Column. Appl. Environ. Microbiol. 74(18): 5695-5703. https://doi.org/10.1128/AEM.00926-08

Bishop P, Jeyakumar P (2021) A comparison of three nitrate leaching mitigation treatments with dicyandiamide using lysimeters. N. Z. J. Agric. Res.: 1-14. https://doi.org/10.1080/00288233.2021.1963289

Bjorneberg DL, Kanwar RS, Melvin SW (1996) Seasonal changes in flow and nitrate-N loss from subsurface drains. Trans. ASAE 39(3): 961-967.

Blakemore LC (1987) Methods for chemicalanalysis of soils. NZ Soil Bureau Sci. Rep. 80: 72-76.

Blöchl E, Rachel R, Burggraf S, Hafenbradl D, Jannasch HW, Stetter KO (1997) Pyrolobus fumarii, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. Extremophiles 1(1): 14-21. https://doi.org/10.1007/s007920050010
Blondel J (2003) Guilds or functional groups: does it matter? Oikos 100(2): 223-231. https://doi.org/10.1034/j.1600-0706.2003.12152.x
Bolan NS, Khan M, Donaldson J, Adriano D, Matthew C (2003) Distribution and bioavailability of copper in farm effluent. Sci. Total Environ. 309(1-3): 225-236. https://doi.org/10.1016/S0048-9697(03)00052-4
Box LA, Edwards GR, Bryant RH (2017) Milk production and urinary nitrogen excretion of dairy cows grazing plantain in early and late lactation. N. Z. J. Agric. Res. 60(4): 470-482. https://doi.org/10.1080/00288233.2017.1366924
Bozal-Leorri A, Corrochano-Monsalve M, Vega-Mas I, Aparicio-Tejo PM, González-Murua C, Marino D (2022) Evidences towards deciphering the mode of action of dimethylpyrazole-based nitrification inhibitors in soil and pure cultures of Nitrosomonas europaea. Chem. Biol. Technol. Agric. 9(1): 56. https://doi.org/10.1186/s40538-022-00321-3
Bristow AW, Whitehead DC, Cockburn JE (1992) Nitrogenous constituents in the urine of cattle, sheep and goats. J. Sci. Food Agric. 59(3): 387-394. https://doi.org/10.1002/jsfa.2740590316
Bryant RH, Edwards GR, Robinson B (2016) Comparing response of ryegrass-white clover pasture to gibberellic acid and nitrogen fertiliser applied in late winter and spring. N. Z. J. Agric. Res. 59(1): 18-31. https://doi.org/10.1080/00288233.2015.1119164
Bryant RH, Miller ME, Greenwood SL, Edwards GR (2017) Milk yield and nitrogen excretion of dairy cows grazing binary and multispecies pastures. Grass Forage Sci. 72(4): 806-817. https://doi.org/10.1111/gfs.12274
Byrne MP, Tobin JT, Forrestal PJ, Danaher M, Nkwonta CG, Richards K, Cummins E, Hogan SA, O'Callaghan TF (2020) Urease and Nitrification Inhibitors—As Mitigation Tools for Greenhouse Gas Emissions in Sustainable Dairy Systems: A Review. Sustainability 12(15): 6018. https://doi.org/10.3390/su12156018
Cai Y, Akiyama H (2016) Nitrogen loss factors of nitrogen trace gas emissions and leaching from excreta patches in grassland ecosystems: A summary of available data. Sci. Total Environ. 572: 185-195. https://doi.org/10.1016/j.scitotenv.2016.07.222
Cameron K, Di HJ, Moir J (2013) Nitrogen losses from the soil/plant system: a review. Ann. Appl. Biol. 162(2): 145-173. https://doi.org/10.1111/aab.12014
Cameron KC, Di HJ, Moir JL (2014) Dicyandiamide (DCD) effect on nitrous oxide emissions, nitrate leaching and pasture yield in Canterbury, New Zealand. N. Z. J. Agric. Res. 57(4): 251-270. https://doi.org/10.1080/00288233.2013.797914
Cameron KC, Smith NP, McLay C, Fraser PM, McPherson R, Harrison D, Harbottle P (1992) Lysimeters without edge flow: an improved design and sampling procedure. Soil Sci. Soc. Am. J. 56(5): 1625-1628. https://doi.org/10.2136/sssaj1992.03615995005600050048x
Carey CJ, Dove NC, Beman JM, Hart SC, Aronson EL (2016) Meta-analysis reveals ammonia-oxidizing bacteria respond more strongly to nitrogen addition than ammonia-oxidizing archaea. Soil Biol. Biochem. 99: 158-166. https://doi.org/10.1016/j.soilbio.2016.05.014
Carlton AJ, Cameron KC, Di HJ, Edwards GR, Clough TJ (2019) Nitrate leaching losses are lower from ryegrass/white clover forages containing plantain than from ryegrass/white clover forages under different irrigation. N. Z. J. Agric. Res. 62(2): 150-172. https://doi.org/10.1080/00288233.2018.1461659
Carrick S, Rogers G, Cameron K, Malcolm B, Payne J (2017) Testing large area lysimeter designs to measure leaching under multiple urine patches. N. Z. J. Agric. Res. 60(2): 205-215. https://doi.org/10.1080/00288233.2017.1291527
Cela S, Sumner ME (2002) Critical concentrations of copper, nickel, lead, and cadmium in soils based on nitrification. Commun. Soil Sci. Plant Anal. 33(1-2): 19-30. https://doi.org/10.1081/CSS-120002374
Chen Q, Qi L, Bi Q, Dai P, Sun D, Sun C, Liu W, Lu L, Ni W, Lin X (2015) Comparative effects of 3, 4-dimethylpyrazole phosphate (DMPP) and dicyandiamide (DCD) on ammonia-oxidizing bacteria and archaea in a vegetable soil. Appl. Microbiol. Biotechnol. 99(1): 477-487. https://doi.org/10.1007/s00253-014-6026-7

Chen Y, Jiang H, Li Y, Liu Y, Chen Y, Chen L, Luo X, Tang P, Yan H, Zhao M, Yuan Y, Hou S (2022) A critical review on EDTA washing in soil remediation for potentially toxic elements (PTEs) pollutants. Rev. Environ. Sci. Biotechnol. 21(2): 399-423. 10.1007/s11157-022-09613-4

Chen Z, Li Y, Xu Y, Lam SK, Xia L, Zhang N, Castellano MJ, Ding W (2021) Spring thaw pulses decrease annual N2O emissions reductions by nitrification inhibitors from a seasonally frozen cropland. Geoderma 403: 115310. https://doi.org/10.1016/j.geoderma.2021.115310

Chibuike G, Saggar S, Palmada T, Luo J (2022) The persistence and efficacy of nitrification inhibitors to mitigate nitrous oxide emissions from New Zealand pasture soils amended with urine. Geordema Reg. 30: e00541. https://doi.org/10.1016/j.geodrs.2022.e00541

Christensen CL, Hedley MJ, Hanly JA, Horne DJ (2019) Duration-controlled grazing of dairy cows. 2: nitrogen losses in sub-surface drainage water and surface runoff. N. Z. J. Agric. Res. 62(1): 48-68. https://doi.org/10.1080/00288233.2017.1418396

Clough T, Ledgard S, Sprosen M, Kear M (1998) Fate of 15 N labelled urine on four soil types. Plant Soil 199(2): 195-203. https://doi.org/10.1023/A:1004361009708

Cookson WR, Cornforth IS (2002) Dicyandiamide slows nitrification in dairy cattle urine patches: effects on soil solution composition, soil pH and pasture yield. Soil Biol. Biochem. 34(10): 1461-1465. https://doi.org/10.1016/S0038-0717(02)00090-1

Corrochano-Monsalve M, González-Murua C, Bozal-Leorri A, Lezama L, Artetxe B (2021) Mechanism of action of nitrification inhibitors based on dimethylpyrazole: A matter of chelation. Sci. Total Environ. 752: 141885. https://doi.org/10.1016/j.scitotenv.2020.141885

Coskun D, Britto DT, Shi W, Kronzucker HJ (2017) Nitrogen transformations in modern agriculture and the role of biological nitrification inhibition. Nat. Plants. 3(6): 17074. https://doi.org/10.1038/nplants.2017.74

Dai Y, Di HJ, Cameron KC, He J-Z (2013) Effects of nitrogen application rate and a nitrification inhibitor dicyandiamide on ammonia oxidizers and N2O emissions in a grazed pasture soil. Sci. Total Environ. 465: 125-135. https://doi.org/10.1016/j.scitotenv.2012.08.091

DairyNZ (2021) New Zealand Dairy Statistics. Dairy New Zealand, Hamilton: pp 9.

Dawson DA, Genco N, Bensinger HM, Guinn D, Il'Giovine ZJ, Wayne Schultz T, Pöch G (2012) Evaluation of an asymmetry parameter for curve-fitting in single-chemical and mixture toxicity assessment. Toxicology 292(2): 156-161. https://doi.org/10.1016/j.tox.2011.12.006

De Klein C, Smith L, Monaghan R (2006) Restricted autumn grazing to reduce nitrous oxide emissions from dairy pastures in Southland, New Zealand. Agric., Ecosyst. Environ. 112(2-3): 192-199.

Di H, Cameron K (2002a) Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. Nutri. Cycl. Agroecosystems 64(3): 237-256. https://doi.org/10.1023/A:1021471531188

Di H, Cameron K (2002b) The use of a nitrification inhibitor, dicyandiamide (DCD), to decrease nitrate leaching and nitrous oxide emissions in a simulated grazed and irrigated grassland. Soil Use Manag 18(4): 395-403. https://doi.org/10.1111/j.1475-2743.2002.tb00258.x

Di H, Cameron K (2005a) Effects of temperature and application rate of a nitrification inhibitor, dicyandiamide (DCD), on nitrification rate and microbial biomass in a grazed pasture soil. Soil Res. 42(8): 927-932. https://doi.org/10.1071/SR04050

Di H, Cameron K (2012) 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, Cameron K, Moore S, Smith N (1999) Contributions to nitrogen leaching and pasture uptake by autumn-applied dairy effluent and ammonium fertilizer labeled with 15 N isotope. Plant Soil 210(2): 189-198.

Di H, Cameron K, Shen J, He J, Winefield C (2009) A lysimeter study of nitrate leaching from grazed grassland as affected by a nitrification inhibitor, dicyandiamide, and relationships with ammonia oxidizing bacteria and archaea. Soil Use Manag 25(4): 454-461. https://doi.org/10.1111/j.1475-2743.2009.00241.x

Di HJ, Cameron KC (2005b) Reducing environmental impacts of agriculture by using a fine particle suspension nitrification inhibitor to decrease nitrate leaching from grazed pastures. Agric., Ecosyst. Environ. 109(3): 202-212. https://doi.org/10.1080/00288233.2004.9513604

Di HJ, Cameron KC (2016) Inhibition of nitrification to mitigate nitrate leaching and nitrous oxide emissions in grazed grassland: a review. J. Soils Sed. 16(5): 1401-1420. https://doi.org/10.1007/s11368-016-1403-8

Di HJ, Cameron KC, Podolyan A, Edwards GR, de Klein CAM, Dynes R, Woods R (2016) The potential of using alternative pastures, forage crops and gibberellic acid to mitigate nitrous oxide emissions. J. Soils Sed. 16(9): 2252-2262. https://doi.org/10.1007/s11368-016-1442-1

Di HJ, Cameron KC, Shen J-P, Winefield CS, O'Callaghan M, Bowatte S, He J-Z (2010) Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. FEMS Microbiol. Ecol. 72(3): 386-394. 10.1111/j.1574-6941.2010.00861.x

Di HJ, Cameron KC, Sherlock RR (2007) Comparison of the effectiveness of a nitrification inhibitor, dicyandiamide, in reducing nitrous oxide emissions in four different soils under different climatic and management conditions. Soil Use Manag 23(1): 1-9. https://doi.org/10.1111/j.1475-2743.2006.00057.x

Dijkstra J, Oenema O, van Groenigen JW, Spek JW, van Vuuren AM, Bannink A (2013) Diet effects on urine composition of cattle and N2O emissions. animal 7(s2): 292-302. 10.1017/S1751731113000578

dos Santos JV, Fregolente LG, Moreira AB, Ferreira OP, Mounier S, Viguier B, Hajjoul H, Bisinoti MC (2020) Humic-like acids from hydrochars: Study of the metal complexation properties compared with humic acids from anthropogenic soils using PARAFAC and time-resolved fluorescence. Sci. Total Environ. 722: 137815. https://doi.org/10.1016/j.scitotenv.2020.137815

Duan P, Fan C, Zhang Q, Xiong Z (2019) Overdose fertilization induced ammonia-oxidizing archaea producing nitrous oxide in intensive vegetable fields. Sci. Total Environ. 650: 1787-1794. https://doi.org/10.1016/j.scitotenv.2018.09.341

Duff AM, Forrestal P, Ikoyi I, Brennan F (2022) Assessing the long-term impact of urease and nitrification inhibitor use on microbial community composition, diversity and function in grassland soil. Soil Biol. Biochem. 170: 108709. https://doi.org/10.1016/j.soilbio.2022.108709

Ensign SA, Hyman MR, Arp DJ (1993) In vitro activation of ammonia monooxygenase from Nitrosomonas europaea by copper. J. Bacteriol. 175(7): 1971-1980. https://doi.org/10.1128/jb.175.7.1971-1980.1993

Fan AM, Steinberg VE (1996) Health Implications of Nitrate and Nitrite in Drinking Water: An Update on Methemoglobinemia Occurrence and Reproductive and Developmental Toxicity. Regul. Toxicol. Pharmacol. 23(1): 35-43. https://doi.org/10.1006/rtph.1996.0006

Feng W, Zhang S, Zhong Q, Wang G, Pan X, Xu X, Zhou W, Li T, Luo L, Zhang Y (2020) Soil washing remediation of heavy metal from contaminated soil with EDTMP and PAA: Properties, optimization, and risk assessment. Journal of Hazardous Materials 381: 120997. https://doi.org/10.1016/j.jhazmat.2019.120997

Ferguson BJ, Foo E, Ross JJ, Reid JB (2011) Relationship between gibberellin, ethylene and nodulation in Pisum sativum. New Phytol. 189(3): 829-842. https://doi.org/10.1111/j.1469-8137.2010.03542.x

Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proceedings of the National Academy of Sciences of the United States of America 102(41): 14683-14688. https://doi.org/10.1073/pnas.0506625102

Frase PM, Cameron KC, Sherlock RR (1994) Lysimeter study of the fate of nitrogen in animal urine returns to irrigated pasture. Eur. J. Soil Sci. 45(4): 439-447. https://doi.org/10.1111/j.1365-2389.1994.tb00529.x

Gao J, Zhao G (2022) Potentials of using dietary plant secondary metabolites to mitigate nitrous oxide emissions from excreta of cattle: Impacts, mechanisms and perspectives. Anim. Nutr. 9: 327-334. https://doi.org/10.1016/j.aninu.2021.12.006

Gao S, Walker WJ, Dahlgren RA, Bold J (1997) Simultaneous sorption of Cd, Cu, Ni, Zn, Pb, and Cr on soils treated with sewage sludge supernatant. Water, Air, and Soil Pollution 93(1): 331-345. https://doi.org/10.1007/BF02404765

Gardiner CA, Clough TJ, Cameron KC, Di HJ, Edwards GR, de Klein CA (2018) Potential inhibition of urine patch nitrous oxide emissions by Plantago lanceolata and its metabolite aucubin. N. Z. J. Agric. Res. 61(4): 495-503. https://doi.org/10.1080/00288233.2017.1411953

Gentile RM, Boldingh HL, Campbell RE, Gee M, Gould N, Lo P, McNally S, Park KC, Richardson AC, Stringer LD, Vereijssen J, Walter M (2022) System nutrient dynamics in orchards: a research roadmap for nutrient management in apple and kiwifruit. A review. Agron. Sustain. Dev. 42(4): 64. https://doi.org/10.1007/s13593-022-00798-0

Glassey CB, Clark CEF, Roach CG, Lee JM (2013) Herbicide application and direct drilling improves establishment and yield of chicory and plantain. Grass Forage Sci. 68(1): 178-185. https://doi.org/10.1111/j.1365-2494.2012.00885.x

Gorman-Lewis D, Martens-Habbena W, Stahl DA (2019) Cu(II) adsorption onto ammonia-oxidizing bacteria and archaea. Geochim. Cosmochim. Acta 255: 127-143. https://doi.org/10.1016/j.gca.2019.04.011

Großkopf R, Janssen PH, Liesack W (1998) Diversity and Structure of the Methanogenic Community in Anoxic Rice Paddy Soil Microcosms as Examined by Cultivation and Direct 16S rRNA Gene Sequence Retrieval. Appl. Environ. Microbiol. 64(3): 960-969. https://doi.org/10.1128/AEM.64.3.960-969.1998

Guardia G, Marsden KA, Vallejo A, Jones DL, Chadwick DR (2018) Determining the influence of environmental and edaphic factors on the fate of the nitrification inhibitors DCD and DMPP in soil. Sci. Total Environ. 624: 1202-1212. https://doi.org/10.1016/j.scitotenv.2017.12.250

Gubry-Rangin C, Nicol GW, Prosser JI (2010) Archaea rather than bacteria control nitrification in two agricultural acidic soils. FEMS Microbiol. Ecol. 74(3): 566-574. https://doi.org/10.1111/j.1574-6941.2010.00971.x

Guo X, Yang Y, Ji L, Zhang G, He Q, Wei Z, Qian T, Wu Q (2019) Revitalization of Mixed Chelator–Washed Soil by Adding of Inorganic and Organic Amendments. Water, Air, Soil Pollut. 230(6): 112. https://doi.org/10.1007/s11270-019-4169-y

Guo YJ, Di HJ, Cameron KC, Li B (2014) Effect of application rate of a nitrification inhibitor, dicyandiamide (DCD), on nitrification rate, and ammonia-oxidizing bacteria and archaea growth in a grazed pasture soil: an incubation study. J. Soils Sed. 14(5): 897-903. https://doi.org/10.1007/s11368-013-0843-7

Gwak J-H, Jung M-Y, Hong H, Kim J-G, Quan Z-X, Reinfelder JR, Spasov E, Neufeld JD, Wagner M, Rhee S-K (2020) Archaeal nitrification is constrained by copper complexation with organic matter in municipal wastewater treatment plants. The ISME Journal 14(2): 335-346. https://doi.org/10.1038/s41396-019-0538-1

Hayatsu M, Katsuyama C, Tago K (2021) Overview of recent researches on nitrifying microorganisms in soil. Soil Sci. Plant Nutr. 67(6): 619-632. 10.1080/00380768.2021.1981119

Haynes R, Williams P (1992) Changes in soil solution composition and pH in urine-affected areas of pasture. J. Soil Sci. 43(2): 323-334. https://doi.org/10.1111/j.1365-2389.1992.tb00140.x

Haynes R, Williams P. (1993). Nutrient cycling and soil fertility in the grazed pasture ecosystem. In *Adv. Agron.* (Vol. 49, pp. 119-199): Elsevier. https://doi.org/10.1016/S0065-2113(08)60794-4

He E, Lü C, He J, Zhao B, Wang J, Zhang R, Ding T (2016) Binding characteristics of Cu 2+ to natural humic acid fractions sequentially extracted from the lake sediments. Environ Sci Pollut Res 23(22): 22667-22677. https://doi.org/10.1007/s11356-016-7487-2

He H, Liu H, Shen T, Wei S, Dai J, Wang R (2018) Influence of Cu application on ammonia oxidizers in fluvo-aquic soil. Geoderma 321: 141-150. https://doi.org/10.1016/j.geoderma.2018.01.037

He T, Xie D, Ni J, Cai X, Li Z (2019) Investigating the effect of copper and magnesium ions on nitrogen removal capacity of pure cultures by modified non-competitive inhibition model. Ecotoxicol. Environ. Saf. 170: 479-487. https://doi.org/10.1016/j.ecoenv.2018.12.019

He Z-Y, Shen J-P, Zhang L-M, Tian H-J, Han B, Di H-J, He J-Z (2020) DNA stable isotope probing revealed no incorporation of 13CO2 into comammox Nitrospira but ammonia-oxidizing archaea in a subtropical acid soil. J. Soils Sed. 20(3): 1297-1308. 10.1007/s11368-019-02540-y

Hewitt A (2010) New Zealand soil classification, Landcare Res. Sci. Ser. N. Zeal 1: 1-136.

Hink L, Gubry-Rangin C, Nicol GW, Prosser JI (2018) The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions. The ISME Journal 12(4): 1084-1093. 10.1038/s41396-017-0025-5

Hooper AB, Vannelli T, Bergmann DJ, Arciero DM (1997) Enzymology of the oxidation of ammonia to nitrite by bacteria. Antonie Van Leeuwenhoek 71(1): 59-67. https://doi.org/10.1023/A:1000133919203

Huang H, Legg W, Cattaneo A (2010) Climate Change and Agriculture: The Policy Challenge for the 21st Century? Changement climatique et agriculture : le défi du 21ème siècle pour l’action publique? Klimawandel und Landwirtschaft: Die politische Herausforderung für das 21. Jahrhundert? EuroChoices 9(3): 9-15. https://doi.org/10.1111/j.1746-692X.2010.00174.x

Huérfano X, Estavillo JM, Torralbo F, Vega-Mas I, González-Murua C, Fuertes-Mendizábal T (2022) Dimethylpyrazole-based nitrification inhibitors have a dual role in N2O emissions mitigation in forage systems under Atlantic climate conditions. Sci. Total Environ. 807: 150670. https://doi.org/10.1016/j.scitotenv.2021.150670

Jeyakumar P, Anderson C, Holmes A, Miller S (2014) Optimising copper sprays on kiwifruit: A review. Mt Maunganui, New Zeal ZESPRI Int Limited: 1-13.

Jez E, Lestan D (2016) EDTA retention and emissions from remediated soil. Chemosphere 151: 202-209. https://doi.org/10.1016/j.chemosphere.2016.02.088

Jia Z, Conrad R (2009) Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. Environ. Microbiol. 11(7): 1658-1671. https://doi.org/10.1111/j.1462-2920.2009.01891.x

Joy MK, Rankin DA, Wöhler L, Boyce P, Canning A, Foote KJ, McNie PM (2022) The grey water footprint of milk due to nitrate leaching from dairy farms in Canterbury, New Zealand. Australasian Journal of Environmental Management: 1-23. https://doi.org/10.1080/14486563.2022.2068685

Kandler O, König H (1998) Cell wall polymers in Archaea (Archaebacteria). Cellular and Molecular Life Sciences CMLS 54(4): 305-308. https://doi.org/10.1007/s000180050156

Kaurin A, Gluhar S, Maček I, Kastelec D, Lestan D (2021) Demonstrational gardens with EDTA-washed soil. Part II: Soil quality assessment using biological indicators. Sci. Total Environ. 792: 148522. https://doi.org/10.1016/j.scitotenv.2021.148522

Kebreab E, France J, Beever DE, Castillo AR (2001) Nitrogen pollution by dairy cows and its mitigation by dietary manipulation. Nutri. Cycl. Agroecosystems 60(1): 275-285. https://doi.org/10.1023/A:1012668109662

Khatami S, Deng Y, Tien M, Hatcher PG (2019) Formation of water-soluble organic matter through fungal degradation of lignin. Org. Geochem. 135: 64-70. https://doi.org/10.1016/j.orggeochem.2019.06.004

Kim D-G, Giltrap D, Saggar S, Palmada T, Berben P, Drysdale D (2012) Fate of the nitrification inhibitor dicyandiamide (DCD) sprayed on a grazed pasture: effect of rate and time of application. Soil Res. 50(4): 337-347. https://doi.org/10.1071/SR12069

Kim DG, Giltrap DL, Saggar S, Hanly JA (2014) Field studies assessing the effect of dicyandiamide (DCD) on N transformations, pasture yields, N2O emissions and N-leaching in the Manawatu region. N. Z. J. Agric. Res. 57(4): 271-293. https://doi.org/10.1080/00288233.2013.855244

Koike K, Smith GJ, Yamamoto-Ikemoto R, Lücker S, Matsuura N (2022) Distinct comammox Nitrospira catalyze ammonia oxidation in a full-scale groundwater treatment bioreactor under copper limited conditions. Water Res. 210: 117986. https://doi.org/10.1016/j.watres.2021.117986

Kumar V, Yadav DV, Yadav DS (1990) Effects of nitrogen sources and copper levels on yield, nitrogen and copper contents of wheat (Triticum aestivum L.). Plant Soil 126(1): 79-83. https://doi.org/10.1007/BF00041371

Larned ST, Snelder T, Unwin MJ, McBride GB (2016) Water quality in New Zealand rivers: current state and trends. N. Z. J. Mar. Freshwat. Res. 50(3): 389-417. https://doi.org/10.1080/00288330.2016.1150309

Laven R, Holmes C (2008) A review of the potential impact of increased use of housing on the health and welfare of dairy cattle in New Zealand. N. Z. Vet. 56(4): 151-157.

Ledgard S, Schils R, Eriksen J, Luo J (2009) Environmental impacts of grazed clover/grass pastures. Irish J. Agric. Food Res. 48(2): 209-226.

Ledgard S, Sprosen M, Judge A, Lindsey S, Jensen R, Clark D, Luo J (2006) Nitrogen leaching as affected by dairy intensification and mitigation practices in the resource efficient dairying

(RED) trial. Implementing sustainable nutrient management strategies in agriculture. Occasional Report 19: 263-268.

Ledgard SF, Luo J, Sprosen MS, Wyatt JB, Balvert SF, Lindsey SB (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. https://doi.org/10.1080/00288233.2014.928642

Ledgard SF, Welten B, Betteridge K (2015) Salt as a mitigation option for decreasing nitrogen leaching losses from grazed pastures. J. Sci. Food Agric. 95(15): 3033-3040. https://doi.org/10.1002/jsfa.7179

Lehtovirta-Morley LE, Stoecker K, Vilcinskas A, Prosser JI, Nicol GW (2011) Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. Proceedings of the National Academy of Sciences 108(38): 15892-15897. doi:10.1073/pnas.1107196108

Li G, Kemp PD. (2005). In *Adv. Agron.* (Vol. 88, pp. 187-222): Academic Press. https://doi.org/10.1016/S0065-2113(05)88005-8

Li X, Wang X, Han T, Hao C, Han S, Fan X (2021) Synthesis of sodium lignosulfonate-guar gum composite hydrogel for the removal of Cu2+ and Co2+. Int. J. Biol. Macromol. 175: 459-472. https://doi.org/10.1016/j.ijbiomac.2021.02.018

Li Y, Chapman SJ, Nicol GW, Yao H (2018) Nitrification and nitrifiers in acidic soils. Soil Biol. Biochem. 116: 290-301. https://doi.org/10.1016/j.soilbio.2017.10.023

Li Y, Yue Q, Gao B (2010) Adsorption kinetics and desorption of Cu(II) and Zn(II) from aqueous solution onto humic acid. Journal of Hazardous Materials 178(1): 455-461. https://doi.org/10.1016/j.jhazmat.2010.01.103

Lieberman RL, Rosenzweig AC (2005) Crystal structure of a membrane-bound metalloenzyme that catalyses the biological oxidation of methane. Nature 434(7030): 177-182. https://doi.org/10.1038/nature03311

Lin Y, Duan C, Fan J, Hu H-W, He Z-Y, Ye G, He J-Z (2022) Nitrification inhibitor 1-octyne inhibits growth of comammox Nitrospira but does not alter their community structure in an acidic soil. J. Soils Sed. https://doi.org/10.1007/s11368-022-03367-w

Lin Y, Hu H-W, Ye G, Fan J, Ding W, He Z-Y, Zheng Y, He J-Z (2021) Ammonia-oxidizing bacteria play an important role in nitrification of acidic soils: A meta-analysis. Geoderma 404: 115395. https://doi.org/10.1016/j.geoderma.2021.115395

Longhurst R, Roberts A, O'Connor M (2000) Farm dairy effluent: a review of published data on chemical and physical characteristics in New Zealand. N. Z. J. Agric. Res. 43(1): 7-14.

López-Alonso M, Miranda M (2020) Copper Supplementation, A Challenge in Cattle. Animals 10(10): 1890. https://doi.org/10.3390/ani10101890

Lourenço KS, Costa OYdA, Cantarella H, Kuramae EE (2022) Ammonia-oxidizing bacteria and fungal denitrifier diversity are associated with N2O production in tropical soils. Soil Biol. Biochem. 166: 108563. https://doi.org/10.1016/j.soilbio.2022.108563

Loveless J, Painter H (1968) The influence of metal ion concentrations and pH value on the growth of a Nitrosomonas strain isolated from activated sludge. Microbiology 52(1): 1-14. https://doi.org/10.1099/00221287-52-1-1

Lu L, Chen C, Ke T, Wang M, Sima M, Huang S (2022) Long-term metal pollution shifts microbial functional profiles of nitrification and denitrification in agricultural soils. Sci. Total Environ. 830: 154732. https://doi.org/10.1016/j.scitotenv.2022.154732

Lu X, Taylor AE, Myrold DD, Neufeld JD (2020) Expanding perspectives of soil nitrification to include ammonia-oxidizing archaea and comammox bacteria. Soil Sci. Soc. Am. J. 84(2): 287-302. https://doi.org/10.1002/saj2.20029

Luo J, Donnison A, Ross C, Ledgard S, Longhurst B. (2006). *Control of pollutants using stand-off pads containing different natural materials.* Paper presented at the PROCEEDINGS OF THE CONFERENCE-NEW ZEALAND GRASSLAND ASSOCIATION.

Lv D, Liu Y, Zhou J, Yang K, Lou Z, Baig SA, Xu X (2018) Application of EDTA-functionalized bamboo activated carbon (BAC) for Pb(II) and Cu(II) removal from aqueous solutions. Applied Surface Science 428: 648-658. https://doi.org/10.1016/j.apsusc.2017.09.151

Malcolm BJ, Cameron KC, Beare MH, Carrick ST, Payne JJ, Maley SC, Di HJ, Richards KK, Dalley DE, de Ruiter JM (2022) Oat catch crop efficacy on nitrogen leaching varies after forage crop grazing. Nutri. Cycl. Agroecosystems 122(3): 273-288. https://doi.org/10.1007/s10705-022-10201-9

Malcolm BJ, Cameron KC, Di HJ, Edwards GR, Moir JL (2014) The effect of four different pasture species compositions on nitrate leaching losses under high N loading. Soil Use Manag 30(1): 58-68. https://doi.org/10.1111/sum.12101

Mangwe M, Bryant R, Beck M, Beale N, Bunt C, Gregorini P (2019) Forage herbs as an alternative to ryegrass-white clover to alter urination patterns in grazing dairy systems. Anim. Feed Sci. Technol. 252: 11-22. https://doi.org/10.1016/j.anifeedsci.2019.04.001

Mao D-P, Zhou Q, Chen C-Y, Quan Z-X (2012) Coverage evaluation of universal bacterial primers using the metagenomic datasets. BMC Microbiol. 12(1): 66. https://doi.org/10.1186/1471-2180-12-66

Marsden KA, Scowen M, Hill PW, Jones DL, Chadwick DR (2015) Plant acquisition and metabolism of the synthetic nitrification inhibitor dicyandiamide and naturally-occurring guanidine from agricultural soils. Plant Soil 395(1): 201-214. https://doi.org/10.1007/s11104-015-2549-7

Martikainen PJ (2022) Heterotrophic nitrification – An eternal mystery in the nitrogen cycle. Soil Biol. Biochem. 168: 108611. https://doi.org/10.1016/j.soilbio.2022.108611

Matse DT, Huang C-H, Huang Y-M, Yen M-Y (2020) Effects of coinoculation of Rhizobium with plant growth promoting rhizobacteria on the nitrogen fixation and nutrient uptake of Trifolium repens in low phosphorus soil. J. Plant Nutr. 43(5): 739-752. https://doi.org/10.1080/01904167.2019.1702205

Matse DT, Jeyakumar P, Bishop P, Anderson CWN (2022a) Bioavailable Cu can influence nitrification rate in New Zealand dairy farm soils. J. Soils Sed. 22(3): 916-930. 10.1007/s11368-021-03113-8

Matse DT, Jeyakumar P, Bishop P, Anderson CWN (2022b) Nitrate Leaching Mitigation Options in Two Dairy Pastoral Soils and Climatic Conditions in New Zealand. Plants 11(18): 2430. https://doi.org/10.3390/plants11182430

Matthew C, Hofmann WA, Osborne MA (2009) Pasture response to gibberellins: A review and recommendations. N. Z. J. Agric. Res. 52(2): 213-225. 10.1080/00288230909510506

Maynard D, Kalra Y, Crumbaugh J (1993) Nitrate and exchangeable ammonium nitrogen. Soil sampling and methods of analysis 1: 25-38.

McCarty G (1999) Modes of action of nitrification inhibitors. Biol. Fertility Soils 29(1): 1-9. https://doi-org.ezproxy.massey.ac.nz/10.1007/s003740050518

McDowell R, Houlbrooke D. (2009). *The effect of DCD on nitrate leaching losses from a winter forage crop receiving applications of sheep or cattle urine.* Paper presented at the Proceedings of the New Zealand Grassland Association.

McKenzie H, Wallace H (1954) The Kjeldahl determination of Nitrogen: A critical study of digestion conditions-Temperature, Catalyst, and Oxidizing agent. Aust. J. Chem. 7(1): 55-70. https://doi.org/10.1071/CH9540055

McLay CDA, Dragten R, Sparling G, Selvarajah N (2001) Predicting groundwater nitrate concentrations in a region of mixed agricultural land use: a comparison of three approaches. Environ. Pollut. 115(2): 191-204. https://doi.org/10.1016/S0269-7491(01)00111-7

McNally SR, Beare MH, Curtin D, Meenken ED, Kelliher FM, Calvelo Pereira R, Shen Q, Baldock J (2017) Soil carbon sequestration potential of permanent pasture and continuous cropping soils in New Zealand. Global Change Biol. 23(11): 4544-4555. https://doi.org/10.1111/gcb.13720

Menció A, Mas-Pla J, Otero N, Regàs O, Boy-Roura M, Puig R, Bach J, Domènech C, Zamorano M, Brusi D, Folch A (2016) Nitrate pollution of groundwater; all right…, but nothing else? Sci. Total Environ. 539: 241-251. https://doi.org/10.1016/j.scitotenv.2015.08.151

Mendes LB, Pieters JG, Snoek D, Ogink NWM, Brusselman E, Demeyer P (2017) Reduction of ammonia emissions from dairy cattle cubicle houses via improved management- or design-based strategies: A modeling approach. Sci. Total Environ. 574: 520-531. https://doi.org/10.1016/j.scitotenv.2016.09.079

Meng Y, Wang JJ, Wei Z, Dodla SK, Fultz LM, Gaston LA, Xiao R, Park J-h, Scaglia G (2021) Nitrification inhibitors reduce nitrogen losses and improve soil health in a subtropical pastureland. Geoderma 388: 114947. https://doi.org/10.1016/j.geoderma.2021.114947

Menneer JC, Ledgard S, Sprosen M (2008) Soil N process inhibitors alter nitrogen leaching dynamics in a pumice soil. Soil Res. 46(4): 323-331.
Mével G, Prieur D (2000) Heterotrophic nitrification by a thermophilic Bacillus species as influenced by different culture conditions. Can J. Microbiol 46(5): 465-473. https://doi.org/10.1139/w00-005
Ministry for the Environment. (2021). *Nitrogen cap guidance for dairy farms* Wellington, New Zealand.
Ministry of Primary Industries. (2013). *Withdrawal of DCD in New Zealand letter of assurance*.
Minneé EMK, Waghorn GC, Lee JM, Clark CEF (2017) Including chicory or plantain in a perennial ryegrass/white clover-based diet of dairy cattle in late lactation: Feed intake, milk production and rumen digestion. Anim. Feed Sci. Technol. 227: 52-61. https://doi.org/10.1016/j.anifeedsci.2017.03.008
Moir JL, Cameron KC, Di HJ, Fertsak U (2011) The spatial coverage of dairy cattle urine patches in an intensively grazed pasture system. J. Agric. Scie. 149(4): 473-485.
Morgan RK, Bowden R (1993) Copper accumulation in soils from two different-aged apricot orchards in Central Otago, New Zealand. Int. J. Environ. Stud. 43(2-3): 161-167. https://doi.org/10.1080/00207239308710823
Muller J, De Rosa D, Friedl J, De Antoni Migliorati M, Rowlings D, Grace P, Scheer C (2022) Combining nitrification inhibitors with a reduced N rate maintains yield and reduces N2O emissions in sweet corn. Nutri. Cycl. Agroecosystems 10.1007/s10705-021-10185-y
Musiani F, Broll V, Evangelisti E, Ciurli S (2020) The model structure of the copper-dependent ammonia monooxygenase. JBIC Journal of Biological Inorganic Chemistry 25(7): 995-1007. https://doi.org/10.1007/s00775-020-01820-0
Nair D, Abalos D, Philippot L, Bru D, Mateo-Marín N, Petersen SO (2021) Soil and temperature effects on nitrification and denitrification modified N2O mitigation by 3,4-dimethylpyrazole phosphate. Soil Biol. Biochem. 157: 108224. https://doi.org/10.1016/j.soilbio.2021.108224
Nguyen TT, Navarrete S, Horne DJ, Donaghy DJ, Kemp PD (2022a) Forage plantain (Plantago lanceolata L.): Meta-analysis quantifying the decrease in nitrogen excretion, the increase in milk production, and the changes in milk composition of dairy cows grazing pastures containing plantain. Anim. Feed Sci. Technol. 285: 115244. https://doi.org/10.1016/j.anifeedsci.2022.115244
Nguyen TT, Navarrete S, Horne DJ, Donaghy DJ, Kemp PD (2022b) Incorporating Plantain with Perennial Ryegrass-White Clover in a Dairy Grazing System: Dry Matter Yield, Botanical Composition, and Nutritive Value Response to Sowing Rate, Plantain Content and Season. Agronomy 12(11): 2789. https://doi.org/10.3390/agronomy12112789
Nicol GW, Leininger S, Schleper C, Prosser JI (2008) The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environ. Microbiol. 10(11): 2966-2978. https://doi.org/10.1111/j.1462-2920.2008.01701.x
NIWA (2016) The climate and weather of Manawatu-Wanganui. The National Institute of Water and Atmospheric Research Annual report(2nd edition): pp 15-24.
Norton J, Ouyang Y (2019) Controls and Adaptive Management of Nitrification in Agricultural Soils. Front. Microbiol. 10: 1931-1931. https://doi.org/10.3389/fmicb.2019.01931
OECD and FAO. (2020). *OECD-FAO Agricultural Outlook 2020-2029*. doi:https://doi.org/10.1787/1112c23b-en
Oenema O, Wrage N, Velthof GL, van Groenigen JW, Dolfing J, Kuikman PJ (2005) Trends in global nitrous oxide emissions from animal production systems. Nutri. Cycl. Agroecosystems 72(1): 51-65. https://doi.org/10.1007/s10705-004-7354-2
Ouyang Y, Norton JM, Stark JM (2017) Ammonium availability and temperature control contributions of ammonia oxidizing bacteria and archaea to nitrification in an agricultural soil. Soil Biol. Biochem. 113: 161-172. https://doi.org/10.1016/j.soilbio.2017.06.010
Pal P, McMillan AMS, Saggar S (2016) Pathways of dicyandiamide uptake in pasture plants: a laboratory study. Biol. Fertility Soils 52(4): 539-546. https://doi.org/10.1007/s00374-016-1096-6
Panagos P, Ballabio C, Lugato E, Jones A, Borrelli P, Scarpa S, Orgiazzi A, Montanarella L (2018) Potential Sources of Anthropogenic Copper Inputs to European Agricultural Soils. Sustainability 10(7): 2380. https://doi.org/10.3390/su10072380

Parsons AJ, Rasmussen S, Liu Q, Xue H, Ball C, Shaw C (2013) Plant growth – resource or strategy limited: insights from responses to gibberellin. Grass Forage Sci. 68(4): 577-588. https://doi.org/10.1111/gfs.12035

Petruzzelli G (1989) Recycling wastes in agriculture: heavy metal bioavailability. Agric., Ecosyst. Environ. 27(1): 493-503. https://doi.org/10.1016/0167-8809(89)90110-2

Pinto ISS, Neto IFF, Soares HMVM (2014) Biodegradable chelating agents for industrial, domestic, and agricultural applications—a review. Environ Sci Pollut Res 21(20): 11893-11906. https://doi.org/10.1007/s11356-014-2592-6

Preena PG, Rejish Kumar VJ, Singh ISB (2021) Nitrification and denitrification in recirculating aquaculture systems: the processes and players. Rev. Aquac. https://doi.org/10.1111/raq.12558

Principi P, Villa F, Giussani B, Zanardini E, Cappitelli F, Sorlini C (2009) The effect of copper on the structure of the ammonia-oxidizing microbial community in an activated sludge wastewater treatment plant. Microb. Ecol. 57(2): 215-220. https://doi.org/10.1007/s00248-008-9432-5

Prosser JI, Hink L, Gubry-Rangin C, Nicol GW (2020) Nitrous oxide production by ammonia oxidizers: Physiological diversity, niche differentiation and potential mitigation strategies. Global Change Biol. 26(1): 103-118. https://doi.org/10.1111/gcb.14877

Prosser JI, Nicol GW (2012) Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. Trends Microbiol. 20(11): 523-531. https://doi.org/10.1016/j.tim.2012.08.001

Qi Y, Zhu J, Fu Q, Hu H, Huang Q (2017) Sorption of Cu by humic acid from the decomposition of rice straw in the absence and presence of clay minerals. J. Environ. Manage. 200: 304-311. https://doi.org/10.1016/j.jenvman.2017.05.087

Qiu Y-R, Mao L-J (2013) Removal of heavy metal ions from aqueous solution by ultrafiltration assisted with copolymer of maleic acid and acrylic acid. Desalination 329: 78-85. https://doi.org/10.1016/j.desal.2013.09.012

Rafique M, Naveed M, Mustafa A, Akhtar S, Munawar M, Kaukab S, Ali HM, Siddiqui MH, Salem MZM (2021) The Combined Effects of Gibberellic Acid and Rhizobium on Growth, Yield and Nutritional Status in Chickpea (Cicer arietinum L.). Agronomy 11(1): 105. https://doi.org/10.3390/agronomy11010105

Ramotowski D, Shi W (2022) Nitrapyrin-based nitrification inhibitors shaped the soil microbial community via controls on soil pH and inorganic N composition. Appl. Soil Ecol. 170: 104295. https://doi.org/10.1016/j.apsoil.2021.104295

Rehman M, Liu L, Wang Q, Saleem MH, Bashir S, Ullah S, Peng D (2019) Copper environmental toxicology, recent advances, and future outlook: a review. Environ Sci Pollut Res 26(18): 18003-18016. https://doi.org/10.1007/s11356-019-05073-6

Rex D, Clough TJ, Lanigan GJ, Jansen-Willems AB, Condron LM, Richards KG, Müller C (2021) Gross N transformations vary with soil moisture and time following urea deposition to a pasture soil. Geoderma 386: 114904. https://doi.org/10.1016/j.geoderma.2020.114904

Reyes C, Hodgskiss LH, Baars O, Kerou M, Bayer B, Schleper C, Kraemer SM (2020) Copper limiting threshold in the terrestrial ammonia oxidizing archaeon Nitrososphaera viennensis. Res. Microbiol. 171(3): 134-142. https://doi.org/10.1016/j.resmic.2020.01.003

Richards J, Chambers T, Hales S, Joy M, Radu T, Woodward A, Humphrey A, Randal E, Baker MG (2021) Nitrate contamination in drinking water and colorectal cancer: Exposure assessment and estimated health burden in New Zealand. Environ. Res.: 112322. https://doi.org/10.1016/j.envres.2021.112322

Rieuwerts JS (2007) The mobility and bioavailability of trace metals in tropical soils: a review. Chemical Speciation & Bioavailability 19(2): 75-85. https://doi.org/10.3184/095422907X211918

Rieuwerts JS, Thornton I, Farago ME, Ashmore MR (1998) Factors influencing metal bioavailability in soils: preliminary investigations for the development of a critical loads approach for metals. Chemical Speciation & Bioavailability 10(2): 61-75. https://doi.org/10.3184/095422998782775835

Rotthauwe JH, Witzel KP, 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. https://doi.org/10.1128/aem.63.12.4704-4712.1997
Ruby MV, Davis A, Schoof R, Eberle S, Sellstone CM (1996) Estimation of lead and arsenic bioavailability using a physiologically based extraction test. Environ. Sci. Technol. 30(2): 422-430. https://doi.org/10.1021/es950057z
Rütting T, Schleusner P, Hink L, Prosser JI (2021) The contribution of ammonia-oxidizing archaea and bacteria to gross nitrification under different substrate availability. Soil Biol. Biochem. 160: 108353. https://doi.org/10.1016/j.soilbio.2021.108353
Saggar S, Bolan N, Bhandral R, Hedley C, 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. https://doi.org/10.1080/00288233.2004.9513618
Sahrawat K (2008) Factors affecting nitrification in soils. Commun. Soil Sci. Plant Anal. 39(9-10): 1436-1446.
Schils R, Verhagen A, Aarts H, Kuikman P, Šebek L (2006) Effect of improved nitrogen management on greenhouse gas emissions from intensive dairy systems in the Netherlands. Global Change Biol. 12(2): 382-391.
Selbie DR, Buckthought LE, Shepherd MA. (2015). The challenge of the urine patch for managing nitrogen in grazed pasture systems. In *Adv. Agron.* (Vol. 129, pp. 229-292): Elsevier.
Shen J-p, Zhang L-m, Zhu Y-g, Zhang J-b, He J-z (2008) Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. Environ. Microbiol. 10(6): 1601-1611. https://doi.org/10.1111/j.1462-2920.2008.01578.x
Shepherd M, Menneer J, Ledgard S, Sarathchandra U (2010) Application of carbon additives to reduce nitrogen leaching from cattle urine patches on pasture. N. Z. J. Agric. Res. 53(3): 263-280. https://doi.org/10.1080/00288233.2010.501520
Shi X, Hu H-W, Kelly K, Chen D, He J-Z, Suter H (2017) Response of ammonia oxidizers and denitrifiers to repeated applications of a nitrification inhibitor and a urease inhibitor in two pasture soils. J. Soils Sed. 17(4): 974-984. https://doi.org/10.1007/s11368-016-1588-x
Shuaib M, Azam N, Bahadur S, Romman M, Yu Q, Xuexiu C (2021) Variation and succession of microbial communities under the conditions of persistent heavy metal and their survival mechanism. Microb. Pathog. 150: 104713. https://doi.org/10.1016/j.micpath.2020.104713
Silva TH, Guimaraes I, Menta PR, Fernandes L, Paiva D, Ribeiro TL, Celestino ML, Netto AS, Ballou MA, Machado VS (2022) Effect of injectable trace mineral supplementation on peripheral polymorphonuclear leukocyte function, antioxidant enzymes, health, and performance in dairy cows in semi-arid conditions. J. Dairy Sci. 105(2): 1649-1660. https://doi.org/10.3168/jds.2021-20624
Smith G, Cornforth I, Henderson H (1985) Critical leaf concentrations for deficiencies of nitrogen, potassium, phosphorus, sulphur, and magnesium in perennial ryegrass. New Phytol. 101(3): 393-409. https://doi.org/10.1111/j.1469-8137.1985.tb02846.x
Soler-Jofra A, Pérez J, van Loosdrecht MCM (2021) Hydroxylamine and the nitrogen cycle: A review. Water Res. 190: 116723. https://doi.org/10.1016/j.watres.2020.116723
Soler-Rovira P, Madejón E, Madejón P, Plaza C (2010) In situ remediation of metal-contaminated soils with organic amendments: role of humic acids in copper bioavailability. Chemosphere 79(8): 844-849.
Soliman M, Eldyasti A (2018) Ammonia-Oxidizing Bacteria (AOB): opportunities and applications—a review. Rev. Environ. Sci. Biotechnol. 17(2): 285-321. https://doi.org/10.1007/s11157-018-9463-4
Spek JW, Bannink A, Gort G, Hendriks WH, Dijkstra J (2012) Effect of sodium chloride intake on urine volume, urinary urea excretion, and milk urea concentration in lactating dairy cattle. J. Dairy Sci. 95(12): 7288-7298. https://doi.org/10.3168/jds.2012-5688
Stafford AD, Anderson CWN, Hedley MJ, McDowell RW (2016) Cadmium accumulation by forage species used in New Zealand livestock grazing systems. Geordema Reg. 7(1): 11-18. https://doi.org/10.1016/j.geodrs.2015.11.003
Stout WL, Fales SL, Muller LD, Schnabel RR, Weaver SR (2000) Water quality implications of nitrate leaching from intensively grazed pasture swards in the northeast US. Agric., Ecosyst. Environ. 77(3): 203-210. https://doi.org/10.1016/S0167-8809(99)00084-5

Suanon F, Sun Q, Dimon B, Mama D, Yu C-P (2016) Heavy metal removal from sludge with organic chelators: Comparative study of N, N-bis(carboxymethyl) glutamic acid and citric acid. J. Environ. Manage. 166: 341-347. https://doi.org/10.1016/j.jenvman.2015.10.035

Sun H, Chen Y, Yi Z (2022) After-Effects of Hydrochar Amendment on Water Spinach Production, N Leaching, and N2O Emission from a Vegetable Soil under Varying N-Inputs. Plants 11(24): 3444.

Sun Z, Lv Y, Liu Y, Ren R (2016) Removal of nitrogen by heterotrophic nitrification-aerobic denitrification of a novel metal resistant bacterium Cupriavidus sp. S1. Bioresour. Technol. 220: 142-150. https://doi.org/10.1016/j.biortech.2016.07.110

Swain SM, Singh DP (2005) Tall tales from sly dwarves: novel functions of gibberellins in plant development. Trends Plant Sci. 10(3): 123-129. https://doi.org/10.1016/j.tplants.2005.01.007

Szostek M, Szpunar-Krok E, Pawlak R, Stanek-Tarkowska J, Ilek A (2022) Effect of Different Tillage Systems on Soil Organic Carbon and Enzymatic Activity. Agronomy 12(1): 208.

Takeda N, Friedl J, Rowlings D, De Rosa D, Scheer C, Grace P (2021) Exponential response of nitrous oxide (N2O) emissions to increasing nitrogen fertiliser rates in a tropical sugarcane cropping system. Agric., Ecosyst. Environ. 313: 107376. https://doi.org/10.1016/j.agee.2021.107376

Talbot WD, Malcolm BJ, Cameron KC, Di HJ, Whitehead D (2021) Effect of timing of cattle urine deposition and pasture composition on nitrogen leaching losses. Soil Use Manag 37(4): 723-735. https://doi.org/10.1111/sum.12652

Tang J, Zhuang L, Yu Z, Liu X, Wang Y, Wen P, Zhou S (2019) Insight into complexation of Cu(II) to hyperthermophilic compost-derived humic acids by EEM-PARAFAC combined with heterospectral two dimensional correlation analyses. Sci. Total Environ. 656: 29-38. https://doi.org/10.1016/j.scitotenv.2018.11.357

Tao R, Wakelin SA, Liang Y, Chu G (2017) Response of ammonia-oxidizing archaea and bacteria in calcareous soil to mineral and organic fertilizer application and their relative contribution to nitrification. Soil Biol. Biochem. 114: 20-30. https://doi.org/10.1016/j.soilbio.2017.06.027

Taylor MD, Kim ND, Hill RB, Chapman R (2010) A review of soil quality indicators and five key issues after 12 yr soil quality monitoring in the Waikato region. Soil Use Manag 26(3): 212-224. https://doi.org/10.1111/j.1475-2743.2010.00276.x

Tomlinson T, Boon A, Trotman C (1966) Inhibition of nitrification in the activated sludge process of sewage disposal. Journal of Applied Bacteriology 29(2): 266-291. https://doi.org/10.1111/j.1365-2672.1966.tb03477.x

Ubeynarayana N, Jeyakumar P, Bishop P, Pereira RC, Anderson CWN (2021) Effect of soil cadmium on root organic acid secretion by forage crops. Environ. Pollut. 268: 115839. https://doi.org/10.1016/j.envpol.2020.115839

van der Weerden TJ, Cox N, Luo J, Di HJ, Podolyan A, Phillips RL, Saggar S, de Klein CAM, Ettema P, Rys G (2016) Refining the New Zealand nitrous oxide emission factor for urea fertiliser and farm dairy effluent. Agric., Ecosyst. Environ. 222: 133-137. https://doi.org/10.1016/j.agee.2016.02.007

van Rossum M, Bryant R, Edwards G (2013) Response of simple grass-white clover and multi-species pastures to gibberellic acid or nitrogen fertiliser in autumn. J. N. Z. Grassl. 75: 145–150. https://doi.org/10.33584/jnzg.2013.75.2935

Vandevivere P, Ficara E, Terras C, Julies E, Verstraete W (1998) Copper-Mediated Selective Removal of Nitrification Inhibitors from Industrial Wastewaters. Environ. Sci. Technol. 32(7): 1000-1006. https://doi.org/10.1021/es970800i

Victorino G, Santos ES, Abreu MM, Viegas W, Nogales A (2021) Detrimental effects of copper and EDTA co-application on grapevine root growth and nutrient balance. Rhizosphere 19: 100392. https://doi.org/10.1016/j.rhisph.2021.100392

Waggoner AL, Bottomley PJ, Taylor AE, Myrold DD (2021) Soil nitrification response to dairy digestate and inorganic ammonium sources depends on soil pH and nitrifier abundances. Soil Sci. Soc. Am. J. 85(6): 1990-2006. https://doi.org/10.1002/saj2.20325

Wagner FB, Diwan V, Dechesne A, Fowler SJ, Smets BF, Albrechtsen H-J (2019) Copper-Induced Stimulation of Nitrification in Biological Rapid Sand Filters for Drinking Water Production by Proliferation of Nitrosomonas spp. Environ. Sci. Technol. 53(21): 12433-12441. https://doi.org/10.1021/acs.est.9b03885

Wagner FB, Nielsen PB, Boe-Hansen R, Albrechtsen H-J (2016) Copper deficiency can limit nitrification in biological rapid sand filters for drinking water production. Water Res. 95: 280-288. https://doi.org/10.1016/j.watres.2016.03.025
Wagner FB, Nielsen PB, Boe-Hansen R, Albrechtsen H-J (2018) Remediation of incomplete nitrification and capacity increase of biofilters at different drinking water treatment plants through copper dosing. Water Res. 132: 42-51. https://doi.org/10.1016/j.watres.2017.12.061
Wei Z, Hoffland E, Zhuang M, Hellegers P, Cui Z (2021) Organic inputs to reduce nitrogen export via leaching and runoff: A global meta-analysis. Environ. Pollut. 291: 118176. https://doi.org/10.1016/j.envpol.2021.118176
Wilcock RJ, Nagels JW, Rodda HJE, O'Connor MB, Thorrold BS, Barnett JW (1999) Water quality of a lowland stream in a New Zealand dairy farming catchment. N. Z. J. Mar. Freshwat. Res. 33(4): 683-696. 10.1080/00288330.1999.9516911
Wild A, Cameron K (1980) Soil nitrogen and nitrate leaching. In'Soils and Agriculture'.(Ed. PB Tinker.) pp. 35-70. In: Blackwell: Oxford.
Williams C, Arnold G (1964) Winter growth stimulation by gibberellin in differentially grazed pastures of <I>Phalaris tuberosa</I>. Aust. J. Exp. Agric. 4(14): 225-230. https://doi.org/10.1071/EA9640225
Williams PH, Haynes RJ (2000) Transformations and plant uptake of urine N and S in long and short-term pastures. Nutri. Cycl. Agroecosystems 56(2): 109-116. 10.1023/A:1009885413823
Woods RR, Cameron KC, Edwards GR, Di HJ, Clough TJ (2016) Effects of forage type and gibberellic acid on nitrate leaching losses. Soil Use Manag 32(4): 565-572. https://doi.org/10.1111/sum.12297
Woods RR, Cameron KC, Edwards GR, Di HJ, Clough Tim J (2018) Reducing nitrogen leaching losses in grazed dairy systems using an Italian ryegrass-plantain-white clover forage mix. Grass Forage Sci. 73(4): 878-887. https://doi.org/10.1111/gfs.12386
Wrage N, Velthof G, Van Beusichem M, Oenema O (2001) Role of nitrifier denitrification in the production of nitrous oxide. Soil Biol. Biochem. 33(12-13): 1723-1732.
Xia Z, Zhang S, Cao Y, Zhong Q, Wang G, Li T, Xu X (2019) Remediation of cadmium, lead and zinc in contaminated soil with CETSA and MA/AA. Journal of Hazardous Materials 366: 177-183. https://doi.org/10.1016/j.jhazmat.2018.11.109
Xu J, Yang L, Wang Z, Dong G, Huang J, Wang Y (2006) Toxicity of copper on rice growth and accumulation of copper in rice grain in copper contaminated soil. Chemosphere 62(4): 602-607. https://doi.org/10.1016/j.chemosphere.2005.05.050
Xu X, Yang Y, Wang G, Zhang S, Cheng Z, Li T, Yang Z, Xian J, Yang Y, Zhou W (2020) Removal of heavy metals from industrial sludge with new plant–based washing agents. Chemosphere 246: 125816. https://doi.org/10.1016/j.chemosphere.2020.125816
Yan M, Benedetti MF, Korshin GV (2013) Study of iron and aluminum binding to Suwannee River fulvic acid using absorbance and fluorescence spectroscopy: Comparison of data interpretation based on NICA-Donnan and Stockholm humic models. Water Res. 47(14): 5439-5446. https://doi.org/10.1016/j.watres.2013.06.022
Yan Y-P, He J-Y, Zhu C, Cheng C, Pan X-B, Sun Z-Y (2006) Accumulation of copper in brown rice and effect of copper on rice growth and grain yield in different rice cultivars. Chemosphere 65(10): 1690-1696. https://doi.org/10.1016/j.chemosphere.2006.05.022
Yang B, Zhu Z, Sun H, Yin W, Hong J, Cao S, Tang Y, Zhao C, Yao J (2020) Improving flotation separation of apatite from dolomite using PAMS as a novel eco-friendly depressant. Miner. Eng. 156: 106492. https://doi.org/10.1016/j.mineng.2020.106492
Yang T, Hodson ME (2019) Investigating the use of synthetic humic-like acid as a soil washing treatment for metal contaminated soil. Sci. Total Environ. 647: 290-300. https://doi.org/10.1016/j.scitotenv.2018.07.457
Yi Z, Zhang Z, Chen G, Rengel Z, Sun H (2023) Microplastics have rice cultivar-dependent impacts on grain yield and quality, and nitrogenous gas losses from paddy, but not on soil properties. Journal of Hazardous Materials 446: 130672. https://doi.org/10.1016/j.jhazmat.2022.130672
Ying J-Y, Zhang L-M, He J-Z (2010) Putative ammonia-oxidizing bacteria and archaea in an acidic red soil with different land utilization patterns. Environ. Microbial. Rep. 2(2): 304-312. https://doi.org/10.1111/j.1758-2229.2009.00130.x

Yu C, Huang X, Chen H, Godfray HCJ, Wright JS, Hall JW, Gong P, Ni S, Qiao S, Huang G, Xiao Y, Zhang J, Feng Z, Ju X, Ciais P, Stenseth NC, Hessen DO, Sun Z, Yu L, Cai W, Fu H, Huang X, Zhang C, Liu H, Taylor J (2019) Managing nitrogen to restore water quality in China. Nature 567(7749): 516-520. https://doi.org/10.1038/s41586-019-1001-1

Zaman M, Blennerhassett JD (2010) Effects of the different rates of urease and nitrification inhibitors on gaseous emissions of ammonia and nitrous oxide, nitrate leaching and pasture production from urine patches in an intensive grazed pasture system. Agric., Ecosyst. Environ. 136(3): 236-246. https://doi.org/10.1016/j.agee.2009.07.010

Zaman M, Ghani A, Kurepin LV, Pharis RP, Khan S, Smith TJ (2014) Improving ryegrass-clover pasture dry matter yield and urea efficiency with gibberellic acid. J. Sci. Food Agric. 94(12): 2521-2528. https://doi.org/10.1002/jsfa.6589

Zaman M, Nguyen M (2012) How application timings of urease and nitrification inhibitors affect N losses from urine patches in pastoral system. Agric., Ecosyst. Environ. 156: 37-48.

Zhang L-M, Hu H-W, Shen J-P, He J-Z (2012a) Ammonia-oxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. The ISME Journal 6(5): 1032-1045. https://doi.org/10.1038/ismej.2011.168

Zhang Q-L, Liu Y, Ai G-M, Miao L-L, Zheng H-Y, Liu Z-P (2012b) The characteristics of a novel heterotrophic nitrification–aerobic denitrification bacterium, Bacillus methylotrophicus strain L7. Bioresour. Technol. 108: 35-44. https://doi.org/10.1016/j.biortech.2011.12.139

Zhang Q, Zou D, Zeng X, Li L, Wang A, Liu F, Wang H, Zeng Q, Xiao Z (2021) Effect of the direct use of biomass in agricultural soil on heavy metals __ activation or immobilization? Environ. Pollut. 272: 115989. https://doi.org/10.1016/j.envpol.2020.115989

Zhang T, Liu J-M, Huang X-F, Xia B, Su C-Y, Luo G-F, Xu Y-W, Wu Y-X, Mao Z-W, Qiu R-L (2013) Chelant extraction of heavy metals from contaminated soils using new selective EDTA derivatives. Journal of Hazardous Materials 262: 464-471. https://doi.org/10.1016/j.jhazmat.2013.08.069

Zhang Y, Cai Z, Zhang J, Müller C (2023) The controlling factors and the role of soil heterotrophic nitrification from a global review. Appl. Soil Ecol. 182: 104698. https://doi.org/10.1016/j.apsoil.2022.104698

Zhang Y, Edwards M (2010) Nutrients and metals effects on nitrification in drinking water systems. J Am Water Works 102(7): 56-66. https://doi.org/10.1002/j.1551-8833.2010.tb10149.x

Zhang Z, Gao Q, Yang J, Li L, Li Y, Liu J, Wang Y, Su H, Wang Y, Wang S, Feng G (2020) Effect of Soil Organic Matter on Adsorption of Nitrification Inhibitor Nitrapyrin in Black Soil. Commun. Soil Sci. Plant Anal. 51(7): 883-895. https://doi.org/10.1080/00103624.2020.1744636

Zhao L, Liu J, Wang H, Dong Y-h (2019) Sorption of copper and norfloxacin onto humic acid: effects of pH, ionic strength, and foreign ions. Environ Sci Pollut Res 26(11): 10685-10694. https://doi.org/10.1007/s11356-019-04515-5

Zhao X, Li X, Zhang H, Chen X, Xu J, Yang J, Zhang H, Hu G (2022) Atomic-dispersed copper simultaneously achieve high-efficiency removal and high-value-added conversion to ammonia of nitrate in sewage. Journal of Hazardous Materials 424: 127319. https://doi.org/10.1016/j.jhazmat.2021.127319

Zhongqi H, Pagliari PH, Waldrip HM (2016) Applied and environmental chemistry of animal manure: A review. Pedosphere 26(6): 779-816.

Zhou H, Shi X, Wu W, An X, Tian Y, Qiao Y (2020) Facile preparation of lignosulfonate/N-methylaniline composite and its application in efficient removal of Cr(VI) from aqueous solutions. Int. J. Biol. Macromol. 154: 1194-1204. https://doi.org/10.1016/j.ijbiomac.2019.10.274

Zupanc V, Kastelec D, Lestan D, Grcman H (2014) Soil physical characteristics after EDTA washing and amendment with inorganic and organic additives. Environ. Pollut. 186: 56-62. https://doi.org/10.1016/j.envpol.2013.11.027

Zvomuya F, Rosen CJ, Russelle MP, Gupta SC (2003) Nitrate Leaching and Nitrogen Recovery Following Application of Polyolefin-Coated Urea to Potato. J.Environ.Qual. 32(2): 480-489. https://doi.org/10.2134/jeq2003.4800

APPENDIX

Appendix 1. Supporting Information for Chapter 4

Table A1.1 Pearson's correlation coefficients between AOB/AOA *amoA* gene abundance and soil bioavailable Cu, NH_4^+ -N concentration, NO_3^- -N concentration, and soil pH for the Pumice soil. Significance difference: ** $P < 0.01$. The relationship was analysed at day 1 because it is where treatment effect was at maximum for all the soils.

Items	Bioavailable Cu	AOA *amoA*	Soil pH	NH_4^+ -N	NO_3^- -N	AOB *amoA*
Bioavailable Cu	1					
AOA *amoA*	0.421	1				
Soil pH	0.654	0.052	1			
NH_4^+ -N	0.029	-0.435	0.158	1		
NO_3^- -N	-0.053	-0.499	-0.057	0.91**	1	
AOB *amoA*	0.067	-0.111	0.551	-0.142	-0.07	1

Table A1.2 Pearson's correlation coefficients between AOB/AOA *amoA* gene abundance and soil bioavailable Cu, NH_4^+ -N concentration, NO_3^- -N concentration, and soil pH for the Pallic soil. Significance difference: ** $P < 0.01$, * $P < 0.05$. The relationship was analysed at day 1 because it is where treatment effect was at maximum for all the soils.

Items	Bioavailable Cu	AOA *amoA*	Soil pH	NH_4^+ -N	NO_3^- -N	AOB *amoA*
Bioavailable Cu	1					
AOA *amoA*	0.096	1				
Soil pH	0.161	0.545	1			
NH_4^+ -N	-0.386	-0.509	-0.511	1		
NO_3^- -N	0.748*	-0.167	-0.407	0.222	1	
AOB *amoA*	0.702*	-0.176	-0.48	0.213	0.982**	1

Table A1.3 Pearson's correlation coefficients between AOB/AOA *amoA* gene abundance and soil bioavailable Cu, NH_4^+ -N concentration, NO_3^- -N concentration, and soil pH for the Recent soil. Significance difference: ** $P < 0.01$, * $P < 0.05$. The relationship was analysed at day 1 because it is where treatment effect was at maximum for all the soils.

Items	Bioavailable Cu	AOA *amoA*	Soil pH	NH_4^+ -N	NO_3^- -N	AOB *amoA*
Bioavailable Cu	1					
AOA *amoA*	0.139	1				
Soil pH	-0.285	0.19	1			
NH_4^+ -N	-0.243	0.116	0.402	1		
NO_3^- -N	0.937**	0.221	-0.191	-0.406	1	
AOB *amoA*	0.940**	0.316	-0.025	-0.224	0.943**	1

Appendix 2. Supporting Information for Chapter 5

Does delay in field Lysimeter leachate samples collection influence nitrate and ammonia concentration results: pre-liminary experiment

This experiment consisted of field stored samples. These field conditions samples were compared to immediately collected and analysed samples. For this experiment, four independent lysimeter leachate samples; Sample A, B, C, and D were randomly selected from an on-going lysimeter study at Manawatu, Massey University farm (40°23'0.95"S175°36'36.16"E). Each bulk sample was collected into 250 ml bottles. The samples bottles were left in the experimental site covered in aluminium foil to replicate the black containers used for leachate collection in real lysimeter experiment.

Leachate Analysis

Each bulk sample was replicated three times. Sampling was done at 0 (control), 7, 14, 21, 28, 56, and 90 days. The initial samples analysis was analysed within 1 hour from collection. All Samples were measured for pH using a pH meter (acumen 910, Fisher Scientific Ltd, Pittsburgh, PA, USA) and analysed for NO_3^- -N and NH_4^+ -N using the Technicon autoanalyzer (Blakemore 1987).

Statistical analysis

Statistical analyses in this experiment were done using Minitab™ 19 and graphs were drawn sigma plot 13. The effect of storage days on each sample was analysed using the ANOVA test and significant ($P < 0.05$) differences between mean were determined using Turkey post hoc test.

Results

There storage period did not have significant effect on each sample relative to control in relation to leachate pH, NO_3^- -N, and NH_4^+ -N concentration (Figure A2.1).

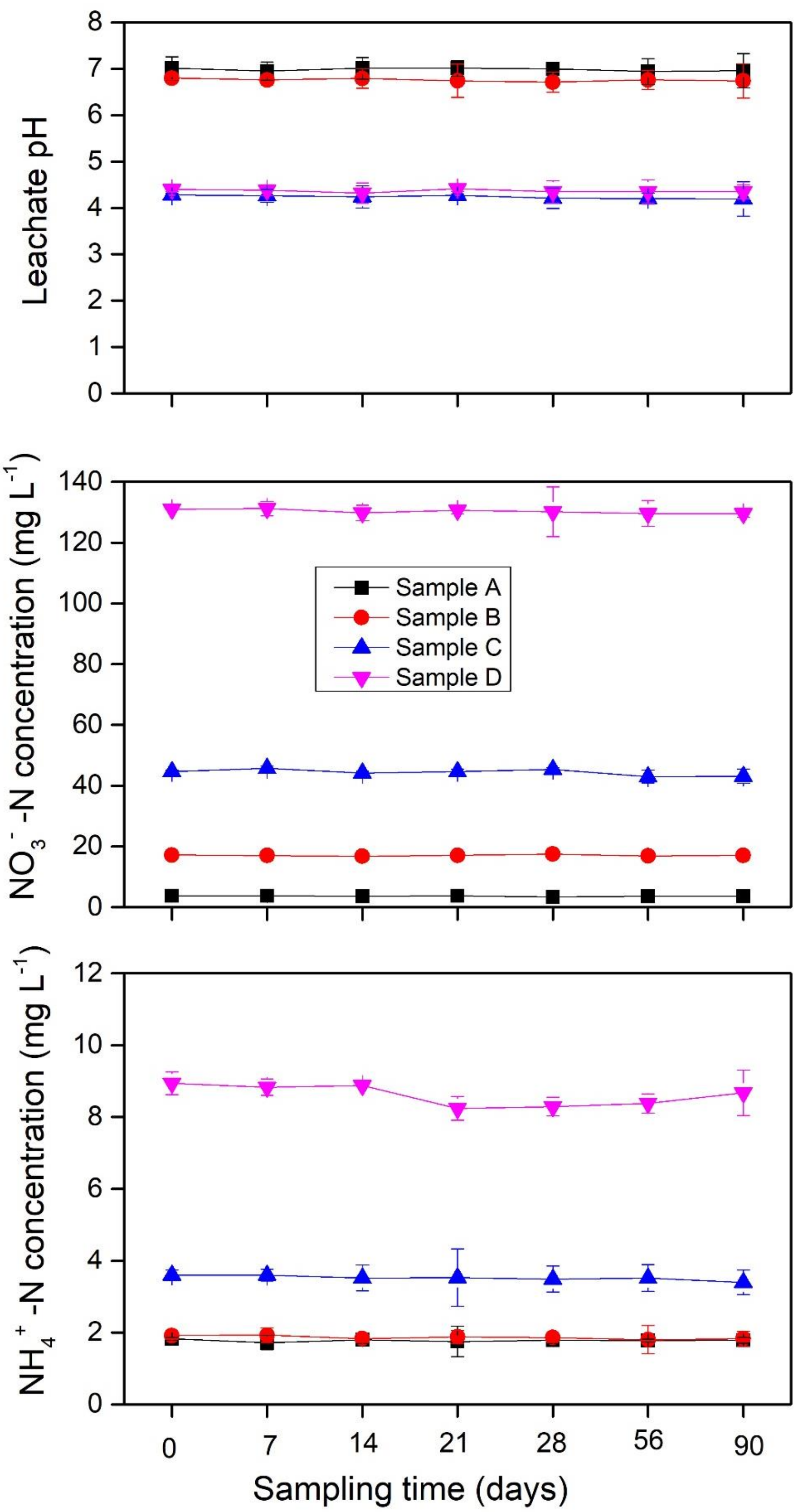


Figure A2.1 The effect of lysimeter leachate collection period in the field on leachate pH, NO_3^- -N, and NH_4^+ -N concentration in different sampling days relative to day 0 (control). There were no significant changes in leachate parameters as influenced by period sample collection in the field.

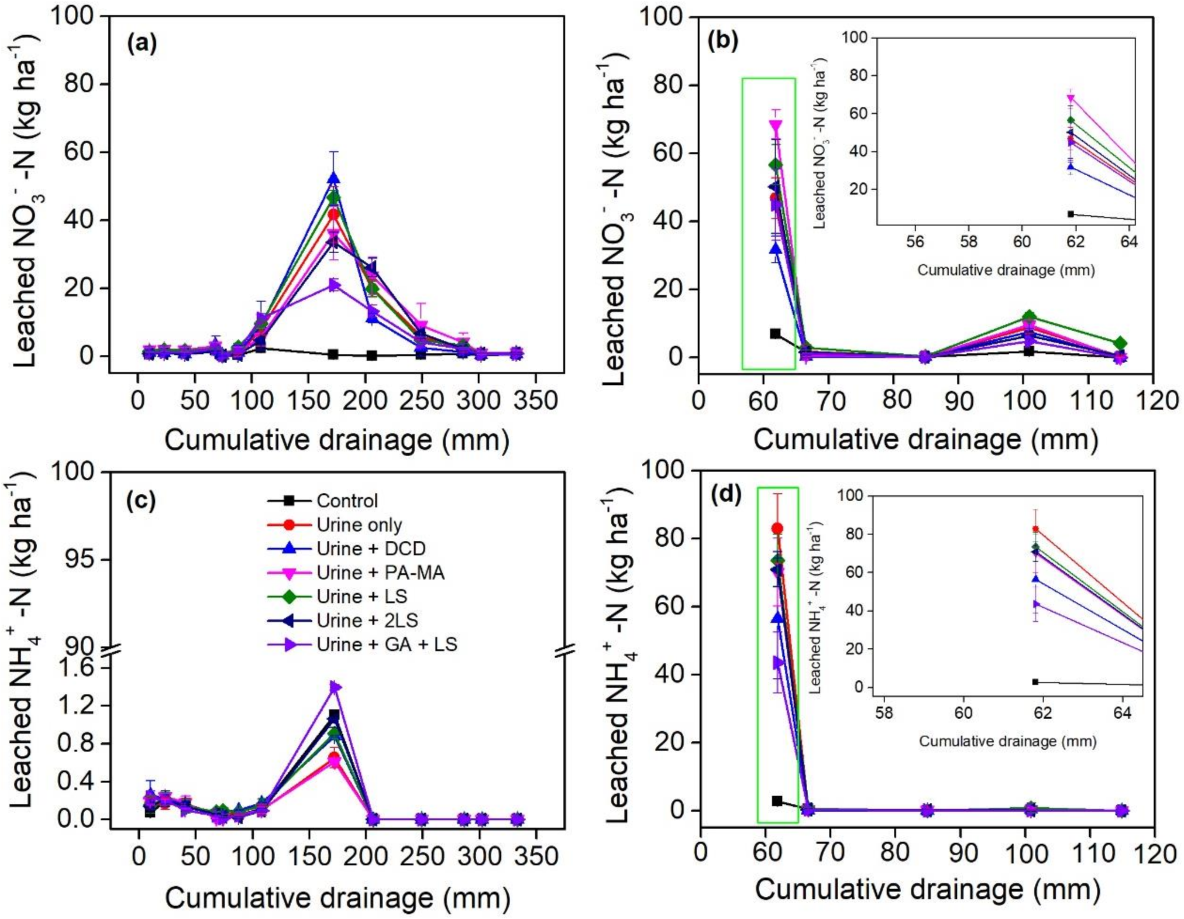


Figure A2.2 Leached NO_3^- -N from the Pumice (**a**) and Pallic soil (**b**), and leached NH_4^+ -N from the Pumice (**c**) and Pallic lysimeters (**d**) as a function of cumulative drainage following late-autumn urine and treatments application to lysimeters. Error bars represent standard deviation of mean (n = 4). Data points for the Pallic soil lysimeters start at 61.8 cumulative drainage and correspond to both the maximum and first collected drainage for the late-autumn treatment application.

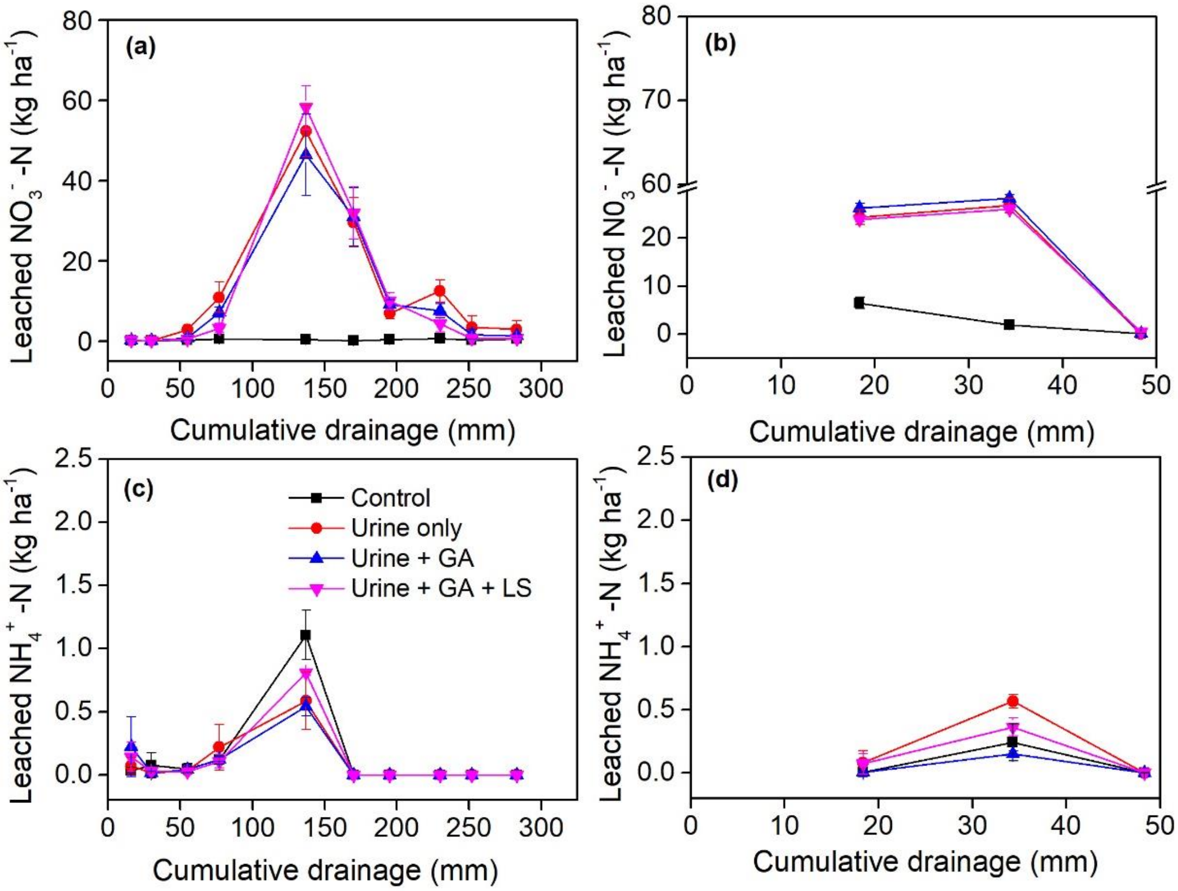


Figure A2.3 Leached NO_3^- -N from the Pumice (**a**) and Pallic soil lysimeters (**b**), and leached NH_4^+ -N from the Pumice (**c**) and Pallic soil lysimeters (**d**) as a function of cumulative drainage following mid-winter urine and treatments application to lysimeters. NH_4^+ -N concentration after 150 mm cumulative drainage from the Pumice soil lysimeters was below detectable levels. Vertical error bars represent standard deviation of means (n = 4). Data points for the Canterbury lysimeters start at 18.4 mm cumulative drainage and correspond to the first collected drainage for the mid-winter treatment application.

Table A2.1 NO_3^- -N in leachate in the late-autumn treatments application in the Pumice and Pallic lysimeters before treatment application.

Treatments	**Pumice soil**	
	05/06/2020	**09/06/202**
	Leachate kg NO_3^- -N ha^{-1}	**Leachate kg NO_3^- -N ha^{-1}**
Control	1.0±0.23a	1.4±0.75a
Urine only	1.4±0.26a	1.5±0.39a
Urine + DCD	1.1±0.37a	1.5±0.09a
Urine + PA-MA	1.6±0.90a	1.1±0.44a
Urine + LS	1.3±0.29a	1.4±0.73a
Urine + 2LS	2.0±0.69a	1.2±0.69a
Urine + GA + LS	0.9±0.27a	0.9±0.19a
Treatments	**Pallic soil**	
	13/05/2020	**20/05/2020**
	Leachate kg NO_3^- -N ha^{-1}	**Leachate kg NO_3^- -N ha^{-1}**
Control	8.5±2.37a	1.0±0.23
Urine only	9.7±1.46a	1.4±0.26
Urine + DCD	9.4±1.38a	1.6±1.33
Urine + PA-MA	11.3±1.91a	1.6±0.90
Urine + LS	9.4±1.73a	1.5±0.61
Urine + 2LS	9.6±1.73a	1.9±0.69
Urine + GA + LS	8.7±1.15a	1.0±0.74

Different small letters in each soil column indicate significant difference at $P < 0.05$. NH_4^+ -N was below detectable concentrations.

Table A2.2 NO_3^- -N in leachate in the mid-winter treatments application in the Pumice and Pallic lysimeters before treatments application.

Treatments	Pumice soil	
	05/06/2020	**09/06/202**
	Leachate kg NO_3^- -N ha^{-1}	**Leachate kg NO_3^- -N ha^{-1}**
Control	1.2±0.21a	1.0±0.30a
Urine only	0.9±0.52a	1.0±0.20a
Urine + GA	1.4±0.93a	1.2±0.18a
Urine + GA + LS	1.3±0.90a	1.0±0.58a
Treatments	**Pallic soil**	
	13/05/2020	**20/05/2020**
	Leachate kg NO_3^- -N ha^{-1}	**Leachate kg NO_3^- -N ha^{-1}**
Control	10.1±1.04a	0.9±0.60a
Urine only	11.2±1.55a	1.0±0.42a
Urine + GA	10.9±1.06a	0.9±0.50a
Urine + GA + LS	9.7±0.35a	1.3±0.90a

Different small letters in each soil column indicate significant difference at $P < 0.05$. NH_4^+ -N was below detectable concentrations.

Table A2.3. Herbage N uptake (kg N/ha) and herbage DM yield (kg DM/ha), following late-autumn urine and treatment application to the Pumice and Pallic soils.

	Pumice soil									
Treatments	11/07/20		11/09/20		10/10/20		11/11/20		11/12/20	
	kg N/ha	kg DM/ha	kg N/ha	kg DM/ha	kg N/ha	kg DM/ha	kg N/ha	kg DM/ha	kg N/ha	kg DM/ha
Control	2.7d	91e	1.8d	121b	11.6c	538d	11.0a	772b	21.4a	1262b
Urine only	11.6c	266d	95.2b	3062a	66.8b	2663c	31.7ab	1968a	24.6a	1520a
Urine + DCD	16.8b	373b	95.3b	3303a	79.5ab	2815bc	35.7a	2088a	27.4a	1697a
Urine + PA-MA	11.7c	274dc	62.9c	3196a	76.2ab	3067ab	28.9b	1888a	24.5a	1515a
Urine + LS	13.9c	320c	67.6c	2857a	72.8ab	2614c	32.2ab	1975a	26.7a	1708a
Urine + 2LS	16.9b	385b	101.8b	3190a	85.4a	3276a	28.7b	1860a	25.3a	1590a
Urine + GA + LS	21.3a	505a	114.9a	2993a	68.5b	2527c	30.8ab	1897a	25.6a	1661a
	Pallic soil									
			27/08/20		17/10/20		19/11/20		16/12/20	
Treatments			kg N/ha	kg DM/ha	kg N/ha	kg DM/ha	kg N/ha	kg DM/ha	kg N/ha	kg DM/ha
Control			11.4c	454d	27.1c	1002b	36.8b	2212b	18.0d	777b
Urine only			76.8a	1964ab	74.6b	3122a	91.9a	3798a	37.6c	1221a
Urine + DCD			80.80a	2125a	93.7a	3098a	103.4a	4114a	46.9b	1634a
Urine + PA-MA			61.8b	1693abc	88.8a	2934a	94.4a	4275a	41.6bc	1321a
Urine + LS			54.8b	1450bc	95.9a	3013a	102.4a	4483a	55.0a	1650a
Urine + 2LS			56.7b	1276c	96.4a	3731a	116.6a	4445a	42.2bc	1440a
Urine + GA + LS			80.7a	2144a	89.1a	3161a	101.8a	4541a	42.8bc	1441a

Values in each column, followed by different small letters within a column for each soil, are significantly different at $P < 0.05$.

Table A2.4 Effect of applied inhibitors on herbage cation uptake in the Pumice and Pallic soil after late-autumn urine application.

Treatments	**Ca**	**Mg**	**Na**	**K**	**P**
			%		
			Pumice soil		
Control	0.48 ± 0.01a	0.19 ± 0.11a	0.05 ± 0.01ab	4.82 ± 0.02b	0.23 ±0 .02a
Urine only	0.36 ± 0.00b	0.13 ± 0.01bc	0.05 ± 0.01ab	5.40 ± 0.16ab	0.18 ± 0.08ab
Urine + DCD	0.34 ± 0.03b	0.13 ± 0.01bc	0.04 ± 0.01ab	5.81 ± 0.20a	0.19 ± 0.06ab
Urine + PA-MA	0.35 ± 0.02b	0.13 ± 0.00c	0.04 ± 0.00ab	5.76 ± 0.19a	0.13 ± 0.01b
Urine + LS	0.35 ± 0.01b	0.13 ± 0.01bc	0.04 ± 0.01ab	5.65 ± 0.48a	0.14 ± 0.00b
Urine + 2LS	0.34 ± 0.01b	0.13 ± 0.01c	0.04 ± 0.01b	5.85 ± 0.40a	0.12 ± 0.01b
Urine + GA + LS	0.36 ± 0.02b	0.15 ± 0.01b	0.06 ± 0.01a	6.05 ± 0.45a	0.13 ± 0.01b
			Pallic soil		
Control	0.57 ± 0.07a	0.15 ± 0.03a	0.06 ± 0.02ab	1.51 ± 0.17a	0.19 ± 0.05a
Urine only	0.46 ± 0.03ab	0.15 ± 0.01a	0.07 ± 0.01a	3.76 ± 0.51a	0.18 ± 0.00a
Urine + DCD	0.41 ± 0.05b	0.13 ± 0.01a	0.03 ± 0.01b	3.33 ± 0.31a	0.18 ± 0.02a
Urine + PA-MA	0.51 ± 0.01ab	0.14 ± 0.01a	0.07 ± 0.03ab	3.21 ± 0.31a	0.16 ± 0.05a
Urine + LS	0.55 ± 0.07a	0.14 ± 0.02a	0.04 ± 0.01ab	3.33 ± 0.32a	0.17 ± 0.01a
Urine + 2LS	0.47 ± 0.02ab	0.14 ± 0.01a	0.04 ± 0.01ab	3.59 ± 0.47a	0.17 ± 0.02a
Urine + GA + LS	0.51 ± 0.04ab	0.15 ± 0.01a	0.05 ± 0.02ab	3.18 ± 0.13a	0.18 ± 0.03a

The values displayed are mean cation concentration from 1st and 2nd herbage harvests. Different small letters following each other in a column in each soil type are significantly different ($P < 0.05$) ($n = 4$). Values after ± represent standard deviation.

Table A2.5. Herbage N uptake (kg N/ha) and herbage DM yield (kg DM/ha) following mid-winter urine and treatment application to the Pumice and Pallic soils.

Pumice soil								
	11/09/20		**10/10/20**		**11/11/20**		**10/12/20**	
Treatments	kg N/ha	kg DM/ha	kg N/ha	kg DM/ha	kg N/ha	kg DM/ha	kg N/ha	kg DM/ha
Control	1.8c	121c	11.6c	538b	11.0b	772c	21.4a	1262b
Urine only	33.6b	867b	94.5a	2986a	47.4a	2682b	25.6a	1527a
Urine + GA	57.2a	1554a	102.5a	3037a	57.8a	3157a	28.4a	1771a
Urine + GA + LS	52.6a	1428a	96.8a	3031a	52.5a	3107a	25.3a	1704a
Pallic soil								
			17/10/20		**16/11/20**		**16/12/20**	
Treatments			kg N/ha	kg DM/ha	kg N/ha	kg DM/ha	kg N/ha	kg DM/ha
Control			27.1c	1002b	36.8c	2212c	18.0c	777c
Urine only			125.6b	3675a	87.6b	3968b	58.3b	1762b
Urine + GA			119.7b	3667a	130.6a	4802a	71.5a	2040a
Urine + GA + LS			139.3a	4032a	124.3a	5123a	72.4a	1993a

Values in each column, followed by different small letters within a column for each soil, are significantly different at $P < 0.05$.

Appendix 3. Supporting Information for Chapter 6

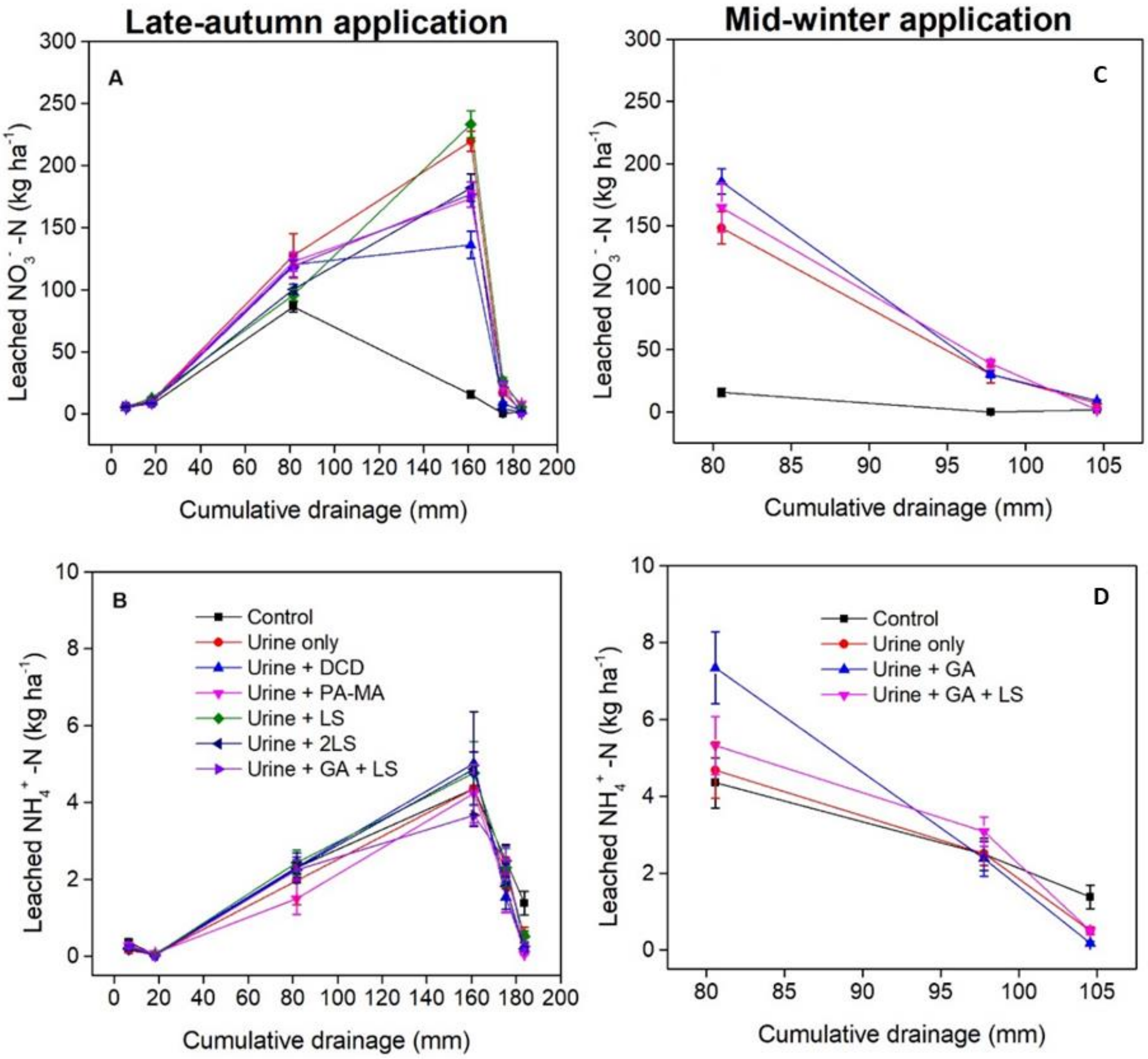


Figure A3.1 Leached NO_3^- -N (**A**) and mid-winter treatment application (**C**). Leached NH_4^+ -N from late-autumn (**C**) and mid-winter treatment application (**D**) as a function of cumulative drainage. Error bars represent standard deviation of mean (n = 4). Data points for the mid-winter treatment application start at 80.5 cumulative drainage and correspond to both the maximum and first collected drainage for the mid-winter treatment application.

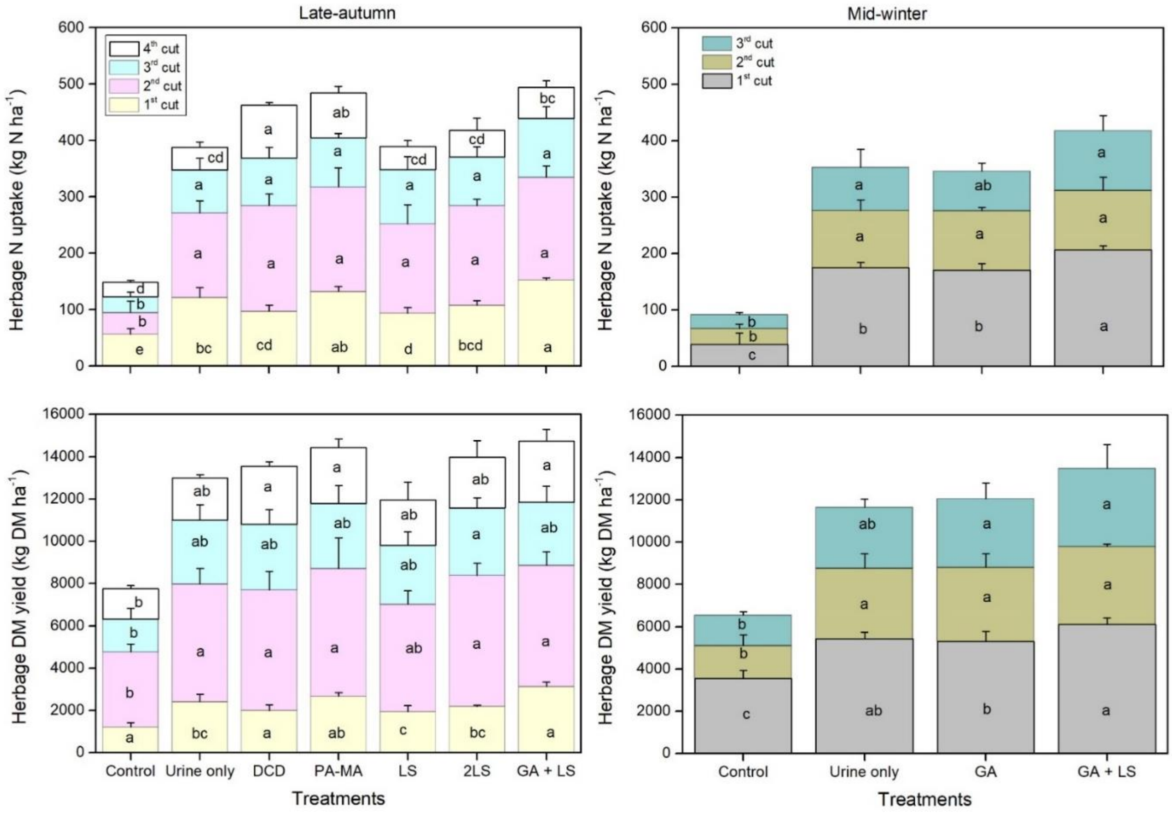


Figure A3.2 Herbage N uptake (kg N ha^{-1}) and Herbage DM yield (kg DM ha^{-1}), following late-autumn and mid-winter treatment application.

DRC 16

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Dumsane Themba Matse
Name/title of Primary Supervisor:	Dr Paramsothy Jeyakumar
In which chapter is the manuscript /published work: Chapter 3	

Please select one of the following three options:

(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Matse, D.T., Jeyakumar, P., Bishop, P., and Anderson, C. W. N., (2022). Bioavailable Copper can influence nitrification rate in New Zealand farm soils. Journal of Soils and Sediments. 22(3), 916-930. doi: 10.1007/s11368-021-03113-8.

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:
- The percentage of the manuscript/published work that was contributed by the candidate:
- Describe the contribution that the candidate has made to the manuscript/published work:

( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Dumsane Themba Matse Digitally signed by Dumsane Themba Matse Date: 2023.01.24 16:27:43 +13'00'
Date:	24-Jan-2023
Primary Supervisor's Signature:	Jeya Jeyakumar Digitally signed by Jeya Jeyakumar Date: 2023.01.24 16:13:55 +13'00'
Date:	24-Jan-2023

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Dumsane Themba Matse
Name/title of Primary Supervisor:	Dr Paramsothy Jeyakumar
In which chapter is the manuscript /published work:	Chapter 4

Please select one of the following three options:

(•) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Matse, D. T., Jeyakumar, P., Bishop, P., & Anderson, C. W. N., (2023). Copper induces nitrification by ammonia-oxidizing bacteria and archaea in pastoral soils. Journal of Environmental Quality. 52, 49-63. doi: 10.1002/jeq2.20440.

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:
- The percentage of the manuscript/published work that was contributed by the candidate:
- Describe the contribution that the candidate has made to the manuscript/published work:

( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Dumsane Themba Matse Digitally signed by Dumsane Themba Matse Date: 2023.01.24 16:27:11 +13'00'
Date:	24-Jan-2023
Primary Supervisor's Signature:	Jeya Jeyakumar Digitally signed by Jeya Jeyakumar Date: 2023.01.24 16:20:32 +13'00'
Date:	24-Jan-2023

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/publication or collected as an appendix at the end of the thesis.

DRC 16

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Dumsane Themba Matse
Name/title of Primary Supervisor:	Dr Paramsothy Jeyakumar

In which chapter is the manuscript /published work: Chapter 5

Please select one of the following three options:

(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Matse, D. T., Jeyakumar, P., Bishop, P., & Anderson, C. W. N., (2022). Nitrate leaching mitigation options in two dairy pastoral soils and climatic conditions in New Zealand. Plants. 11(8): 24-30. doi: 10.3390/plants11182430.

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:
- The percentage of the manuscript/published work that was contributed by the candidate:
- Describe the contribution that the candidate has made to the manuscript/published work:

( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Dumsane Themba Matse — Digitally signed by Dumsane Themba Matse Date: 2023.01.24 16:28:46 +13'00'
Date:	24-Jan-2023
Primary Supervisor's Signature:	Jeya Jeyakumar — Digitally signed by Jeya Jeyakumar Date: 2023.01.24 16:21:21 +13'00'
Date:	24-Jan-2023

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

DRC 16

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Dumsane Themba Matse
Name/title of Primary Supervisor:	Dr Paramsothy Jeyakumar
In which chapter is the manuscript /published work: Chapter 6	

Please select one of the following three options:

(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Matse, D. T., Jeyakumar, P., Bishop, P., & Anderson, C. W. N., (2023). Nitrification in dairy urine patches can be inhibited by changing soil bioavailable Cu concentration. Environmental Pollution. 320: 121107. doi: 10.1016/j.envpol.2023.121107

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:
- The percentage of the manuscript/published work that was contributed by the candidate:
- Describe the contribution that the candidate has made to the manuscript/published work:

( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Dumsane Themba Matse — Digitally signed by Dumsane Themba Matse Date: 2023.01.24 16:25:10 +13'00'
Date:	24-Jan-2023
Primary Supervisor's Signature:	Jeya Jeyakumar — Digitally signed by Jeya Jeyakumar Date: 2023.01.24 16:32:03 +13'00'
Date:	24-Jan-2023

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.