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Title:
Effects of urease and nitrification inhibitors to soil microbial communities and nitrogen use efficiency

Date:
2020

Persistent Link:
https://hdl.handle.net/11343/240388

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Doctor of Philosophy

March 2020

School of Agriculture and Food

Faculty of Veterinary and Agricultural Sciences

The University of Melbourne

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A thesis submitted in fulfilment of the requirement of the degree

of

Doctor of Philosophy

March 2020

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Abstract

Soil microbial organisms are involved in many soil processes including nutrient cycling, impacting on soil quality and health. Anthropogenic activities such as nutrient addition influence soil ecosystems and soil environment. It is widely known that the contribution of urease inhibitors (UI) e.g. *N*-(*n*-butyl) thiophosphoric triamide (NBPT) and nitrification inhibitors (NIs) e.g. 3, 4-dimethylpyrazole phosphate (DMPP) in reducing N losses is associated with urea hydrolysis (and ammonia volatilization) and ammonia oxidization processes, respectively. However, little attention has been given to the understanding of the effect of NBPT on nitrification and urease producing microbes. Further, although the effects of NIs on ammonia oxidizers have been studied, there is limited information on how they influence non-targeted microbes or the newly discovered complete ammonia oxidizers (comammox *Nitrospira*). Some studies have reported that targeting mitigation of N losses through one pathway of the nitrogen cycle may lead to losses in another pathway. Therefore, the use of a combination of mitigation measures was suggested including combining UIs and NIs. Limited bio-molecular studies have investigated the effect of combined UIs and NIs on the soil N cycling microbes. In an incubation study on five soils from different parts of Victoria, Australia, using the terminal restriction fragment length polymorphisms (T-RFLP) and quantitative polymerase chain reaction (qPCR) techniques, we reported that the abundances of ammonia-oxidizing bacteria (AOB) and complete ammonia oxidizers (comammox *Nitrospira*), but not ammonia-oxidizing archaea (AOA), were significantly influenced by the application of NBPT, DMPP, and DMPP + NBPT. The structures and community composition of both AOA and AOB were influenced by NBPT, DMPP, or their combination. AOA, AOB, and comammox *Nitrospira* clade B might be significant contributors to

nitrification in the studied soils. However, the contribution and responses of these microbes to nutrients, UI, or NI could be controlled by soil properties like soil pH. This study for the first time provided some new knowledge about ureolytic microbes and complete ammonia oxidizers (comammox *Nitrospira*) as influenced by NBPT and DMPP. However, there is a need for more information on how to develop a dual inhibitor compound whose individual compounds will work together effectively to target both hydrolysis and nitrification processes. It is also important to understand how *comammox Nitrospira* responds to different UI and NIs or their combinations, and the suitable rates of application of the UIs, NIs or their combinations for effective inhibition of *comammox Nitrospira.*

Further, more studies have concentrated on understanding the molecular mechanisms of inhibition of ammonia oxidation by the NIs in short-term laboratory and field experiments. However, little is known about the effect of DMPP on soil enzyme activities, N cycling microbes (involved in nitrification and denitrification), or non-targeted microbes in soil following a production period in repeated chemical fertilizer and NI application regimes. Such a study has great implications for soil quality and health. Microbial communities were analyzed using Illumina sequencing and qPCR, from soil sampled 1 week after harvesting from a 4.5-year field experiment with repeated application of urea (U), urea + DMPP (UE) at 40, 80 and 120 kg N ha^{-1}. This analysis revealed that the AOB gene abundance increased as the N application rate increased (from 0 to 120 kg N ha^{-1}). The use of DMPP significantly reduced AOB and *nirK* gene copy numbers compared to urea alone at an application rate of 120 kg N ha^{-1}. There was no effect on the abundance of either ammonia-oxidizing archaea (AOA), comammox *Nitrospira* clade A and B, *nosZ,* or bacterial *16S rRNA* genes. The community composition of AOB and AOA changed with N addition and use of DMPP while increasing the N application rate only changed the composition of AOB. Potential

nitrification rates increased after the addition of N at 80 and 120 kg N ha^{-1}. There was no significant treatment effect on the relative abundance of total bacteria at the phylum level, indicating no residual effects of urea and DMPP at different rates on the non-targeted microbes. This experiment demonstrated that the application of N (with or without DMPP) at lower than 120 kg N ha^{-1} would not result in a significant impact on soil archaeal or bacterial ecology.

Repeated application or overuse of chemical fertilizer can lead to environmental issues like soil acidification. The temporal effects of NBPT on soil ureolytic and ammonia-oxidizing microbes, crop yield, and nitrogen use efficiency (NUE) following repeated applications are not well understood. A perennial ryegrass (*Lolium perenne* L.) experimental site, received fertilizer treatments of urea applied alone at 40 kg N ha^{-1} and 80 kg N ha^{-1} or urea applied with NBPT (as Green Urea NV® at 40 kg N ha^{-1}) in respective plots that had received the same treatments since 2014. The qPCR analysis of soil samples collected on a weekly basis for 45 days confirmed that the abundance of ureolytic microbes was higher in control (CK) compared to all N treatments on all sampling days, which was associated with changes in soil pH. Despite the reduced soil pH following repeated applications of urea with NBPT, *ureC* gene copy numbers were significantly reduced in the NBPT treatment plots applied at 40 kg N ha^{-1} compared to those in urea alone applied at the same rate. NBPT had no significant effect on the abundance of ammonia oxidizers. However, increasing the urea application rate significantly increased the abundance of ammonia-oxidizing bacteria (AOB) and complete ammonia oxidizers (comammox *Nitrospira* clade B). NBPT had no significant effect on pasture dry matter (DM) yield, N-uptake, or NUE. Increasing N application rate significantly increased pasture DM yield and N- uptake but this did not influence the pasture NUE.

From the two field experiments, this thesis confirmed that repeated application of urea with NBPT or DMPP led to changes in soil physiochemical properties which included decreasing soil pH, and this controlled the response of soil microbes to chemical inhibitor applications. In both experiments with repeated applications, it was confirmed that AOB could be major players to nitrification in acidic soils with repeated chemical fertilizer applications with UI or NI. Future work should consider understanding the interaction between plants and soil microbial communities especially around the rhizosphere and how these may influence the efficacy of UI and NI on a short and long-term basis. Also, there is a need in the future to investigate how the inhibitor compounds and enzyme activities in different soils change following the application of these inhibitor compounds.

Keywords: ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), *N*-(*n*-butyl) thiophosphoric triamide (NBPT), 3, 4-dimethylpyrazole phosphate (DMPP), comammox *Nitrospira*, urea hydrolysis, ureolytic microbes, nitrification, nitrifying microbes, nitrogen use efficiency (NUE).

Declaration

I declare that

i) This thesis consists of only my original work towards the PhD except where indicated in the preface;

ii) due acknowledgment has been made in the text to all other material used; and

iii) the thesis has less than 80,000 words exclusive of tables, maps, bibliographies, and appendices

Aineah Obed Luchibia

13th March 2020

Preface

This PhD thesis is my original work and has six chapters: introduction, literature review, followed by three experimental chapters and a general discussion and conclusions chapter. Two of the three experimental chapters have been published and are in the format of the respective journals. The other experimental chapter has been submitted to the journal for publication and has also been presented in the format of the journal. Aineah Obed Luchibia is the first author of all the experimental chapters. The co-authors included Helen Suter, Shu Kee Lam, Hang-Wei Hu, Qinglin Chen, Bede O'Mara, Lee Menhenett, and Ji-Zheng He. Aineah Obed Luchibia was responsible for identifying the research gaps, designing the experiment, collecting and analyzing the data, and writing this thesis. The co-authors supervised the different stages of this PhD research and contributed in data analyses and improving the manuscripts. The experimental chapters of this thesis include:-

Chapter 3:

Luchibia AO, Suter H, Hu H-W, Lam SK, He J-Z (2020): Responses of ureolytic and nitrifying microbes to urease and nitrification inhibitors in selected agricultural soils in Victoria, Australia. Journal of Soils and Sediments 20, 1309-1322

Chapter 4: Luchibia AO, Lam SK, Suter H, Chen Q, O'Mara B, He J-Z (2020): Effects of repeated applications of urea with DMPP on ammonia oxidizers, denitrifiers, and non-targeted microbial communities of agricultural soil in Queensland, Australia. Applied Soil Ecology 147, 103392

Chapter 5

Luchibia AO, Suter H, Lam SK, Menhenett L, He J-Z (2020). Temporal response of ureolytic, nitrifying microbes, and pasture yield to urea and NBPT in Ballarat Victoria, Australia. Applied Soil Ecology (under review)

Acknowledgements

This PhD project was funded by the Australian Research Council (ARC-LP160101134). First and foremost, I would like to thank Prof Jim He for allowing me to pursue my PhD in his lab under his supervision at the University of Melbourne. It's through this opportunity that I managed to benefit from the Melbourne International Research and Melbourne International Fee Remission Scholarships which supported my PhD studies. Without these scholarships, I would not have been able to pursue my PhD and I am therefore very grateful.

I sincerely wish to thank my PhD supervisors, Professor Ji-Zheng (Jim) He, A/Professor Helen Suter, and Dr. Shu Kee Lam for their continuous guidance and feedback during my entire PhD candidature.

I would like to thank Dr. Hang-Wei Hu and Dr. Xiuzhen Shi and Dr. Qinglin Chen for their support and guidance during my molecular work experiments. Prof. Deli Chen, the chair of my advisory committee and Dr. Hang-Wei Hu a member of the advisory committee were always available to listen, guide, and support me during my PhD candidature and I am very grateful for their sacrifice and support.

I would like to thank the members of the soils group for their support and company: Dr. Yu Jing Zhang, Dr. Hang Gao, Dr. Bhawana Bhatta, and Dr. Dona Thushari Wijesinghe: Mr. Michael Hall and Najib (TrACEES) for the analysis of my samples. I am also grateful for the good company and moral support from my friends Mr. Robert Impraim and Mr. MD. Shahinur Rahman, Mr. Eric Ireland, and Dr. Arjun, and Mr. Jose Valentin Palacios Zevallos.

I am also grateful to the staff of Incitec Pivot, particularly Mr. Bede O'Mara, and Lee Menhenett for allowing us to use soil samples from their long-term field experiments for the molecular studies of this PhD and for the support they offered whenever I requested.

Special thanks to Mr. Robert Impraim, Mr. Jose Valentin Palacios Zevallos, and Dr. Xiufang Gao for their assistance during field sampling.

I would like to thank Dr. (Eng). James Messo Raude for being a good friend and a mentor to guide and support.

I dedicate this thesis to my dear late mother Jenifer Ayuma Blasio, for her struggles and sacrifice throughout my life and her guidance and discipline which enabled me to reach this point in my academic life. My late younger brother Cpl. Stanley Ben Ambokah was always supportive to me during my studies and I will always be grateful for his support and love.

I wish to thank my wife Jael Kayosa Akhaya and my children for their sacrifice and support to me during my studies.

Finally, I thank God for the gift of life and the ability to reach this far.

Table of Contents

List of tables

Chapter 2.

Chapter 3.

Chapter 4

Chapter 5.

List of figures

Chapter 2

Chapter 3.

List of figures in supplementary materials

Chapter 3.

Chapter 1: Introduction

1.1. Background information

Urea nitrogen (N) is the most preferred form of N in agriculture globally (Khan et al. 2013, Modolo et al. 2015). However, once applied to the soil, it undergoes microbial mediated transformations like hydrolysis, nitrification, and denitrification, which are characterized by leakages, reduced N retention and use by the crop (Rosmarina et al. 2016). This occurs through gas losses via ammonia (NH_3) volatilization (during hydrolysis), nitric oxide (NO), and nitrous oxide (N_2O) emission from both nitrification and denitrification apart from the nitrate (NO_3) leaching and surface run-off (Cameron et al. 2013, Chen et al. 2008, Pan et al. 2016). Such losses have economic (due to lower N retention and use by the crop) and environmental implications.

One of the approaches to reduce these N losses and enhance crop N uptake and nitrogen use efficiency (NUE) is the use of fertilizers coated with urease inhibitors (UIs) or nitrification inhibitors (NIs) which work by reducing the activity of ureolytic microbes and ammonia oxidizers respectively (Akiyama et al. 2010, Cameron et al. 2013). While *N*-(*n*-butyl) thiophosphoric triamide (NBPT) is the only commercially available and efficient UI (Cantarella et al. 2018, Trenkel 2010), several NIs are available for commercial use including dicyandiamide (DCD or cyanoguanidine), Nitrapyrin (2-chloro-6-[trichloromethyl] pyridine), and 3, 4-dimethylpyrazole phosphate (DMPP), (Doran et al. 2018, Franzen & Committee 2017, Zaman et al. 2008). However, DMPP could offer some advantages of longer persistence in soil compared to other NIs. A recent review and a meta-analysis showed the ability of the urease inhibitor NBPT to reduce soil NH_3 emissions due to delaying urea hydrolysis (Cantarella et al. 2018, Silva et al. 2017). Moreover, because NBPT inhibits the urea hydrolysis process, it is likely to influence ammonia oxidation and

subsequent denitrification process (Ding et al. 2011), by controlling the rate of substrate availability. For example, some studies have shown that NBPT could significantly reduce N_2O and NO emission (Sanz Cobena et al. 2012, Thapa et al. 2016), an indication of its influence on the nitrification and denitrification processes. Despite the quantified benefits of NBPT in reducing N losses in some cases, limited studies have investigated the effect of NBPT on the communities of ureolytic microbes which are associated with urea hydrolysis and NH_3 losses (Fan et al. 2018). Further, some ammonia oxidizers have also been reported to possess the urease enzyme and use urea directly as an energy source. This would then imply that NBPT could influence these ureolytic ammonia oxidizers directly by inhibiting their intracellular urease. However, the molecular mechanisms involving urea hydrolysis and its inhibition by NBPT or the influence of NBPT on nitrification and ammonia oxidizers are not well understood. Few studies have investigated the effect of NBPT on ammonia oxidizers (Fan et al. 2018, Shi et al. 2017, Xi et al. 2017). Therefore, there is a need for further investigation to understand the mechanism of NBPT effects on ammonia oxidizers.

NIs inhibit the ammonia oxidation process and have been reported to significantly reduce N_2O and NO emission (Akiyama et al. 2010, Gilsanz et al. 2016, Lam et al. 2017). However, the use of NIs could increase NH_3 losses if used alone due to prolonged retention of NH_4^+ in soil (e.g. Kim et al. 2012, Lam et al. 2017), reducing their anticipated benefits. Therefore, the approaches that target multiple pathways have been suggested to effectively control N losses (Drury et al. 2017, Lam et al. 2017), e.g. combining UI and NI. In line with this suggestion, several studies have been conducted to investigate the effects of UI + NI on NH_3 and N_2O emissions albeit with inconsistent findings on the efficacy of the individual inhibitor compounds compared to when they are combined (e.g. Zhao et al. 2017, Sanz Cobena et al. 2012, Frame 2017, Pereira et al. 2013, Soares

et al. 2012, Ding et al. 2011). Therefore, there is no clear understanding on how the combined UI+NI influences the N-dynamics once applied with urea on soil and there is a need for further investigation on this aspect. Moreover, the effects of such combinations on soil N cycling microbial communities involved in hydrolysis and ammonia oxidation have received little attention, and more studies are required (Dong et al. 2018).

Previously, studies have investigated the effects of NIs on ammonia oxidizers with emphasis on ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) microbial communities (Chen et al. 2015, Shi et al. 2016). Recently, complete ammonia oxidation within a single organism has been shown to be carried out by the complete ammonia oxidizers (comammox *Nitrospira*) (Daims et al. 2015, van Kessel et al. 2015). There is still a need to understand the contribution of these microbes to nitrification, and how they respond to the application of NIs like DMPP and UIs like NBPT.

Some production systems such as pasture production receive N input throughout the whole year (Abalos et al. 2014). However, concerns of long-term/ repeated effects of N application on soil health have been raised (Hartmann et al. 2015). These concerns include changes in soil chemical properties like soil pH. Such changes in soil chemical properties could influence soil microbial community (e.g. Geisseler & Scow 2014, Zhou et al. 2015), or determine the extent of inhibition and contribution of UI or NIs to reducing N losses in subsequent seasons when used repeatedly. It is unclear how the repeated use of urea with UIs and NIs affects the soil environment and the ecosystem. Several studies have investigated the effects of repeated application of N fertilizer on soil microbial communities (Chen et al. 2016, Hartmann et al. 2015, Wang et al. 2019, Ye et al. 2019). However, the effects of repeated and long-term use of UIs and NIs on N cycling microbes involved in nitrification, denitrification together with non-targeted microbial communities have

rarely been studied, which justifies further investigation, especially at the end of a cropping season when these compounds have been used (Shi et al. 2017). Such a study would highlight any potential effects of repeated use of the UI and NI on the soil environment and soil health.

1.2. Research aims, questions, and hypothesis

The aims of this thesis study were:

i. To understand the responses of ureolytic and nitrifying microbes to urease and nitrification inhibitors;

ii. To determine the effects of repeated application of urea with DMPP, at different application rates, on soil N-cycling and non-targeted microbial communities; and

iii. To quantify temporal responses of soil microbial communities and pasture yield to repeated use of urea and NBPT

The thesis answered the following three scientific questions to achieve each of the above aims respectively:

1) What is the response of ureolytic and nitrifying microbes to the application of the urease and nitrification inhibitor in selected agricultural soils?

   The following hypotheses were tested to answer this research question

   i. Use of NBPT and DMPP will reduce the abundance of N cycling microbes in soil and alter the microbial community composition

   ii. The performance of these chemicals will vary with edaphic conditions.

2) How does the repeated application of urea with DMPP at different N application rates influence soil physiochemical properties, the N-cycling and non-targeted microbial communities?

The following hypotheses were tested to answer this research question: -

i. Repeated applications of urea and DMPP will significantly reduce soil pH and increase soil total carbon (TC), total nitrogen (TN).

ii. The levels increased soil TC, TN, and reduced pH will decrease soil total bacterial composition and increase gene copy numbers of soil microbial communities.

3) How does the repeated applications of urea alone at different N application rates or with NBPT influence soil microbial communities, and pasture yield?

The following hypotheses were tested to answer this research question: -

i. The application of NBPT will reduce ureolytic and nitrifying microbes.

ii. Use of NBPT will improve pasture dry matter (DM) yield, N uptake and NUE due to its effects of delaying urea hydrolysis and therefore reducing N losses

iii. Increasing the N application rate will increase pasture DM yield, the abundance of *ureC*, and AOB genes but reduce the abundance of comammox *Nitrospira.*

1.3. **Thesis outline**

This thesis is with publication and contains six chapters with three experimental chapters that were aimed to address the above scientific questions. Two of the experimental chapters have been published and another one has been submitted to the journal for publication.

Chapter 1. Introduction: This chapter includes background information, knowledge gaps, research aims, questions, hypotheses, thesis outline, and the significance of the study.

Chapter 2. Literature review: This chapter is a review of the literature on the responses of soil N cycling microbial communities and crop yield to the use of urease and nitrification inhibitors.

Chapter 3. Responses of ureolytic and nitrifying microbes to urease and nitrification inhibitors: This is an experimental chapter reporting the results of a microcosm incubation experiment that was established using five soils from different agricultural farms in Victoria Australia to address the first research question of this thesis. This study was established with the following treatments: urea (U), U + NBPT (UI), U + DMPP (NI), U + NBPT + DMPP treatments. Urea was applied at 200mg kg^{-1} soil, while DMPP and NBPT were applied at 0.1 % N and 0.5 % N respectively. During the 28-day incubation period, soil sampling was done to measure mineral N dynamics, gene abundance, and community structure of ureolytic and ammonia oxidizers. The study demonstrated that the initial soil pH and organic carbon had the greatest influence on the effectiveness of both NBPT and DMPP inhibitors and that ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB) and complete ammonia oxidizers (comammox *Nitrospira*) clade B were significant contributors to nitrification. The abundance of AOB and comammox *Nitrospira*, rather than that of AOA, were reported to be significantly influenced by the application of the urease inhibitor *N*-(*n*-butyl) thiophosphoric triamide (NBPT) and the nitrification inhibitor 3, 4-dimethylpyrazole phosphate (DMPP). This study highlighted that the contribution of NBPT and DMPP to N fertilizer efficiency was attributed to their inhibition of the growth of AOB and comammox organisms in the tested soils. The study further highlighted a reduction in the *ureC* gene copy numbers in NBPT and DMPP treatments towards the end of the incubation period in Dookie wheat (DW) and Clyde vegetable (CV) soils and associated the findings with the reduction in soil pH resulting from the use of these inhibitors. This chapter has been published.

Luchibia AO, Suter H, Hu H-W, Lam SK, He J-Z (2020): Responses of ureolytic and nitrifying microbes to urease and nitrification inhibitors in selected agricultural soils in Victoria, Australia. Journal of Soils and Sediments 20, 1309-1322

Chapter 4. Effects of repeated applications of urea with DMPP on soil microbial communities. This is an experimental chapter reporting the results of the effects of repeated applications urea with DMPP at different N rates on soil properties and soil microbial communities of soil from a field trial site in Queensland Australia. The sampling and analysis were conducted to address the second research question of this thesis. Soil sampling was done one week after crop harvesting from a 4.5-year field experiment with plots under crop rotation with repeated applications of seven treatments, namely control (CK), Urea (U), Urea + DMPP (UE) applied at 40, 80 and 120 kg N ha^{-1}, each treatment with three replicates. Analysis of soil physiochemical properties; gene abundance of ammonia oxidizers denitrifiers, and total bacteria; and Illumina MiSeq sequencing of the 16S rRNA gene were performed. The study demonstrated that soil pH was significantly reduced with increasing application of urea and urea + DMPP. The abundance of ammonia-oxidizing bacteria (AOB) increased as the N application rate increased (from 0 to 120 kg N ha^{-1}). Use of DMPP significantly reduced AOB and *nirK* gene copy numbers compared to urea alone at an application rate of 120 kg N ha^{-1} while no treatment effect was reported on the abundance of ammonia-oxidizing archaea (AOA), comammox *Nitrospira* clade A and B, *nosZ* and bacterial *16S rRNA* genes. The community composition of AOB and AOA changed with N addition and use of DMPP but increasing N addition rate changed the composition of AOB only. Addition of N increased potential nitrification rates at 80 and 120 kg N ha^{-1}. There was no significant treatment effect on the relative abundance of bacteria at the phylum level. This

experiment demonstrated that repeated application of DMPP at lower N rates can be done without significant impacts on soil microbial ecology. This chapter has been published.

Luchibia AO, Lam SK, Suter H, Chen Q, O'Mara B, He J-Z (2020): Effects of repeated applications of urea with DMPP on ammonia oxidizers, denitrifiers, and non-targeted microbial communities of agricultural soil in Queensland, Australia. Applied Soil Ecology 147, 103392

Chapter 5. Temporal response of soil microbial communities and pasture yield to urea and NBPT: This is an experimental chapter reporting the results of a field experiment that was established by the application of urea alone at 40 and 80 kg N ha^{-1} (40U and 80 U) respectively or urea with NBPT at 40 kg N ha^{-1} as green urea (40 GU), in pasture plots that had received the same treatments on a repeated basis since 5th October 2014 at Leigh Creek Victoria. The analysis was done on soil sampled from day 0 to day 45 on 7-day intervals and plant samples collected once at pasture harvesting time 45 days after treatment application, to address the third research question of this thesis. Measurements included soil pH, pasture DM yield, N uptake, and NUE gene abundance of ureolytic and ammonia oxidizers.

The study reported that NBPT significantly reduced *ureC* gene copy numbers compared to urea applied alone at 40kg N ha^{-1} on sampling days 7 and 45. NBPT had no significant effect on the abundance of ammonia oxidizers but increasing N application rate significantly increased the abundance of ammonia-oxidizing bacteria (AOB) on days 7 and 45, and complete ammonia oxidizers (comammox *Nitrospira* clade B) on day 7 compared to urea applied at 40 kg N ha^{-1}. The use of NBPT with urea had no significant effect on pasture DM, N uptake, or NUE compared to urea alone. Increasing the N application rate increased pasture DM yield but not N-uptake or NUE. The experiment demonstrated that AOB were the main contributors to nitrification in this

experimental site as compared to AOA, and comammox *Nitrospira* and could be important players to nitrification in acidic soils or soils with a history of repeated applications of urea with NBPT.

This chapter has been submitted for publication to the Journal of Applied Soil Ecology.
Luchibia AO, Suter H, Lam SK, Menhenett L, He J-Z (2020). Temporal response of ureolytic, nitrifying microbes, and pasture yield to urea and NBPT in Ballarat Victoria, Australia. Under review

Chapter 6. General discussion, perspectives, and conclusions: This chapter presents the synthesis of the findings from Chapters 3, 4, and 5, and discusses the implications of the research outcomes.

1.4. **The significance of the study**

The results of this research will contribute to the knowledge of understanding how the soil N-cycling microbial communities contribute to hydrolysis and nitrification processes. Such information is relevant to understand how to manipulate soil N cycling microbial communities to minimize N losses and improve the crop N utility. Specific new knowledge contributed by this study was the investigation into ureolytic microbes (encoded by *ureC* genes) and complete ammonia oxidizers (comammox *Nitrospira*) as influenced by NBPT and DMPP. Previous studies concentrated on investigating the effects of NIs on AOA and AOB. This study also contributed to the understanding of the molecular mechanism of NBPT effects on hydrolysis and nitrification, an area that has received little attention. Studies have previously concentrated on investigating the effects of NIs but not UIs on N cycling microbial communities.

The information from chapters 4 and 5 of this research involved the understanding of the effects of repeated field applications of NBPT and DMPP on soil microbial community structure, size,

and function. This information is key to understand how such anthropogenic activities may change soil properties such as pH, total C, total N, NH_4^+−N, and NO_3^-−N, which in turn influence the function and structure of soil microbial communities that are key to nutrient cycling and soil functions. This is valuable knowledge to soil health contributed by this research as soil biodiversity is key to healthy soils.

This study, therefore, has economic and environmental benefits from maintaining soil biodiversity to controlling the rates of N cycling processes leading to reduced N leakage to the environment. The saved N may then be utilized by the crop to improve productivity and the farmer's income.

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Chapter 2. Literature review

2.1. **World population and fertilizer use**

The world population keeps increasing and is projected to be >9 billion in 2050 (Desa 2009, 2015, Glenn et al. 2007).To keep up with this population increase, food production is expected to increase by 70 % by the same time (Glenn et al. 2010, Glenn et al. 2011). This increased food production is expected from the limited and decreasing arable land resource (Malhi et al. 2001, Roberts 2009). Therefore, the use of modern technologies like high-yielding crop varieties, pesticides, irrigation, mechanization, plant breeding, and chemical fertilizers among others (Erisman et al. 2008, Matson et al. 1997) is key to ensuring adequate food productivity.

Global fertilizer consumption has risen from 168.4 Mt in 2007/08 (100.8 Mt N, 38.5 Mt P_2O_5, and 29.1 Mt K_2O) (Heffer & Prud'homme 2013) to 191.5 Mt in 2017/18(107.9 Mt N, 46.7 Mt P_2O_5, and 36.9 Mt K_2O) (IFA2019). The world fertilizer demand is estimated to reach 203.6 Mt (114.6 Mt N, 49.6 Mt P_2O_5, and 39.4 Mt K_2O) in 2023/24, (IFA 2019) (Table 2.1). Of all the nutrients, nitrogen (N) consumption is highest (Table 2.1), because N is the most limiting crop nutrient required in large quantities (Grant et al. 2012, Stark & Richards 2008).

Table 2.1. Global fertilizer consumption (Mt)

Year	N (Mt)	P_2O_5 (Mt)	K_2O (Mt)	Total globally (Mt)	Reference
2007/08	100.8	38.5	29.1	168.4	Heffer & Prud'homme 2013
2008/09	98.3	33.9	23.1	155.3	
2009/10	102.2	37.6	23.6	163.5	
2010/11	104.2	40.6	27.5	172.2	
2011/12	107.8	40.6	27.7	176.1	
2012/13	108.6	41.4	29.2	179.1	Heffer & Prud'homme 2015a
2013/14	109.9	40.5	30.4	180.7	
2014/15	111.8	41.3	31.5	184.6	Heffer & Prud'homme 2015b
2016/17	107.7	46.0	35.3	189.1	IFA 2019
2017/18	107.9	46.7	36.9	191.5	
2018/19(forecast)	119.2	45.7	35.3	200.2	FAO 2015, Heffer & Prud'homme 2015b
2023/24(forecast)	114.6	49.6	39.4	203.6	IFA 2019

Urea is the most used form of N in agriculture globally (Khan et al. 2013, Modolo et al. 2015), accounting for 50% of the world N consumption (Sanz-Cobena et al. 2008, Zaman et al. 2012). Furthermore, urea use has been increasing in the last 4 decades, a situation that is expected to continue (Kiss & Simihaian 2013).

The rapid adoption of the use of urea is because of its high (46%) N content, low cost compared to other N sources, high solubility in water, ease of handling, transport and storage safety, suitability for production of compound fertilizers, ability to be applied in solid or in liquid forms, or as a foliar spray in combination with many compatible pesticides (Chen et al. 2008, Khan et al. 2014, Kiss & Simihaian 2013).

2.2. **Fertilizer N –Transformations and associated effects.**

i. Hydrolysis

Urea applied to soil as fertilizer or via animal urine deposition is hydrolyzed rapidly within the initial days of application to ammonium (NH_4^+) and ammonia (NH_3), hydroxyl (OH^-), and carbonate (CO_2) ions by the urease enzyme (equation 1).

$$(NH_2)_2CO + 2H_2O \xrightarrow{\text{Urease}} (NH_4)_2CO_3 \rightarrow NH_4^+ + NH_3(gas) + CO_2 + OH^- \quad (1)$$

The hydrolysis process leads to an increase in NH_4^+ and NH_3 and a rise in pH around the reaction site due to OH^- and this could increase the loss of N through NH_3 volatilization depending on the pH change (Singh et al. 2008a). Losses of applied N due to ammonia volatilization in the range of 20-30% from the pasture system (emissions from urea fertilizer and urine deposition) (Laubach et al. 2013, Laubach et al. 2012, Schwenke et al. 2014, Suter et al. 2013), 4.8-10% from the cropping system (Schwenke et al. 2014, Turner et al. 2012) and 24-32 % in flooded rice production systems (Griggs et al. 2007, Norman et al. 2009, Shang et al. 2014) have been reported.

Volatilization losses could generally be realized from urea fertilizers, ammoniacal fertilizers, animal urine, manure, ammoniacal wastes, and mineralization of organic matter or plant residues (Cameron et al. 2013, Laubach et al. 2013). Besides causing a great economic loss to the farmer, NH_3 emissions have a negative impact on the environment (Chen et al. 2008, Tian & Niu 2015). For instance, NH_3 is an atmospheric pollutant since it contributes to the formation of fine particulate matter (aerosols) and ground-level ozone which may impact human health negatively (Bittman & Mikkelsen 2009, Congreves et al. 2016). The NH_3 emitted to the environment may subsequently be deposited on land or water contributing to soil acidification, eutrophication of water bodies (Bittman & Mikkelsen 2009, Cameron et al. 2013, Sutton et al. 2008), and increased N_2O emission through subsequent nitrification and denitrification (Lam et al. 2017, Rosmarina et al. 2016).

ii. Nitrification and denitrification

Nitrification involves the microbial oxidation of ammonia (NH_3) to nitrate (NO_3^-) by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (He et al. 2007, Subbarao et al. 2013), through a series of steps (equation 2). The first step involves the NH_4^+ oxidation to

hydroxylamine (NH_2OH) catalyzed by the enzyme ammonia monooxygenase (AMO) (equation 2) encoded by the *amoA* gene found in both AOA and AOB (Ensign et al. 1993, He et al. 2012a). The AMO is a membrane-bound enzyme containing copper (Arp et al. 2002, McCarty 1999).

$$NH_3 \xrightarrow[Monooxygenase]{Amonia} NH_2OH \xrightarrow[Oxidoreductase]{Hydroxylamine} [NHO] \xrightarrow[H_2O]{} NO_2 \xrightarrow[H_2O]{} NO_3 \quad (2)$$

The second step involves the oxidation of NO_2^- to NO_3^- using the nitric oxide reductase (NOR) (Ruser & Schulz 2015). Complete NH_3 oxidization to NO_3^- by the comammox *Nitrospira*, which can carry out the oxidation of NH_3 to NH_2OH and NO_2 to NO_3 within the same cell could also occur (Daims et al. 2015, van Kessel et al. 2015).

During nitrification, direct N losses via nitric oxide (NO) and nitrous oxide (N_2O) emissions have been associated with both AOA and AOB, by nitrifier denitrification (e.g. Gilsanz et al. 2016, Stieglmeier et al. 2014, Wrage-Mönnig et al. 2018, Wrage et al. 2001). The incomplete NH_2OH oxidation could also produce NO and N_2O under conditions of low oxygen availability (e.g. Caranto & Lancaster 2017, Stein 2011).

The NO_3^- form of N is more mobile compared to NH_4^+ and therefore more prone to leaching (Liu et al. 2015, Maqsood et al. 2016), posing a threat of contaminating the ground and surface waters which has become an important global environmental concern due to increasing N fertilizer application (Di & Cameron 2002b, a). This is because the leached NO_3^- leads to excessive growth of aquatic plants and algae, leading to eutrophication in surface waters, and/or to human and animal health problems in underground and drinking water like methemoglobinemia (reduced oxygen-carrying capacity syndrome of the hemoglobin) when ingested, and cancer in the gut of mammals (Camargo et al. 2005, Jadhao 2013).

Further N-transformation involves the reduction of the NO_3^- in the absence of oxygen to NO_2^-, NO, N_2O and dinitrogen (N_2), (equation 3), catalyzed by the nitrate-, nitrite-, nitric oxide-, and nitrous oxide-reductase enzymes and encoded by the *napA*/*narG*, *nirK*/*nirS*, *norB*, and *nosZ* genes respectively, with N_2O as an intermediate product through the process of denitrification (Benckiser et al. 2013, Di & Cameron 2016, Hallin et al. 2018, Junejo et al. 2013).

$$NO_3^- \xrightarrow{\substack{nitrate \\ reductase}} NO_2^- \xrightarrow{\substack{nitrite \\ reductase}} NO \xrightarrow{\substack{nitric\ oxide \\ reductase}} N_2O \xrightarrow{\substack{nitrous\ oxide \\ reductase}} N_2 \qquad (3)$$

Therefore, nitrification and denitrification are biological sources of N_2O (Bateman et al. 2004, Butterbach Bahl et al. 2013). Nitrous oxide is a greenhouse gas (GHG) about 310 times more potent than carbon dioxide, with an estimated lifetime of 120 years and is involved in the depletion of the ozone layer (Davidson & Kanter 2014, IPCC 2014, Portmann et al. 2012, Ravishankara et al. 2009, Singh et al. 2008a).

Apart from substrate availability, water-filled pore space (WFPS) is a controlling factor to N_2O emission which determines oxygen availability thereby affecting the microbial nitrification and denitrification processes (Akiyama et al. 2020, Butterbach Bahl et al. 2013, Dobbie & Smith 2003). Nitrous oxide production from nitrification occurs when the water-filled pore space WFPS is in the range of 50- 60%) and adequate air while N_2O from denitrification occurs at WFPS of >60 %, (Bateman & Baggs 2005, Liu et al. 2016, Zaman et al. 2009). Other indirect sources of N_2O include denitrification of the leached nitrate, and the subsequent nitrification and denitrification of emitted and redeposited NH_3 (Di & Cameron 2012, Lam et al. 2017, Rosmarina et al. 2016).

Globally, agriculture is the highest source of N_2O, accounting for about 60 % of total gross anthropogenic emissions (Davidson & Kanter 2014, Reay et al. 2012, Syakila & Kroeze 2011).

Nitric oxide also accounts for further nutrient losses, although generally small amounts are emitted from the soil but still, with significant environmental impacts (Cameron et al. 2013).

The estimated total annual denitrification losses from the global agriculture are in the range of 10% to 33% of applied N (Aulakh et al. 2001, Buresh et al. 2008, Hofstra & Bouwman 2005).

Because of these losses, plant agronomic N use efficiency (NUE) (kg yield per kg of applied N in soil) is low (Khan et al. 2014, Zaman et al. 2010) rarely exceeding 50% (Abalos et al. 2014, Chen et al. 2008, Huddell et al. 2020, Zhaohui et al. 2012). Because these N leakages account for environmental degradation and the threat to global biodiversity (e.g. De Klein & Eckard 2008, Hartmann et al. 2015, Huddell et al. 2020) there is an increasing concern of intensification of agricultural productivity. Therefore, intensification of agricultural production is occurring at the same time as increasing awareness of the environmental impacts of for example chemical fertilizer use (e.g. Johnston & Bruulsema 2014). N should be used more efficiently.

2.3. **Mitigation of N losses and improvement of NUE**

Nitrogen use efficiency of crops could be improved and N-emissions reduced by the use of the best management practices (BMPs) including right rate, right source, right place, the right time (4Rs), (Ghosh et al. 2015, Harold F. Reetz 2016, IPNI 2016). The right rate involves supplying the required quantity of nutrients to the crop, but it requires soil testing which requires facilities that may not be available or accessible everywhere (Ghosh et al. 2015). The right source involves utilizing formulations that would improve NUE and reduce environmental consequences by considering the crop needs and the soil properties (Harold F. Reetz 2016, Johnston & Bruulsema 2014, Johnston & Bruulsema 2014). For example, the use of slow-release fertilizers, (which include polymer and sulfur coated fertilizers), or urease inhibitors (UIs) and nitrification inhibitors (NIs) (Ghosh et al. 2015, Trenkel 2010). However, most of the slow-release fertilizers are

expensive and may limit the growth of the fast-growing crops by restricting nutrient availability (Ghosh et al. 2015, Wade 2010). The right place refers to where the nutrient is placed which depends on crop type and soil conditions, e.g. banding (Ghosh et al. 2015, Grant & Brandon 2004, Grant et al. 1996). However, banding may lead to an increased cost of fertilizer application, soil disturbance in reduced tillage systems, and a possibility of moisture loss and reduced seedbed quality and may not be applicable in all production systems (Grant & Brandon 2004, Grant et al. 1996, Wade 2010). Right time involves matching nutrient supply with the crop requirement (Johnston & Bruulsema 2014). This would involve the use of technology that allows prior determination of the nutrient requirement of the crop (Johnston & Bruulsema 2014).

UIs and NIs seem to be more efficient as they simplify and reduce the number of required fertilizer applications, which allows flexibility in the timing of fertilizer applications, saving time, fuel and labour, they allow the application of fertilizer at any time regardless of poor environmental conditions (Grant 2005, Grant & Brandon 2004, Pasda et al. 2001, Wade 2010)

Urease inhibitors, their interaction with fertilizers, soil and microbial communities

The urease enzyme in soil could originate from soil microbes, plants, or animals (Kiss & Simihaian 2002). The urease enzyme is either bound to the soil particles (extracellular), or within (intracellular) urease-producing microbes, known as ureolytic and both intracellular and extracellular ureases are involved in urea hydrolysis (Harder Nielsen et al. 1998, Hasan 2000, Qin et al. 2010, Sigurdarson et al. 2018, Singh et al. 2009). The active site of the urease enzyme consists of two nickel atoms which are important for the activity of the enzyme (Benini et al. 1999, Domínguez et al. 2008, Manunza et al. 1999).

Urease inhibitors are compounds that delay urea hydrolysis to NH_4^+/NH_3 by inhibiting the urease enzyme, thus prevent localized zones of high pH that favor volatilization (Akiyama et al. 2010,

Harty et al. 2016, Sigurdarson et al. 2018, Watson et al. 1994b). This allows urea diffusion into the soil before hydrolysis, which eventually reduces NH_3 loss and improves crop NUE (Cancellier et al. 2016, Chen et al. 2008, Hube et al. 2017, Rosmarina et al. 2016, Sigurdarson et al. 2018).

Among the many compounds tested for inhibiting urea hydrolysis, N-(n-butyl) thiophosphoric triamide (NBPT) is the only commercially available UI that is most promising and effective in a wide range of soils (Harty et al. 2016, Khan et al. 2013, Sigurdarson et al. 2018, Zanin et al. 2016) at very low concentrations of <0.01% of applied Nitrogen (Carmona et al. 1990, Watson et al. 1994c). NBPT can inhibit hydrolysis for 3-14 days based on soil and environmental conditions (Cantarella et al. 2008, Khan et al. 2013, Trenkel 2010, Wang et al. 2020).

Research findings have shown that NBPT (and other UIs) delayed urea hydrolysis from cattle and swine waste, cattle urine, urea, and reduced ammonia emission (e.g. Engel et al. 2011, Affendi et al. 2020, Cantarella et al. 2018, Ni et al. 2014, Sanz-Cobena et al. 2008, Silva et al. 2017, Tian et al. 2015, Wang et al. 2020, Zaman et al. 2009). However, their effects on N_2O emissions are variable with no effects (Dougherty 2016, Hube et al. 2017, van der Weerden et al. 2016) to reduced (e.g. Dawar et al. 2011, Sanz Cobena et al. 2012, Singh et al. 2004, Suter et al. 2020), and increased N_2O emissions (e.g. Fan et al. 2018b, Suter et al. 2016). Despite the positive contribution of NBPT in some cases, the effects of NBPT on yield, N-uptake, and NUE, are variable with some increase (e.g. Cantarella et al. 2018, Drury et al. 2017, Hube et al. 2017, Silva et al. 2017), to no significant effect (e.g. Dougherty 2016, Frame et al. 2013, Menendez et al. 2009, Suter et al. 2016, Suter et al. 2013,) compared to N alone.

The urease enzyme is encoded by the urease gene (*ure*) which has 3 catalytic subunits *ureA*, *ureB*, and *ureC* encoding the γ, β, and α subunits (Nim & Wong 2019). The *ureC* has been used as a

functional gene marker to study ureolytic microbes since it is the largest subunit with several conserved regions (Gresham et al. 2007, Nim & Wong 2019, Oshiki et al. 2018, Singh et al. 2009).

Apart from the NH_3 derived from hydrolysis being the substrate for nitrification, some ammonia oxidizers including AOB, AOA, and comammox have been reported to possess the urease enzyme and the ureolytic capability to use urea as a source of nitrogen (Koch et al. 2015, Koper et al. 2004, Lehtovirta-Morley et al. 2016, Palatinszky et al. 2015, Pommerening-Röser & Koops 2005, Santoro 2016, van Kessel et al. 2015). This would, therefore, mean that NBPT could potentially influence N losses derived from nitrification by influencing the ammonia oxidizers directly through inhibiting the intracellular urease in these ammonia oxidizers. Few studies have investigated the effect of NBPT on ureolytic and ammonia oxidizers

Mechanisms of urease inhibition by NBPT

The explanation of the mechanism of NBPT inhibition or other UI to the urease enzyme activity is based on the understanding of the active site of the UI and the urease enzyme. NBPT is converted to its active inhibitory chemical species, the oxon analog *N*-(*n*-butyl) phosphoric triamide (NBPTO) (Figure 2.1) which then forms a tridentate ligand with the urease enzyme via both nickel (Ni) atoms of the urease enzyme active site and the oxygen atom of the urea-derived carbamate, blocking the enzyme and inhibiting urea hydrolysis (Cantarella et al. 2018, Font et al. 2008, Manunza et al. 1999, Sigurdarson et al. 2018, Watson et al. 2008). Therefore, NBPT inhibits urease enzyme activity by competing with urea for the Ni active sites of the urease enzyme (e.g. Chien et al. 2014). This is made possible as NBPT is a structural analog of urea (Chien et al. 2009).

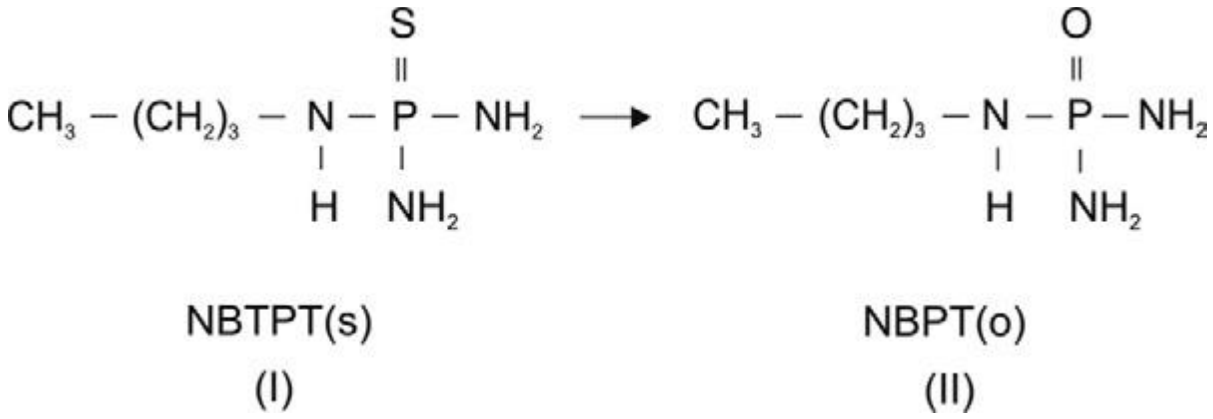

Figure 2.1. Structure and conversion of N-(n-butyl) thiophosphoric triamide (NBPT) to *N*-(*n*-butyl) phosphoric triamide (NBPTO) (Chien et al. 2014).

Factors affecting the effectiveness of UIs

The effectiveness of NBPT varies with several factors including temperatures, soil pH, organic matter, soil WFPS, and % clay content. For instance, higher ammonia volatilization is expected in alkaline soils (Martens & Bremner 1989, Sha et al. 2019, Watson et al. 1994a). This then implies that the contribution of UIs to reduce ammonia volatilization resulting from urea hydrolysis would be relatively higher in alkaline soil conditions (Sha et al. 2019). NBPT degradation was shown to reduce with increasing pH (e.g. Engel et al. 2015, Engel et al. 2013). Similarly, a reduction of about 17-86 % in urea hydrolysis by NBPT was reported in acidic soil compared to a 53- 92 % reduction in alkaline soil (Engel et al. 2013). Hendrickson and Douglass (1993) reported that the reduction of urea hydrolysis by NBPT and its oxon analog (NBPTO), and the disappearance of these compounds was slower in neutral than in acidic soils. Therefore, soil pH determines the efficacy and persistence of NBPT.

Temperature is also an influencing factor influencing NBPT activity and persistence in soil. For example, one study reported that at 32^{o}C, higher rates of NBPT were needed for effective inhibition of urea hydrolysis in soils than at 18^{o}C (Carmona et al. 1990). In another study, 55% urea remained in soil after 2 weeks of inhibition of urea hydrolysis by NBPT at 5-15^{o}C while only <2% of urea remained in the same soil within the same period of NBPT inhibition at 25^{o}C (Suter et al. 2011). As temperature increases, urease enzyme activity also increases up to some point influencing the persistence of NBPT in soil (Bremner et al. 1991, Carmona et al. 1990, Dharmakeerthi & Thenabadu 1996 and references therein). Therefore, the efficacy of NBPT reduces at higher temperatures since the rate of hydrolysis could be higher than the conversion of NBPT to its oxon analog, or there could be increased degradation of NBPT.

At higher WFPS (>60%), the effectiveness of NBPT has also been shown to reduce compared to lower WFPS (<60%) (Dougherty 2016, Sanz-Cobena et al. 2014). This has been linked to the inability of conversion of NBPT to its oxon analog NBPTO (Sanz-Cobena et al. 2014).

The recovery of NBPT was reduced by the addition of organic matter and this was linked to its increased adsorption (Gioacchini et al. 2000). Therefore, the effectiveness of NBPT and other UIs may be reduced with increasing organic matter (Asgedom et al. 2014, Bremner & Chai 1986, Carmona et al. 1990, Gill et al. 1999, Watson et al. 1994a, Zhengping et al. 1991). This has been linked to increased microbial activity, which increases the degradation and reduces the efficacy of the UIs (Gill et al. 1999).

The other factor that has been suggested to influence UI activity includes soil texture. For example, a study reported that NBPT led reduced soil pH and NH_4 concentration around the reaction site in silty loam soil (with 20% clay) allowing diffusion of urea from the placement site, while in clay soil (with 46% clay), the NBPT effect was low which led to increased NH_4 concentration around

the fertilizer placement site (Christianson et al. 1993). Therefore, soil texture influences the diffusion of the UI which is important for its activity in the soil.

Nitrification inhibitors, their interaction with fertilizers, soil, and microbial communities.

Nitrification inhibitors are compounds that delay the first and rate-limiting step of nitrification, the conversion of NH_4^+ to NH_2OH by inhibiting the activity of AMO enzyme (Di & Cameron 2011, Hube et al. 2017, Vallejo et al. 2005). This results in the accumulation of NH_4^+ that can then be adsorbed onto the soil cation exchange sites reducing the risk of being lost via leaching, as occurs with NO_3- (e.g. Di & Cameron 2002a, Nair et al. 2020, Qiao et al. 2015). Further, the inhibition of NH_3 oxidation by NIs could reduce denitrification losses indirectly by suppressing the substrate (NO_3-) availability for denitrification (Akiyama et al. 2010, Yang et al. 2016, Duan et al. 2017). For instance, NIs have been shown to significantly reduce N_2O, NO emissions in both chemical and organic fertilizers (e.g. Akiyama et al. 2010, Lam et al. 2015, Lam et al. 2017, Hube et al. 2017). The accumulated NH_4^+ due to NI effect can be utilized by the crop, therefore, NIs can contribute to improved crop nitrogen use efficiency and productivity in some cases (e.g. Freney et al. 1993, Zaman et al. 2009, Akiyama et al. 2010, Cui et al. 2011, Di & Cameron 2012, Di & Cameron 2016, Wallace et al. 2020). Further, the delayed nitrification could allow the crop to develop and utilize the NO_3^- better once nitrification takes place contributing to increased crop productivity. However, in some studies, there were no reported effects of NIs on crop productivity (e.g. Asing et al. 2008, Huérfano et al. 2015, Misselbrook et al. 2014, Suter et al. 2014, Suter et al. 2016).

Studies have shown that NIs influence the activity and growth of AOB (e.g. Dai et al. 2013, Di et al. 2010, Fu et al. 2020, Kou et al. 2015, Qiuhui Chen et al. 2015, Shi et al. 2016b), and AOA (e.g. Florio et al. 2014, Liu et al. 2015), significantly reducing nitrification rates and associated losses

(Chen et al. 2008, Subbarao et al. 2010). It is however not understood how the available NIs influence the newly discovered comammox *Nitrospira* in soil. Nitrification inhibitors have also been shown to significantly alter soil properties e.g. causing a significant increase in soil pH relative to fertilizer without NIs (O'Callaghan et al. 2010, Yang et al. 2013, Zaman & Nguyen 2012), which has been linked to their effect to increase NH_3 loss (Zaman & Nguyen 2012). For sustainable long-term use of the NIs, there is a need to investigate how their use could influence the soil non-targeted microbes, especially after the production period. This is important for understanding the possible soil health implications of their use. However, only a handful of studies have investigated the effects of long-term use NIs on non-targeted microbes in the long-term e.g. (Guo et al. 2013, Shi et al. 2017). 3, 4-dimethylpyrazol phosphate (DMPP) and Dicyandiamide (DCD) are two NIs that have gained commercial application. However, DMPP has some advantages that make it preferred over DCD e.g. efficacy for about 4-10 weeks, very low application rate, less affected by temperature, it is less mobile in soil and the fact that it is unlikely to have phytotoxic effects on crops (e.g. Macadam et al. 2003, Menéndez et al. 2012, Reeves & Touchton 1986, Wissemeier et al. 2001, Zerulla et al. 2001).

Mechanism of DMPP inhibition

One of the suggested mechanisms of action of several NIs against the AMO enzyme include the direct binding and interaction with AMO which could lead to inhibition by competing for the enzyme active site with NH_3, binding at a site other than the AMO active site, or through the NI acting as metal chelators and removing the co-factors (e.g. Cu) from the AMO, therefore, inactivating it (e.g. McCarty 1999, Subbarao et al. 2006). DMPP has also been believed to inhibit nitrification by acting as a metal chelator which removes the Cu co-factor making the AMO inactive (Ruser & Schulz 2015).

Factors affecting the effectiveness of NIs.

The extent of inhibition by the NIs depends on several factors e.g. soil pH (Guardia et al. 2018, Shi et al. 2016a), soil temperature (Di & Cameron 2005, Kelliher et al. 2014, Lan et al. 2018) soil organic matter (OM) (Fisk et al. 2015, McGeough et al. 2016, Singh et al. 2008b, Zhu et al. 2019), texture and % clay content (McGeough et al. 2016, Pasda et al. 2001, Sha et al. 2020, Wu et al. 2007), soil aeration (Balaine et al. 2015, Kim et al. 2011, Menéndez et al. 2012).

Soil organic matter (OM) and clay content have also been shown to influence NI efficacy and persistence in soil with their effects being attributed to the adsorption of the NIs to the OM, which affects their availability and the fact that soil organic carbon and N are a substrate for soil microbial communities, increasing their activity and degradation of the NIs (Asgedom et al. 2014, Asing et al. 2008, Fisk et al. 2015, McGeough et al. 2016, Shi et al. 2016a).

Soil temperature has been reported to affect the duration and activity of NIs through its influence on their degradation which could also be indirectly influenced by temperature effect on soil microbial activity (Di & Cameron 2005, Kelliher et al. 2014, Marsden et al. 2017, McGeough et al. 2016). Soil aeration also influences the duration and activity of the NIs through its influence on the microbial activities, affecting the degradation of the NIs (e.g. Balaine et al. 2015, Kim et al. 2011, Marsden et al. 2017, Menéndez et al. 2012).

Soil pH has been shown to influence the distribution and abundance of ammonia oxidizers (e.g. He et al. 2012a, He et al. 2012b, Liu et al. 2017, Prosser et al. 2020, Prosser & Nicol 2012, Yao et al. 2011, Zhongjun et al. 2020), which influences the efficacy NIs. Other factors like the uptake of the NIs by the microbial communities and enzyme activities (e.g. catalase enzyme activity and potential nitrification activity) (Barth et al. 2001, Marsden et al. 2016), fertilizer application rate

and N forms (Barth et al. 2001, Barth et al. 2008, Marsden et al. 2016, Yang et al. 2016) have also been shown to greatly influence the effectiveness of NIs.

Use of combined UIs and NIs

The nitrogen use efficiency of crops can be increased by minimizing N losses. IFA (2007) noted that minimizing losses through one pathway could increase losses in the other. For example, it has been shown that when NBPT is used alone, there is inhibition of hydrolysis but the NH_4^+ is exposed to nitrification and denitrification losses (Dougherty 2016, Lam et al. 2018b). For instance, some studies reported an increase in N_2O emissions with the use of UI (e.g. Suter et al. 2016, Lam et al. 2018b, Fan et al. 2018b), which could be linked to this phenomenon. On the other hand, when NIs are used alone, there is prolonged retention of NH_4^+ in soil due to their inhibitory effect on NH_3 oxidation and this might increase the chances for volatilization. For example, it has been reported that NIs increased volatilization losses compared to N alone (e.g. Soares et al. 2012, Pan et al. 2016, Frame 2017, Lam 2018a, Fan et al. 2018a, Castellano-Hinojosa et al. 2019). This means that the use of NIs may not be beneficial in areas with concerns about NH_3 emissions. Therefore, the use of an approach that simultaneously targets all the N pathways for an overall environmental benefit has been suggested. A good example is combining the UIs and NIs for use with ammoniacal fertilizers aimed to simultaneously minimize the losses from the N cycling pathways of hydrolysis, nitrification, and denitrification (Cantarella et al. 2018, Drury et al. 2017, Frame 2017).

Indeed, some studies have reported the effect of combined use of UI and NI on N losses and crop productivity (e.g. Khan et al. 2013, Li et al. 2018, Soares et al. 2012, Zaman et al. 2013). However, the effects of the combined inhibitor compared to the individual compounds have been variable. For instance, a study reported that combining NBPT with DMPP reduced the effectiveness of DMPP to reduce N_2O emission (Zhao et al. 2017). Another study showed that NBPT was more

effective alone than when combined with a NI in reducing N_2O emissions (Sanz Cobena et al. 2012) or NH_3 volatilization (Frame 2017, Pereira et al. 2013, Soares et al. 2012). On the other hand, other studies reported that combining NBPT with DMPP produced better results than NBPT alone in reducing N_2O emission but this combination increased NH_3 emissions (Ding et al. 2011). This highlights the need to understand further the effects of the combined inhibitor compounds on N-dynamics in soil. Further, little attention has been given to the understanding of the effect of such a combination to N cycling microbes (e.g. Fu et al. 2020) and this warrants an understanding from such a study.

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Chapter 3. Responses of ureolytic and nitrifying microbes to urease and nitrification inhibitors in selected agricultural soils in Victoria, Australia.

This chapter is based on an article published in the Journal of Soils and Sediments 20, 1309-1322.

Journal of Soils and Sediments (2020) 20:1309–1322
https://doi.org/10.1007/s11368-020-02562-x

SOILS, SEC 1 • SOIL ORGANIC MATTER DYNAMICS AND NUTRIENT CYCLING • RESEARCH ARTICLE

Responses of ureolytic and nitrifying microbes to urease and nitrification inhibitors in selected agricultural soils in Victoria, Australia

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Received: 9 August 2019 / Accepted: 1 January 2020 / Published online: 10 January 2020

Abstract

Purpose Urease inhibitors (UIs) such as *N*-(*n*-butyl)thiophosphoric triamide (NBPT) and nitrification inhibitors (NIs) such as 3,4-dimethylpyrazole phosphate (DMPP) have been reported to improve the efficiency of nitrogen (N) fertilizers. However, their effects on the ureolytic and ammonia-oxidizing microbes in agricultural soils are uncertain. This study aimed to investigate the effects of DMPP and NBPT on the abundance and community composition of ureolytic and nitrifying microbes in selected agricultural soils in Australia.

Materials and methods Soil was collected from five agricultural farms and used to establish an incubation experiment. Urea, urea + NBPT, urea + DMPP, and urea + NBPT + DMPP were applied on the soils which were incubated under 25 °C and 60% water-filled pore space for 28 days. Sampling was done on different days for DNA extraction and measurement of ammonium (NH_4^+) and nitrate (NO_3^-) concentration.

Results and discussion NBPT inhibited NH_4^+ accumulation in all the soils but had no significant effect on nitrification in any soil. DMPP alone or DMPP + NBPT significantly inhibited nitrification. The abundances of ammonia-oxidizing bacteria (AOB) and complete ammonia oxidizers (comammox *Nitrospira*), but not ammonia-oxidizing archaea (AOA), were significantly influenced by the application of NBPT, DMPP, or DMPP + NBPT. AOA, AOB, and comammox *Nitrospira* clade B might be significant contributors to nitrification in the studied soils.

Conclusions These findings suggest that NBPT and DMPP can reduce N losses and improve N fertilizer efficiency by directly inhibiting the growth of AOB and comammox organisms in the soils, with implications for our mechanistic understanding of the molecular mechanisms of urease and nitrification inhibitors.

Keywords 3,4-Dimethylpyrazole phosphate (DMPP) · *N*-(*n*-Butyl)thiophosphoric triamide (NBPT) · Ammonia-oxidizing archaea · Ammonia-oxidizing bacteria · Comammox

1 Introduction

Urea is the most used form of nitrogen (N) fertilizers in agriculture globally, accounting for 50% of the world N consumption (Zaman et al. 2012). Soil-applied urea fertilizer or animal urine is rapidly hydrolyzed to NH_4^+ and ammonia (NH_3) by the urease enzyme, leading a pH increase around the urea granule, which drives NH_3 loss through volatilization. Subsequent transformation of NH_3 or NH_4^+ in the soil involves nitrification which converts NH_4^+ to NO_3^- via nitrite (NO_2^-) (He et al. 2012). Nitrification has previously been thought to be a two-step process involving ammonia oxidation

Responsible editor: Huaiying Yao

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11368-020-02562-x) contains supplementary material, which is available to authorized users.

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and nitrite oxidation. Ammonia oxidation to NO_2^- is the rate-limiting step of nitrification, regulated by the *amoA* gene encoding the alpha subunit of the NH_3 monooxygenase (AMO) within ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Prosser and Embley 2002; He et al. 2012). Nitrite oxidation to NO_3^- is the second step of nitrification and is regulated by nitrite-oxidizing bacteria (NOB). Recently, a group of bacteria within the *Nitrospira* genus was discovered with the capacity of completely oxidizing NH_3 to NO_3^- in a single organism (comammox *Nitrospira*) (Daims et al. 2015; van Kessel et al. 2015). These comammox organisms have been shown to be widely distributed in soils and water and might have an advantage over AOB and AOA under substrate limiting conditions (van Kessel et al. 2015; Hu and He 2017). The NO_3^- produced by nitrification provides a substrate for denitrification, a biological pathway in which N is returned to the atmosphere from soil through the reduction of NO_3^- or NO_2^- to N_2 via nitrous oxide (N_2O)/nitric oxide (NO) by the nitrite reductase enzyme (Prosser and Embley 2002; Philippot et al. 2007).

Nitrogen use efficiency (NUE) is generally low, rarely exceeding 50% (Chen et al. 2008; Khan et al. 2014) as a consequence of N loss mainly via the nitrification and denitrification pathways (Hu et al. 2015a). These account for economic losses to the farmer and environmental pollution. Technologies developed to improve urea efficiency and minimize N losses include the use of urea coated with urease and/or nitrification inhibitors (Chen et al. 2008). Urease inhibitors (UIs) are compounds that delay urea hydrolysis by inhibiting the urease enzyme activity, thus preventing localized zones of high pH that drive NH_3 volatilization (Watson et al. 2008; Harty et al. 2016). *N*-(*n*-Butyl)thiophosphoric triamide (NBPT) is the only urease inhibitor that has been developed for commercial use and is effective in a wide range of soils (Watson et al. 2008; Harty et al. 2016; Cantarella et al. 2018). Once applied to the soil, NBPT is considered to be converted to its oxon analog *N*-(*n*-butyl)phosphoric triamide (NBPTO) (active form), forming a tridentate ligand with the urease enzyme, inactivating the enzyme, and inhibiting urea hydrolysis (Manunza et al. 1999; Watson et al. 2008). NBPT can inhibit hydrolysis for a period of up to 2 weeks depending on soil and environmental conditions (Cantarella et al. 2008; Suter et al. 2011).

Nitrification inhibitors (NIs) are compounds that delay the oxidation of NH_4^+ to NO_2^-, reducing NO_3^- concentration for some days (Trenkel 1997) by suppressing the activity of AMO in AOA and AOB (Di and Cameron 2011; Florio et al. 2014). This reduces nitrification rates and associated N losses (Chen et al. 2008; Recio et al. 2018). NIs could also reduce denitrification losses indirectly by influencing substrate availability (Florio et al. 2014; Ruser and Schulz 2015). DMPP and dicyandiamide (DCD) are two NIs that are commercially available. DMPP has some advantages over DCD, e.g., longer efficacy (about 4–10 weeks), lower application rate, no deleterious effects on crops, and less mobile in soil (Wissemeier et al. 2001; Zerulla et al. 2001). Some factors that have been reported to influence the effectiveness of UIs and NIs include soil pH, organic matter content, soil texture, and microbial activity (Barth et al. 2001; San Francisco et al. 2011; Thapa et al. 2016).

The NUE of crops can be increased by minimizing N losses but minimizing losses through one pathway could increase losses of another (IFA 2007). For example, when NBPT is used alone, there is inhibition of urea hydrolysis which initially reduces the NH_4^+ concentration, but any NH_4^+ formed is subject to nitrification and denitrification losses (Dougherty 2016). NIs can inhibit NH_3 oxidation but this could lead to prolonged retention of NH_4^+ in soil and increase the risk of volatilization (Kim et al. 2012). Therefore, in systems with multiple N loss pathways, an approach that simultaneously targets all N pathways for an overall economic and environmental benefit is required (IFA 2007). Combining UIs and NIs for use with fertilizers would simultaneously minimize losses from hydrolysis, nitrification, and denitrification. However, limited molecular studies have investigated the effect of combined UIs and NIs on the soil N-cycling microbes. Although some studies exist on the effect of NIs on nitrifying microbes (Florio et al. 2014; Kong et al. 2016), there is limited information on the effect of NIs on the urease producing microbes. The effect of NBPT on nitrifying microbes and urease producing microbes has also received little attention (Fan et al. 2018).

The objectives of this study were to investigate (a) the effects of DMPP and NBPT on soil N-cycling microbes and (b) how soil properties influence the efficiency of NBPT and DMPP. We hypothesized that (a) use of NBPT and DMPP will reduce the abundance of N-cycling microbes in soil and alter the microbial community composition and (b) soil properties will influence the performance of these inhibitors.

2 Materials and methods

2.1 Site description, sampling, and physicochemical characterization

The soil was collected in March 2017 from five agricultural sites in Victoria, Australia: Dookie (DW), 36° 22′ S, 145° 22′ E; Horsham (HW) 36° 45′ S, 142° 07′ E; Warrnambool (WP) 38° 24′ S, 142° 38′ E; Panmure (PP) 38° 20′ S, 142° 44′ E; and Clyde (CV) 38° 08′ S, 145° 20′ E. The DW soil was classified as sandy loam with a pH of 6.4, HW was classified as clay with a pH of 8.8, WP was classified as loamy sand with a pH of 7.3, PP was classified as sandy loam with a pH of 5.5, and CV was classified as loamy sand with a pH of 7.2. At each site, five soil cores (7.6 cm in diameter and 10 cm in depth) were homogenized to a composite sample and transported to

the laboratory on ice. The soil was air-dried, ground, and passed through a 2-mm sieve for physical and chemical analyses. Gravimetric water content was determined by oven drying of sub-samples at 105 °C for 24 h. Soil NH_4^+-N and NO_3^--N were extracted using a ratio of 1:5 (fresh soil, 2 M KCl, *w*/*v*) by shaking at 175 rpm for 1 h. The solution was filtered through a Whatman 42 filter paper and measured by a Segmented Flow Analyzer (SAN++, Skalar). Details of the selected soil physical and chemical properties are shown in Table 1.

2.2 Microcosm incubation

For each site, sieved (2 mm) soil (55 g air-dried) was placed into a 500-ml vial, wetted to 60% water-filled pore space (WFPS) and incubated at 25 °C for 1 week prior to the commencement of the experiment to stabilize the microbial activity. The microcosm incubation experiment was established with the following treatments with three replicates: control (CK), urea (U), urea + NBPT (UN), urea + DMPP (UD), and urea + NBPT + DMPP (UND). Urea was applied at 200 mg N kg^{-1} soil as a solution. NBPT and DMPP were applied in solution form, at a rate of 0.5% and 1% of applied N, respectively. Two milliliters of each solution were applied to the soil to make the final WFPS to 60%. The vials were closed immediately after treatment application and incubated at 25 °C in the dark for 28 days. During the incubation, the microcosms were aerated every 3 days and moisture was replenished as required. The soils were destructively sampled at days 0 (4 h), 1, 2, 4, 7, 14, and 28 after treatment application. On the sampling days, a subsample of 5 g (air-dried equivalent) of soil was collected from each vial and stored separately at − 20 °C for DNA extraction while the rest of the soil (50 g) was used for soil NH_4^+-N and NO_3^--N extraction.

2.3 Net nitrification rate

Soil NH_4-N and NO_3-N were extracted from 50 g soil using 2 M KCl as described above. The equation developed by Persson and Wirén (1995) was used to calculate the net nitrification rates in the first week of incubation (0–7 days).

$$n(\mathrm{d0}-\mathrm{d7}) = \left[(NO_3^-\text{–}N)_{d7} - (NO_3^-\text{–}N)_{d0}/7\right]$$

where n is the net nitrification (mg N kg^{-1} soil day^{-1}) within a specified period, $(NO_3^-\text{-}N)_{d0}$ and $(NO_3^-\text{-}N)_{d7}$ are the NO_3^--N concentrations (mg kg^{-1} soil) of the soil on days 0 and 7, respectively.

2.4 Soil DNA extraction

DNA was extracted from 0.25 g frozen soil sample using the MoBio PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions with slight modifications where a Fastprep beating system (Bio-101 Vista CA, USA) at a speed of 5.5 m s^{-1} for 30 s was used for the initial cell lysis step (Hu et al. 2015b). The DNA concentration was assessed photometrically using the NanoDrop® ND-2000c Spectrophotometer UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.5 Quantitative PCR analyses of N-cycling functional genes

Abundances of the urea-hydrolyzing and nitrifying genes in the soil samples were quantified on a Bio-Rad CFX384 optical real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the primer sets and thermal cycling conditions shown in Table 2. The 10-μl reaction mixture contained 5 μl of Sensimix (Bioline, Sydney, NSW, Australia),

Table 1 Basic properties of the soils used in this study

Soil property	Units	Dookie wheat (DW)	Horsham wheat (HW)	Warrnambool pasture (WP)	Panmure pasture (PP)	Clyde vegetable (CV)
Location		36° 22′ S, 145° 22′ E	36° 45′ S, 142° 07′ E	38° 24′ S, 142° 38′ E	38° 20′ S, 142° 44′ E	38° 08′ S, 145° 20′ E
Cation exchange capacity (CEC)	Cmol kg^{-1}	21	45	19.4	10.6	11.0
pH (1:5 $cacl_2$)		5.7	7.9	6.8	4.7	6.5
pH (1:5 water)		6.4	8.8	7.3	5.5	7.2
Organic carbon	g kg^{-1}	2.7	7.3	33	60	18
Nitrate-N	mg kg^{-1}	32	7.2	110	38	51
Ammonium-N	mg kg^{-1}	5.9	0.95	5.8	16	2.2
Particle size						
Sand (0.02–2 mm)	%	59.9	35.7	86.1	68.9	83.7
Silt (0.002–0.02 mm)	%	21.3	10.1	6.3	17.4	7.5
Clay (< 0.002 mm)	%	18.8	54.2	7.6	13.7	8.8

0.25 μl of each primer (10 μM), and 1 μl of template DNA. Standard curves were generated using 10-fold serial dilutions of plasmids containing correct inserts of the target genes. Melting curve analysis was performed between 72 and 94.5 °C at the end of each amplification assay to evaluate the specificity of quantitative PCR (qPCR) products, and the amplification efficiencies for all qPCR runs ranged between 80 and 110% with R^2 of 0.99.

2.6 Terminal restriction fragment length polymorphism (T-RFLP) analyses

T-RFLP analysis was performed to investigate the changes in community structure of nitrifying microbes focusing on the *amoA* genes of AOA and AOB using fluorescently labeled primers (Hu et al. 2015b). A 25-μl PCR reaction mixture contained 2 μl of diluted template DNA (1–10 ng), 0.5 μl of each primer (10 μM), 5 μl MyTaq buffer, and 1.5 U of MyTaq polymerase (Bioline, Sydney, Australia). The thermal cycling conditions for AOA and AOB *amoA* genes were as follows: 95 °C for 5 min, 35 cycles of 30 s at 95 °C, 30 s at 56 °C, and 60 s at 72 °C, followed by 10 min at 72 °C. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA, USA) and quantified using the NanoDrop ND-2000c Spectrophotometer. The restriction digestion was carried out in a 10-μl mixture containing 200 ng of purified PCR products, 0.1 μl of BSA,1 μl of × 10 NE buffer, and 5 U of the restriction enzymes MspI for AOB and RsaI (BioLabs, Sydney, Australia) for AOA. The digests were incubated at 37 °C for 3 h and denatured for 10 min at 95 °C. Terminal restriction fragments (TRFs) were size separated with an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, CA, USA) and analyzed using a local southern size-calling method (peaks > 50 bp) and a peak amplitude threshold setting of 50, using GeneMapper version 4.0 (Applied Biosystems). TRFs with peak height comprising less than 1% of the total peak height were removed, and peaks that differed by less than 1 bp were combined into the same TRF (Hu et al. 2015b). The relative fluorescence abundances of all TRFs were exported for further analysis of the community composition.

2.7 Statistical analysis

Data are represented as the means of three replicates. The gene copy numbers were calculated using the equation described in Behrens et al. (2008). Data were analyzed using analysis of variance (ANOVA) at $p < 0.05$ followed by the Fisher test to compare treatment means only where there was a significant effect as shown by ANOVA in the Minitab 18 statistical software. Pearson's correlation was performed to assess the correlation between the *amoA* gene copy numbers and NO_3^--N concentrations. Non-metric multidimensional scaling (NMDS) plots were used to visualize the Bray–Curtis dissimilarity matrices based on the T-RFLP data of AOA and AOB.

3 Results

3.1 Mineral N and net nitrification rate under different treatments

Urea addition significantly ($p < 0.05$) increased NH_4^+-N concentration compared to CK in all the soils and the NH_4^+-N

Table 2 Primers used for quantification of the key N-cycling functional genes

Primers	Sequence	Length (bp)	Reference	Thermocycling conditions
ureC gene				
L2F	ATHGGYAARGCNGGNAAYCC	390	Gresham et al. 2007	10 min at 95 °C, 40 cycles of (30 s at 95 °C, 45 s at 55 °C, and 45 s at 72 °C), 10 min at 72 °C
L2R	GTBSHNCCCCARTCYTCRTG			
AOA *amoA*				
CrenamoA-23f	ATGGTCTGGCTWAGACG	629	Tourna et al. 2008	10 min at 95 °C, 40 cycles of (30 s at 95 °C, 45 s at 55 °C, and 45 s at 72 °C), 10 min at 72 °C
CrenamoA-616r	GCCATC CATCTGTATGTCCA			
AOB *amoA*				
amoA-1F	GGGGTTTCTACTGGTGGT	491	Rotthauwe et al. 1997	10 min at 95 °C, 40 cycles of (30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C), 10 min at 72 °C
amoA-2R	CCCCTCKGSAAAGCCTTCTTC			
Comammox A				
comaA-244F	TAYAAYTGGGTSAAYTA	415	Pjevac et al. 2017	10 min at 95 °C, 25 cycles of (30 s at 94 °C, 45 s at 42–52 °C, and 60s at 72 °C), 10 min at 72 °C
comaA-659R	ARATCATSGTGCTRTG			
Comammox B				
comaB-244F	TAYTTCTGGACRTTYTA	415	Pjevac et al. 2017	10 min at 95 °C, 25 cycles of (30 s at 94 °C, 45 s at 42–52 °C, and 60s at 72 °C), 10 min at 72 °C
comaB-659R	ARATCCARACDGTGTG			

concentration in the U treatment remained significantly higher than CK for 2 weeks in all soils except in WP and CV (Fig. 1 and Fig. S1). Addition of NBPT (UN) significantly reduced NH_4^+-N accumulation compared to U ($p < 0.05$). The reduction of NH_4^+-N concentration by NBPT varied with 58–69%, 47–55%, 47.4%, 26–29%, and 18% in HW, DW, CV, PP, and WP soils, respectively. Addition of DMPP (UD) or DMPP + NBPT (UND) resulted in a significant reduction in NH_4^+-N accumulation compared to U in the initial days for DW and HW and to a small extent in CV and WP (Fig. 1 and Fig. S1).

The NO_3^--N concentration in the CK treatment remained almost unchanged in all the soils throughout the 28-day incubation period. Urea addition significantly increased NO_3^--N concentration compared to CK in all the five soils. Addition of DMPP (UD) alone or DMPP + NBPT (UND) significantly reduced NO_3^--N concentration compared to U in all the soils. However, the addition of NBPT alone did not significantly affect NO_3^--N concentration in any soil compared to U. The significant reduction in NO_3^--N concentration by DMPP alone (UD) and DMPP + NBPT (UND) ranged between 44–48.7 and 28.7–46.3% for HW soil, 38.2 and 23.9–28.9% for DW soil, 13.2–36.4 and 12.7–34.7% for PP soil, 25.5–39 and 7–38.6% for CV soil, and 13.5–30.6 and 11.9–23.6% for WP soil, respectively (Fig. 1 and Fig. S1).

In the first week, net nitrification rates were significantly higher in U treatment of all the soils compared to CK. Use of DMPP alone (UD) or DMPP + NBPT (UND) significantly reduced the net nitrification rates by 59.6 and 55.8% in HW soil, 31.6 and 31.9% in PP soil, and 33.4 and 28.4% in WP soil, respectively, compared to U (Table 3) and 43.4% reduction by DMPP (UD) in CV soil compared to U. In DW soil, net nitrification rates were not significantly different between UD and UND compared to U during the first week of incubation. NBPT had no significant influence on net nitrification rates in all the soils compared to U (Table 3).

Overall, our results showed that accumulation of NH_4^+ was significantly suppressed by NBPT but stimulated by DMPP alone or combined with NBPT in all soils. On the contrary, the nitrification process was suppressed by DMPP alone or combined with NBPT but not by NBPT alone in all the soils. The efficacy of NBPT and DMPP varied among the soils.

3.2 Copy numbers of the *ureC* and *amoA* genes

The addition of urea with or without inhibitors had no significant effect on the *ureC* gene copy numbers in WP, HW, and PP soils ($p > 0.05$) (Fig. 2 and Fig. S2). UN, UD, and UND treatments had significantly lower gene copy numbers compared to U in DW and CV soils on the last day of incubation ($p < 0.05$) (Fig. 2 and Fig. S2).

There was no significant treatment effect on AOA abundance in all the soils during the sampling period (Fig. 3 and Fig. S3). However, there seemed to be growth of AOA in PP soil during the incubation period compared with much smaller or no growth of AOB or comammox (Fig. S3). Urea addition significantly increased AOB *amoA* gene copy numbers ($p < 0.05$) compared to CK on some incubation days in DW, WP, HW, and CV soils. Addition of DMPP alone or DMPP + NBPT significantly ($p < 0.05$) reduced AOB abundance compared to urea on some incubation days in DW, WP, HW, and CV soils compared to U (Fig. 3 and Fig. S3). NBPT addition significantly reduced AOB abundance compared to U alone on day 7 in WP soil (Fig. 3). However, there was no significant treatment effect on AOB abundance in PP soil (Fig. S3). There was an increase in AOA/AOB ratio on day 7 with the addition of DMPP alone or DMPP + NBPT except in WP soil.

Urea addition with or without inhibitors had no significant effect on comammox *Nitrospira* clade A *amoA* gene copy numbers in WP and DW soils on any sampling day (Fig. 4). NBPT significantly reduced comammox *Nitrospira* clade A abundance compared to urea on the last day of incubation in PP soil (Fig. S4). The addition of NBPT + DMPP significantly reduced comammox *Nitrospira* clade A abundance compared to urea on some incubation days in the HW, PP, and CV soils (Fig. S4). Urea addition significantly increased comammox *Nitrospira* clade B abundance compared to CK on day 7 in DW (Fig. 4). Addition of NBPT, DMPP, or DMPP + NBPT significantly reduced comammox *Nitrospira* clade B abundance on some days in WP, CV, DW, and HW soils compared to urea (Fig. 4 and Fig. S4). However, there was no significant treatment effect on comammox *Nitrospira* clade B gene copy numbers in PP soil (Fig. S4) on any day during the incubation.

Our results showed that except for AOA, all the treatments significantly suppressed the growth of ammonia oxidizers in some soils. A trend of increasing ratio of AOA/AOB was observed when DMPP alone or DMPP + NBPT were added. The *ureC* gene copy numbers were suppressed by all the treatments towards the end of the experiment in most soils.

3.3 Treatment effects on community composition and structure of AOA and AOB

For the T-RFLP analysis, digestion of AOA and AOB *amoA* genes with RsaI and mspI restriction enzymes, respectively, produced different TRFs in the five soils, suggesting that AOA and AOB had different responses to treatment application on day 7. The changes in the relative abundance of major fragments were used to depict treatment effects on AOA and AOB community composition in the five soils.

AOA digestion in DW and WP soils produced a total of five and three distinct TRFs, respectively, with TRFs 41 bp and 56 bp being the most abundant, respectively (Fig. 5). Three distinct TRFs were produced in HW with TRFs 41 bp and 56 bp being the most abundant (Fig. S5). Both PP and CV soils produced five distinct TRFs each with TRF 36 bp in PP soil and TRF 56 bp in CV soil being the most abundant (Fig.

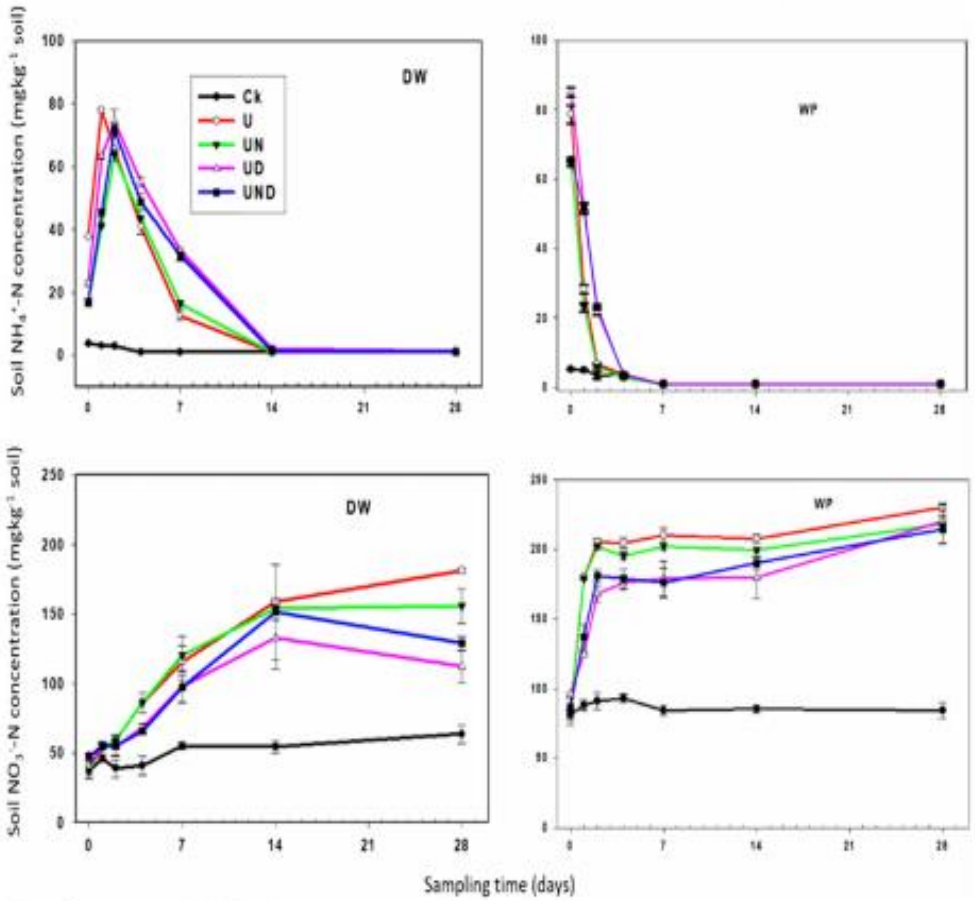

Fig. 1 Soil NH_4^+-N (top) and NO_3^--N (bottom) concentrations during the 28-day microcosm incubation in the Dookie wheat (DW) and Warrnambool pasture (WP) soils across the five treatments of CK (control), U (urea), UN (urea + NBPT), UD (urea + DMPP), and UND (urea + NBPT + DMPP). Error bars represent standard errors from three replicates

S5). In DW soil, the relative abundance of TRF 41 bp was reduced by U compared to CK, and increased with the addition of NBPT, DMPP, or NBPT + DMPP compared to U (Fig. 5). In WP soil, the relative abundance of TRF 56 bp was increased by U compared to CK and reduced by the addition of NBPT or DMPP + NBPT compared to U (Fig. 5). In HW soil, the relative abundance of TRF 41 bp was increased when U was added compared to CK but reduced when DMPP + NBPT was added compared to U. The relative abundance of TRF 56 bp was reduced by U compared to CK and reduced by

Table 3 Net nitrification rates (mg N kg^{-1} soil day^{-1}) of Horsham wheat (HW), Dookie wheat (DW), Clyde vegetable (CV), Panmure pasture (PP), and Warrnambool pasture (WP) soils during the period from day 0 to day 7. Treatments: control, urea, urea + NBPT, urea + NBPT + DMPP

Soil type	Net nitrification rate (mg N kg^{-1} soil day^{-1})				
	Control	Urea	Urea + NBPT	Urea + DMPP	Urea + NBPT + DMPP
DW	2.55 ± 1.04 b	9.64 ± 1.81 a	11.92 ± 2.39 a (NR)	8.26 ± 1.34 a (NR)	7.14 ± 1.31 ab (NR)
HW	0.89 ± 0.09 b	8.44 ± 1.19 a	7.98 ± 1.2 a (NR)	3.41 ± 0.57 b (59.6)	3.74 ± 1.06 b (55.8)
WP	1.2 ± 0.93 c	17.8 ± 2.33 a	17.09 ± 0.51 a (NR)	11.85 ± 1.64 b (33.4)	12.78 ± 0.59 b (28.2)
PP	0.65 ± 0.41 c	6.89 ± 0.75 a	6.10 ± 0.97 ab (NR)	4.72 ± 0.4 b (31.6)	4.7 ± 0.64 b (31.9)
CV	1.45 ± 0.11 d	9.90 ± 1.36 ab	13.45 ± 1.44 a (NR)	5.6 ± 1.38 c (43.4%)	6.78 ± 1.21 bc (NR)

Values are means ± standard error ($n = 3$). Values within the same row followed by the different letters indicate a significant difference ($p < 0.05$). The values in the parentheses show the % inhibition by NBPT, DMPP, or a combination of both used with urea. Assuming nil inhibition where there is "NR" in parenthesis

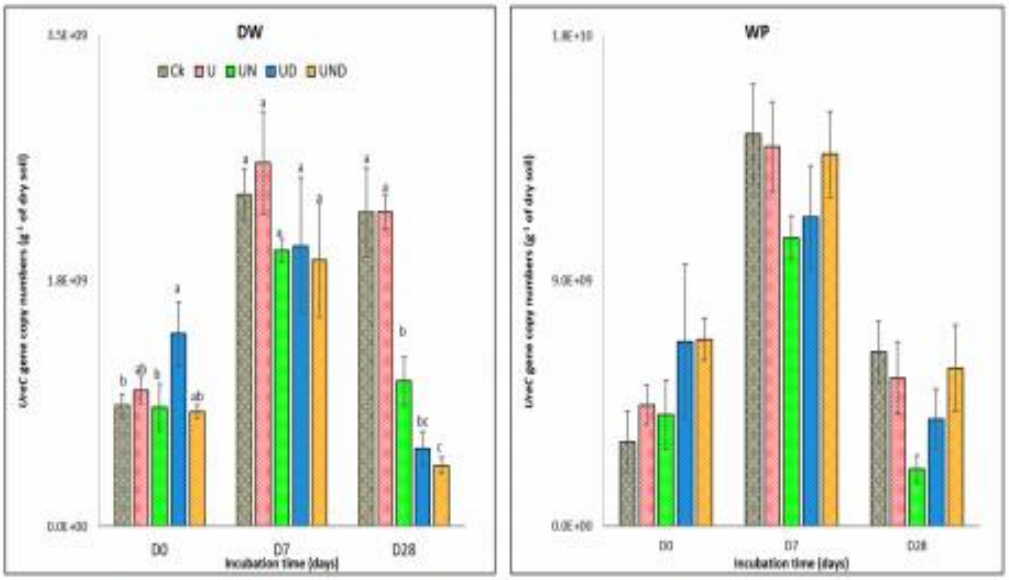

Fig. 2 The *ureC* gene abundance during the 28-day microcosm incubation of Dookie wheat (DW) and Warrnambool pasture (WP) soils across the five treatments of CK (control), U (urea), UN (urea + NBPT), UD (urea + DMPP), and UND (urea + NBPT + DMPP). Error bars represent the standard errors of three replicates. Means that do not share a letter are significantly different at any sampling time at $p \leq 0.05$ level (Fisher test). Note that *y*-axes scales differ between charts. There was no significant difference between the treatments on all the sampling days in WP soil

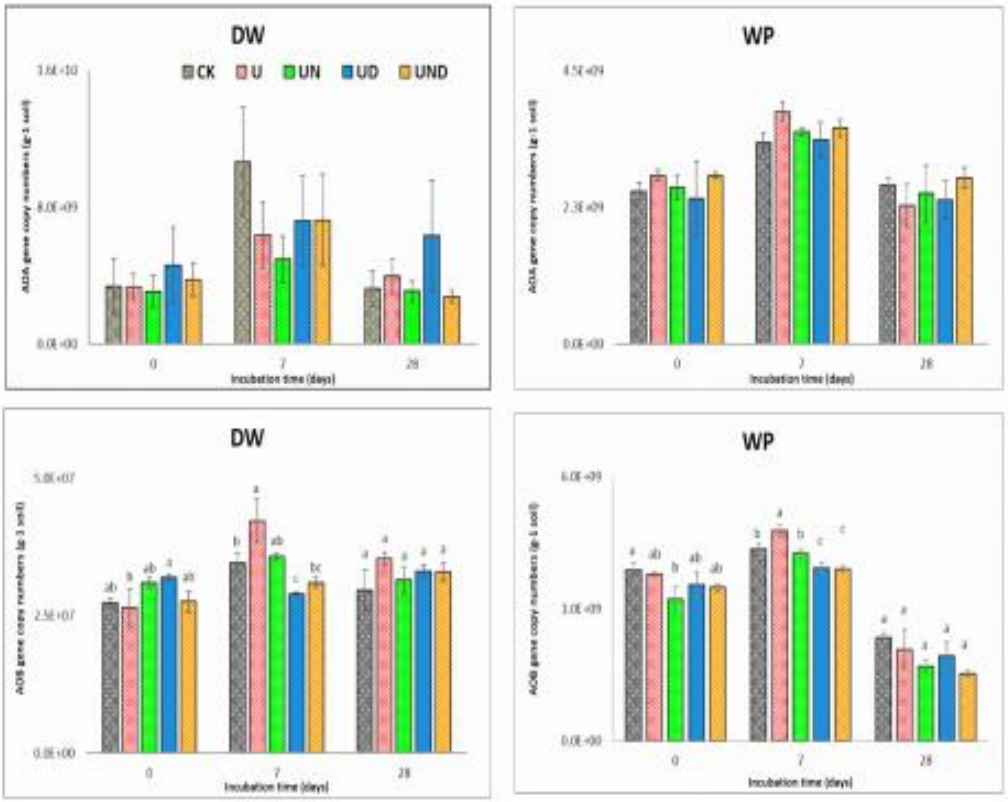

Fig. 3 The abundance of AOA and AOB during the 28-day microcosm incubation of Dookie wheat (DW) and Warrnambool pasture (WP) soils across the five treatments of CK (control), U (urea), UN (urea + NBPT), UD (urea + DMPP), and UND (urea + NBPT + DMPP). Error bars represent the standard errors of three replicates. Means that do not share a letter are significantly different at any sampling time at $p \leq 0.05$ level (Fisher test). There was no significant difference between the treatments on all the sampling days in the two soils on AOA gene copy numbers. Note that *y*-axes scales differ between charts

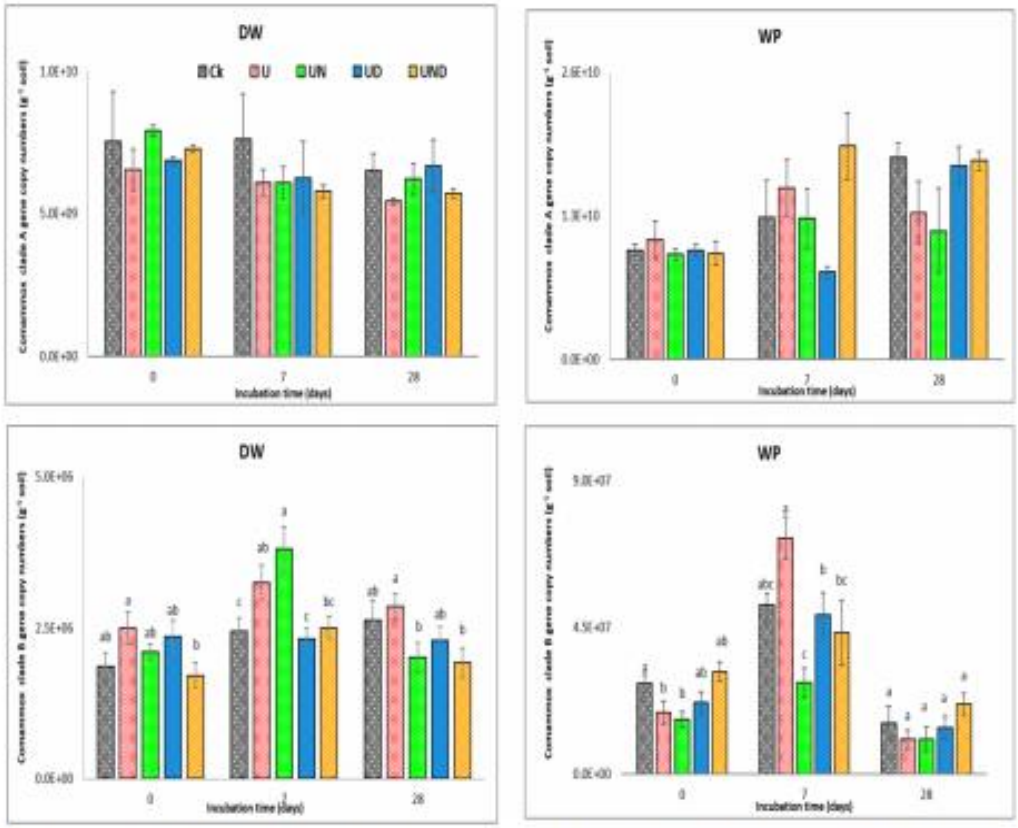

Fig. 4 The abundance of comammox *Nitrospira* clade A and clade B during the 28-day microcosm incubation of Dookie wheat (DW) and Warrnambool pasture (WP) soils across the five treatments of CK (control), U (urea), UN (urea + NBPT), UD (urea + DMPP), and UND (urea + NBPT + DMPP). Error bars represent the standard errors of three replicates. Means that do not share a letter are significantly different at any sampling time at $p \leq 0.05$ level (Fisher test). There was no significant difference between the treatments on all the sampling days in the two soils on comammox *Nitrospira* clade A gene copy numbers. Note that *y*-axes scales differ between charts

DMPP compared to U (Fig. S5). In PP soil, the relative abundance of TRF 36 bp was increased by U compared to CK but reduced by NBPT compared to U (Fig. S5). In CV soil, the relative abundance of TRF 56 bp was reduced by DMPP or NBPT + DMPP compared to U (Fig. S5).

Similarly, digestion of AOB produced 8, 6, 9, 9, and 7 distinct TRFs, with 232 bp; 55 bp and 231 bp; 37 bp, 54 bp, and 152 bp; 54 bp, 152 bp, and 253 bp; and 253 bp being the most abundant in DW, WP, HW, PP, and CV soils, respectively (Fig. 6 and Fig. S6). In DW soil, the relative abundance of TRF 232 bp was reduced by U compared to CK but increased by NBPT, DMPP, or DMPP + NBPT compared to U (Fig. 6). In WP soil, there was no treatment effect compared to urea on the TRF relative abundance (Fig. 6). In HW soil, the relative abundance of TRF 37 bp was reduced by U compared to CK but increased by NBPT, DMPP, or DMPP + NBPT compared to U. TRF 152 bp in HW soil was increased by U compared to CK and reduced by NBPT, DMPP, or DMPP + NBPT compared to U (Fig. S6). In PP soil, the abundance of TRF 54 bp was increased by U compared to CK and reduced by DMPP or DMPP + NBPT compared to urea (Fig. S6). In CV soil, the relative abundance of TRF 253 bp was reduced by U compared to CK but reduced by NBPT and increased by DMPP compared to U (Fig. S6).

The NMDS analysis was used to visualize changes in community structure for both AOA and AOB in the soils following treatment application. In DW and WP AOA NMDS plots, there was no clustering into different groups based on treatment (Fig. 5). In HW-AOA NMDS plot, UD and UND clustered separately from U. However, there was no clear separation of CK or UN from U (Fig. S5). PP-AOA NMDS plot showed that U clustered separately from CK, UN, UD, and UND treatments (Fig. S5). CV AOA NMDS plot showed that UND and UD clustered separately from U (Fig. S5). HW, CV, and PP-AOB NMDS plots showed that U clustered separately from UD, UND, and CK but not from the UN (Fig. S6). There was no clustering based on treatment in DW and WP soils (Fig. 6).

The T-RFLP results generally indicated that NBPT alone, DMPP alone, or their combination had an influence on the structure and community composition of both AOA and AOB.

4 Discussion

4.1 Treatment effects on mineral N dynamics and net nitrification

The addition of NBPT significantly reduced the accumulation of NH_4^+-N ($p < 0.05$) in the five soils compared to urea alone in the initial days, indicative of urea hydrolysis (Fig. 1 and Fig. S1). This is because NBPT inhibited hydrolysis through suppressing the activity of urease enzymes (San Francisco et al. 2011). Similar results were reported by other researchers who showed that NBPT significantly reduced the accumulation of soil NH_4^+-N (Zaman et al. 2009; Sanz-Cobena et al. 2014). Our results showed varying efficacies of NBPT in inhibiting hydrolysis across the five soils, indicating that NBPT efficacy varied with soil physical and chemical properties. We noted a general trend of a reduction in NBPT efficacy with reducing pH except for WP soil, which can be attributed to increased chemical degradation of NBPT at lower pH (Engel et al. 2013, 2015). In line with our findings, other studies also reported a decrease in NBPT efficacy with reducing pH (Frame 2017; Sunderlage and Cook 2018). Our results also showed that apart from the pH, other factors also determined NBPT efficacy. For example, both PP and WP soils had the highest OC compared to other soils (Table 1), which may account for reduced efficiency of NBPT in these two soils compared to other soils. Moreover, HW soil with the lowest OC (7.3 g kg^{-1}) had the best NBPT performance compared to other soils. Urease activity has been shown to increase with increasing OC, resulting in reduced NBPT efficacy (San Francisco et al. 2011; Suter et al. 2011). This could be attributed to increased microbial degradation of NBPT in high OC soils.

The high NH_4^+-N concentration in DMPP alone (UD) or DMPP + NBPT indicated the suppression of nitrification in these treatments which showed the effectiveness of DMPP in retarding the conversion of NH_4^+ to NO_2^-. This was also supported by the lower NO_3^- concentration in DMPP alone (UD) or DMPP + NBPT compared to U (Fig. 1 and Fig. S1) and the net nitrification rates (Table 3). An increase in soil NH_4^+-N content due to NIs has also been reported by other researches (Zaman et al. 2009; Fisk et al. 2015). Previous

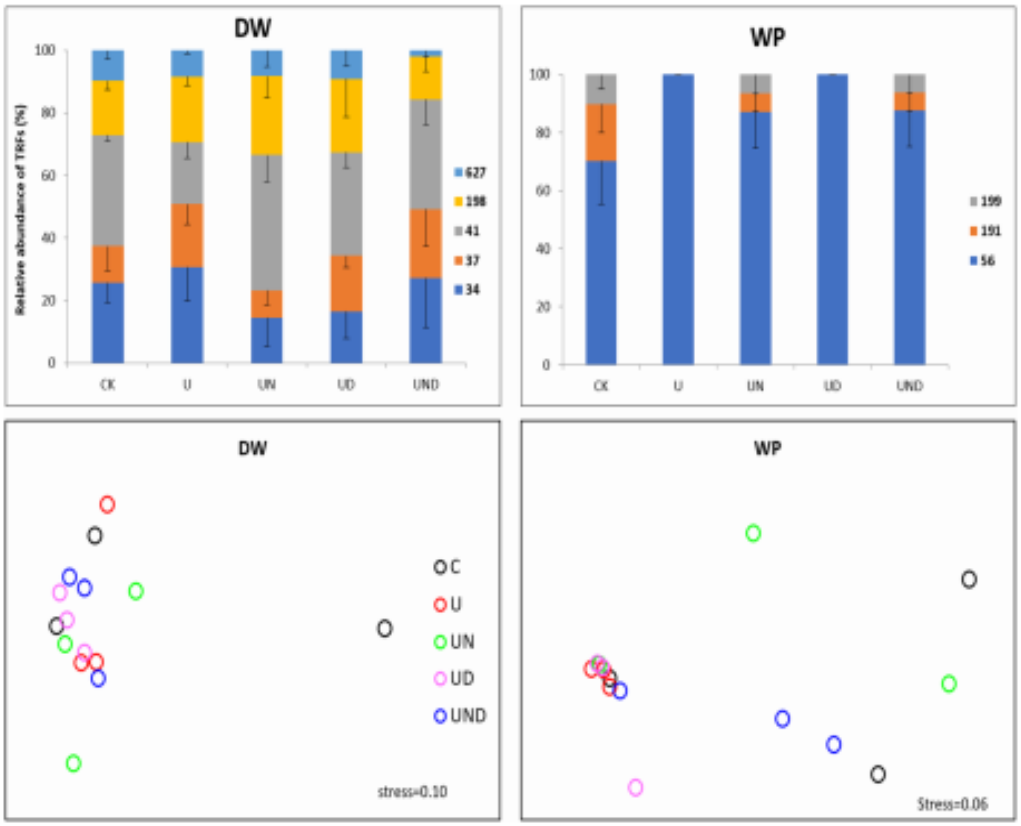

Fig. 5 Terminal restriction fragment length polymorphism fingerprint TRFs (top) and non-metric multidimensional scaling ordinations (bottom) based on the Bray–Curtis dissimilarity matrices of the T-RFLP data of AOA *amoA* genes digested by the RsaI restriction enzyme for Dookie wheat (DW) and Warrnambool pasture (WP) soils across the five treatments of CK (control), U (urea), UN (urea + NBPT), UD (urea + DMPP), and UND (urea + NBPT + DMPP) on day 7 of incubation

reports have shown that where a NI was combined with NBPT, there was either a reduction in effectiveness of NBPT for reducing NH_4^+-N accumulation/NH_3 emissions compared to NBPT alone or an increase in NH_4^+-N accumulation compared to urea alone (Soares et al. 2012; Pereira et al. 2013). Therefore, our results for increasing NH_4^+-N accumulation in the NBPT + DMPP treatment could be related to the increased NH_4^+-N accumulation by DMPP making NBPT to appear ineffective.

The performance of DMPP in reducing NO_3^--N accumulation in the soils varied among the five soils and reduced with increasing OC. This can be linked to the limited mobility of DMPP due to increased adsorption in high OC soils (Ruser and Schulz 2015; Marsden et al. 2016). Other studies have reported increased performance of NIs when OC was low or removed from soils (Yan et al. 2012; Fisk et al. 2015; Shi et al. 2016). We also noted a reduction in DMPP efficacy with reducing pH except for WP soil. This could be associated with increased degradation of DMPP at lower pH. The other reason for the reduced performance of DMPP at lower pH could be due to heterotrophic nitrification that has been reported to occur in acidic soils (Li et al. 2018; Zhang et al. 2018b). Previous studies have reported an increase in DMPP performance or reduced degradation at high soil pH (Yan et al. 2012; Suter et al. 2016).

4.2 Treatment effect on the *ureC* and *amoA* gene copy numbers

AOA abundance showed no significant response to treatment addition compared to other ammonia oxidizers. However, the growth of AOA in PP soil during the sampling period compared to other ammonia oxidizers suggested their potential contribution to nitrification in PP soil. Previous studies have concentrated on studying the effects of fertilizer addition with nitrification inhibitors on AOA and AOB abundances. Some

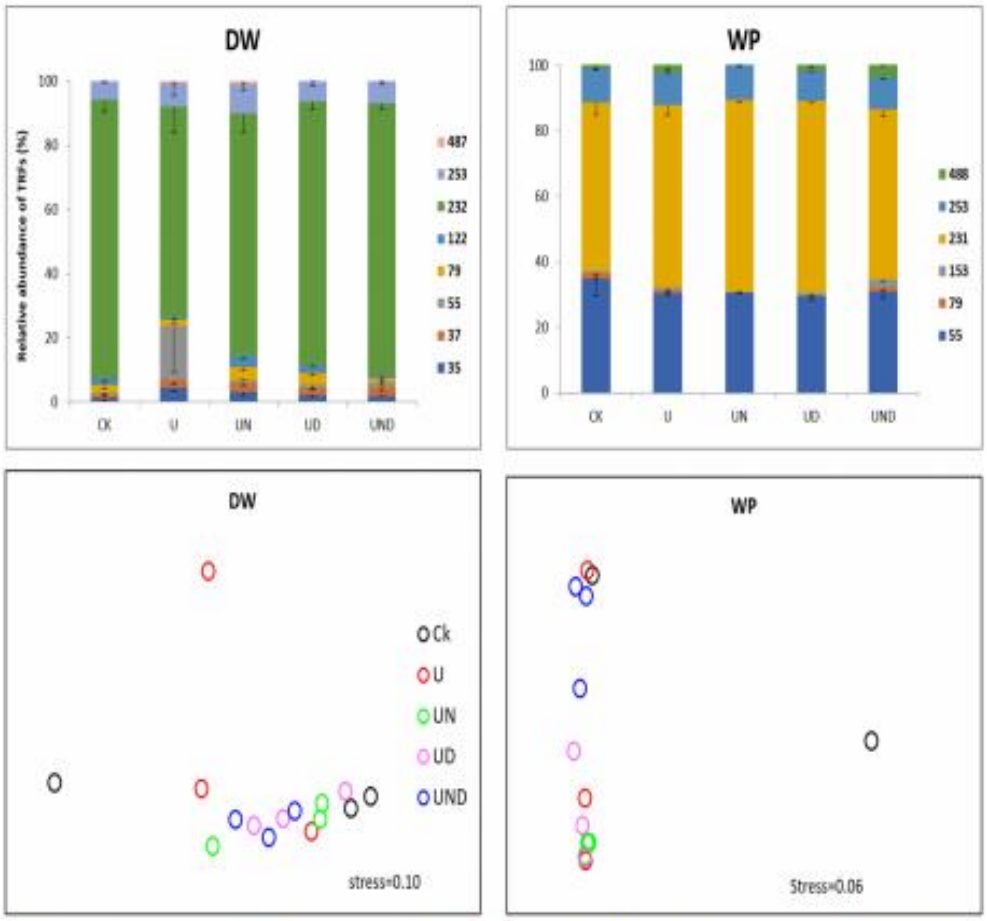

Fig. 6 Terminal restriction fragment length polymorphism fingerprint TRFs (top) of AOB and non-metric multidimensional scaling ordinations (bottom) based on the Bray–Curtis dissimilarity matrices of the T-RFLP data of AOB (bottom) genes digested by the mspI restriction enzyme for Dookie wheat (DW) and Warrnambool pasture (WP) soils across the five treatments of CK (control), U (urea), UN (urea + NBPT), UD (urea + DMPP), and UND (urea + NBPT + DMPP) on day 7 of incubation. All the stress values for the NMDS plots were lower than 0.20, an indication that these data were well-represented by the two-dimensional ordinations

studies reported that growth of AOB, but not AOA, was increased by fertilizer addition (Beeckman et al. 2018; Ouyang et al. 2018) or reduced by DMPP addition (Beeckman et al. 2018; Zhang et al. 2018a). Other studies, however, reported a significant reduction in both AOA and AOB abundances by DMPP (Liu et al. 2015). The significant treatment effect on AOB but not AOA abundance reported in our study could be due to the structural difference between AOA and AOB and the potential heterotrophic growth of AOA (He et al. 2012; Ruser and Schulz 2015; Beeckman et al. 2018; Zhang et al. 2018a). This accounts for less susceptibility of AOA to environmental changes (O'Callaghan et al. 2010). Little is known about the response of comammox organisms to either urease or nitrification inhibitors but inhibition of nitrification in comammox organisms by the NI allylthiourea has been reported (van Kessel et al. 2015; Li et al. 2019). Moreover, DMPP has a similar inhibition mechanism as allylthiourea, which inhibits nitrification by binding to copper, an important cofactor for the *amoA* gene (Ruser and Schulz 2015; Beeckman et al. 2018; Li et al. 2019). Therefore, it could be possible for DMPP to inhibit nitrification by reducing the growth of comammox organisms as reported in some of our soils.

The reported increase in the ratio of AOA/AOB due to DMPP alone or DMPP + NBPT compared to urea in some soils on day 7 may suggest that the AOB population in these treatments was reduced leading to an increase in AOA population. This is consistent with the findings of other researchers who reported that AOA would thrive when AOB is inhibited due to niche differentiation (Hink et al. 2018; Fan et al. 2019).

The reduction of AOB and comammox *Nitrospira* abundances by NBPT in this study could be explained by the fact that NBPT inhibits urea hydrolysis, therefore, avoiding the accumulation of NH_4^+ that is required as a substrate by these microbes to initiate the nitrification process. In addition, some AOA and AOB may have the ability to utilize urea as they possess some ureolytic genes and activities enabling their dependence on urea as a substrate for hydrolysis and subsequent nitrification (Burton and Prosser 2001). Ureolytic capacity has also been reported in some comammox organisms (Palomo et al. 2018; Koch et al. 2019). The reduction of comammox *Nitrospira* and AOB by NBPT would therefore be assumed to be linked to the direct effect of NBPT on urease genes in these ammonia oxidizers, influencing the degradation of urea a source of nitrification. Another possible explanation is that NBPT could inhibit the intracellular urease in ammonia oxidizers, blocking hydrolysis and subsequent nitrification due to limited NH_3 availability (Shi et al. 2017). Other studies have shown that NBPT either inhibited both AOA and AOB or stimulated AOA but inhibited AOB or had no effect on ammonia oxidizers (Shi et al. 2017; Fan et al. 2018).

Pearson's correlation results revealed that significant contributors to nitrification in CV soil might be AOA and comammox *Nitrospira* clade B, while for HW, DW, and WP, AOB might be a significant contributor to nitrification. For PP soil, AOA might be the dominant contributor to nitrification. Soil pH could be a contributing factor that favored activity and distribution of ammonia oxidizers in these soils. Our results agree with previous findings that AOA are an important player of nitrification in acidic soils compared to AOB which are dominant in alkaline soils (Zhang et al. 2012; Di and Cameron 2016). However, our results also showed positive significant correlations for AOB with nitrification in acidic DW soil, which is not unexpected as some acidophilic AOB strains have also been reported to contribute to nitrification in acidic soils (Li et al. 2018; Zhang et al. 2019). Distribution, abundance, and activity of AOB shown in our results could have also been determined by the soil NH_4^+-N concentration which was high for both DW and WP compared to other soils at the beginning of the incubation.

The *ureC* gene copy numbers tended to decrease significantly in some soils towards the end of the incubation period in NBPT, DMPP, or DMPP + NBPT, compared to U ($p < 0.05$). In line with our findings, other researchers reported a reduction in the abundance of *ureC*-containing bacteria in both acid and alkaline soils when they added NBPT to urea in a 25-day incubation experiment (Fan et al. 2018). However, on day 28 of incubation, we expect hydrolysis to have been finished in the soils. Urease enzyme has been shown to exist both inside and outside the cells on release when the cell dies (Fisher et al. 2017; Fan et al. 2018). We would speculate that most of the hydrolysis was conducted by extracellular urease which may absorb and keep NBPT stable in cells for long (Fan et al. 2018).

There is currently no information regarding the effect of DMPP on *ureC* genes. However, the significant reduction of the *ureC* gene copy numbers by UD and UND compared to U as reported in some soils in this study could be explained by the acidic effect of DMPP on soil pH in both treatments. It has been reported that *ureC* gene copy numbers increase with increasing soil pH, while nitrification inhibitors increase NH_4^+-N concentration which enhances acidification (Smoleń et al. 2012; Fisher et al. 2017). Based on these reports and given the fact that the reduction occurred towards the end of incubation, it is possible that the effect of DMPP or DMPP + NBPT on the *ureC* gene abundance was because of the effect of these treatments on soil pH.

5 Conclusions

We reported a significant increase in NH_3 and NO_3 accumulation due to urea addition. NBPT inhibited NH_3 accumulation in all tested soils but had no significant effect on nitrification in any soil. DMPP alone or DMPP + NBPT significantly inhibited nitrification. The abundances of comammox

organisms and AOB but not AOA were significantly influenced by NBPT, DMPP, or DMPP + NBPT. Soil pH and organic carbon might be key factors influencing the activity of urease and nitrification inhibitors in the studied soils. We also found that AOA, AOB, and comammox *Nitrospira* clade B might be significant contributors to nitrification in the studied soils. These findings suggest that NBPT and DMPP can reduce N losses and improve N fertilizer efficiency by directly inhibiting the growth of AOB and comammox organisms in the soils, with implications for our mechanistic understanding of the molecular mechanisms of urease and nitrification inhibitors.

Acknowledgments We acknowledge the financial support from the Australian Research Council (LP160101134) and the Melbourne Trace Analysis for Chemical, Earth, and Environmental Sciences (TrACEES), The University of Melbourne, for the analytical support.

Abbreviations UIs, Urease inhibitors; NIs, Nitrification inhibitors; DMPP, 3,4-Dimethylpyrazole phosphate; NBPT, *N*-(*n*-Butyl)thiophosphoric triamide; AOA, Ammonia-oxidizing archaea; AOB, Ammonia-oxidizing bacteria; Comammox, Complete ammonia oxidizers; PCR, Polymerase chain reaction; qPCR, Quantitative polymerase chain reaction; T-RFLP, Terminal restriction fragment length polymorphism

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ELECTRONIC SUPPLEMENTARY MATERIAL

SOILS, SEC 1 • SOIL ORGANIC MATTER DYNAMICS AND NUTRIENT CYCLING • RESEARCH ARTICLE

Responses of ureolytic and nitrifying microbes to urease and nitrification inhibitors in selected agricultural soils in Victoria, Australia

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Received: 9 August 2019 / Accepted: 30 December 2019

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Fig. S1 Soil NH_4^+-N (top) and NO_3^--N (bottom) concentrations during the 28-day microcosm incubation in the Horsham wheat (HW) and Panmure pasture (PP), and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent standard errors of three replicates

Fig. S2 The *ureC* gene abundance during the 28-day microcosm incubation of Horsham wheat (HW), Panmure pasture (PP), and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent the standard errors of three replicates. Means that do not share a letter are significantly different at any sampling time at $p< 0.05$ level (Fisher test). Note that y-axes scales differ between charts. There was no significant difference between the treatments on all the sampling days in HW and PP soils

Fig. S3 The abundance of ammonia oxidizers (AOA, (top), AOB (bottom), during the 28-day microcosm incubation of Horsham wheat (HW), Panmure pasture (PP) and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent the standard errors of three replicates. Means that do not share a letter are significantly different at any sampling time at $p< 0.05$ level (Fisher test). There was no significant difference between the treatments on all the sampling days in the HW, PP and CV soils on AOA, and in PP soil on AOB gene copy numbers. Note that y-axes scales differ between charts

Fig. S4 The abundance of ammonia oxidizers (comammox Clade A, (top), comammox Clade B (bottom), during the 28-day microcosm incubation of Horsham wheat (HW), Panmure pasture (PP) and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea + NBPT +DMPP. Error bars represent the standard errors of three replicates. Means that do not share a letter are significantly different at

any sampling time at $p < 0.05$ level (Fisher test). There was no significant difference between the treatments on all the sampling days in the PP soil on comammox Clade B gene copy numbers. Note that y-axes scales differ between charts

Fig. S5 Terminal restriction fragment length polymorphism fingerprint TRFs (top), and Non-metric multidimensional scaling ordinations (NMDS (bottom)) based on the Bray-Curtis dissimilarity matrices of the T-RFLP data of AOA genes digested by the RsaI restriction enzyme for Horsham wheat (HW), Panmure pasture (PP) and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea+ DMPP, *UND* Urea+ NBPT +DMPP on day 7 of incubation. All the stress values for the NMDS plots were lower than 0.20, an indication that these data were well-represented by the two-dimensional ordinations

Fig. S6 Terminal restriction fragment length polymorphism fingerprint TRFs (top), and Non-metric multidimensional scaling ordinations (NMDS (bottom)) based on the Bray-Curtis dissimilarity matrices of the T-RFLP data of AOB genes digested by the msp1 restriction enzyme for Horsham wheat (HW), Panmure pasture (PP) and Clyde vegetable (CV) soils across the five treatments of *CK* control, *U* urea, *UN* Urea + NBPT, *UD* urea + DMPP, *UND* Urea + NBPT + DMPP on day 7 of incubation. All the stress values for the NMDS plots were lower than 0.20, an indication that these data were well-represented by the two-dimensional ordinations

Fig. S1

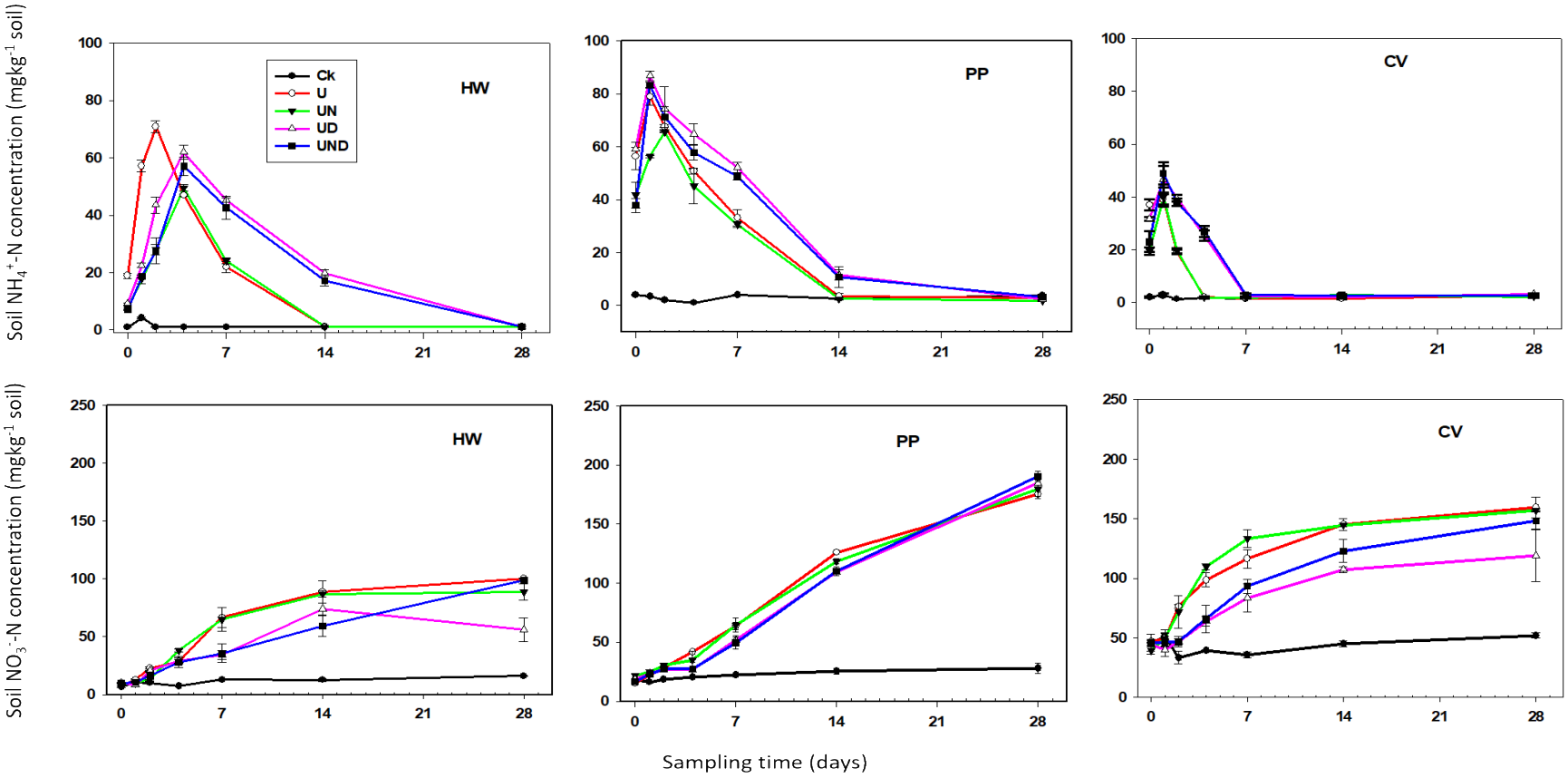

Soil NH_4^+-N concentration (mgkg^{-1} soil)
Soil NO_3^--N concentration (mgkg^{-1} soil)
Ck
U
UN
UD
UND
HW
PP
CV
Sampling time (days)

Fig. S2

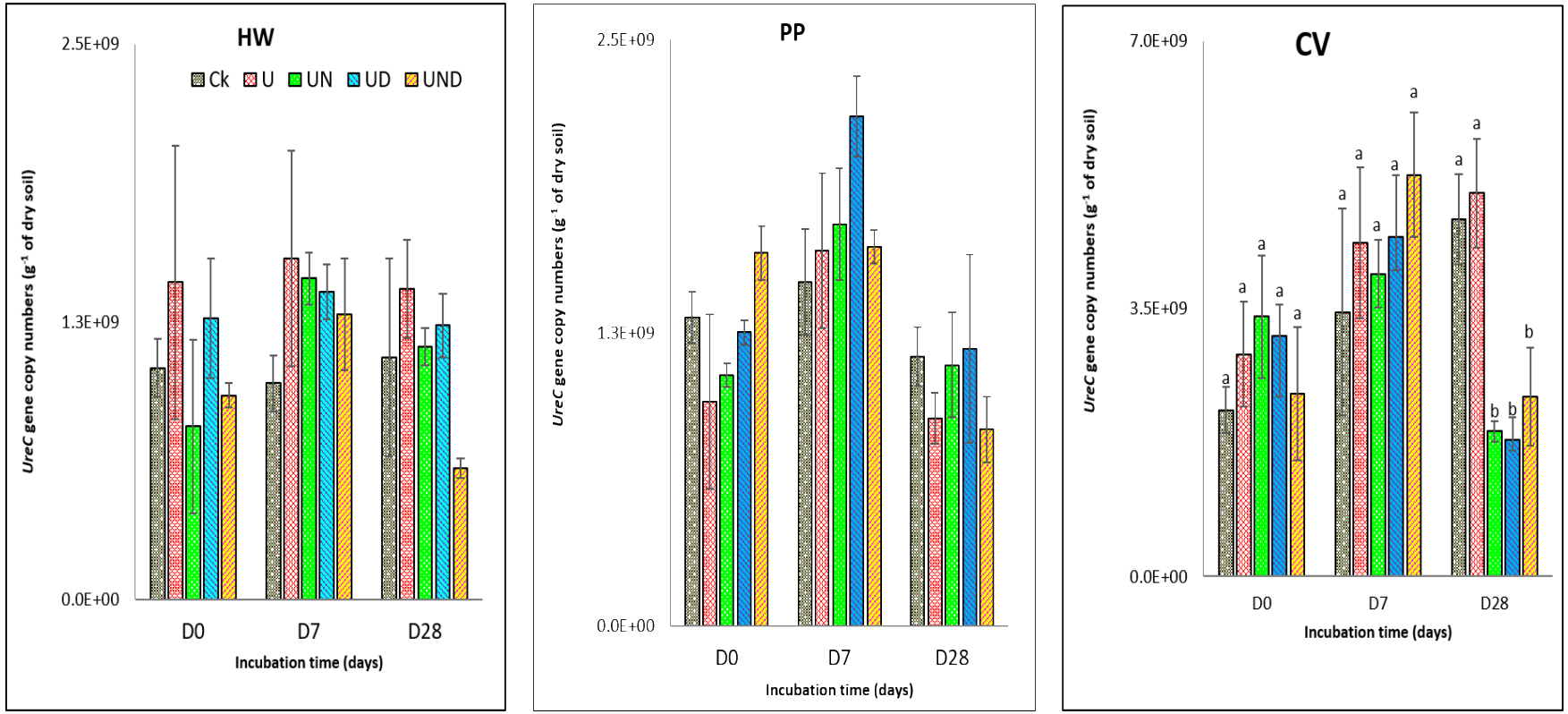

HW
Ck
U
UN
UD
UND
2.5E+09
1.3E+09
0.0E+00
UreC gene copy numbers (g-1 of dry soil)
D0
D7
D28
Incubation time (days)
PP
2.5E+09
1.3E+09
0.0E+00
UreC gene copy numbers (g-1 of dry soil)
D0
D7
D28
Incubation time (days)
CV
7.0E+09
3.5E+09
0.0E+00
UreC gene copy numbers (g-1 of dry soil)
a
a
a
a
a
a
a
a
a
a
a
a
b
b
b
D0
D7
D28
Incubation time (days)

Fig. S3

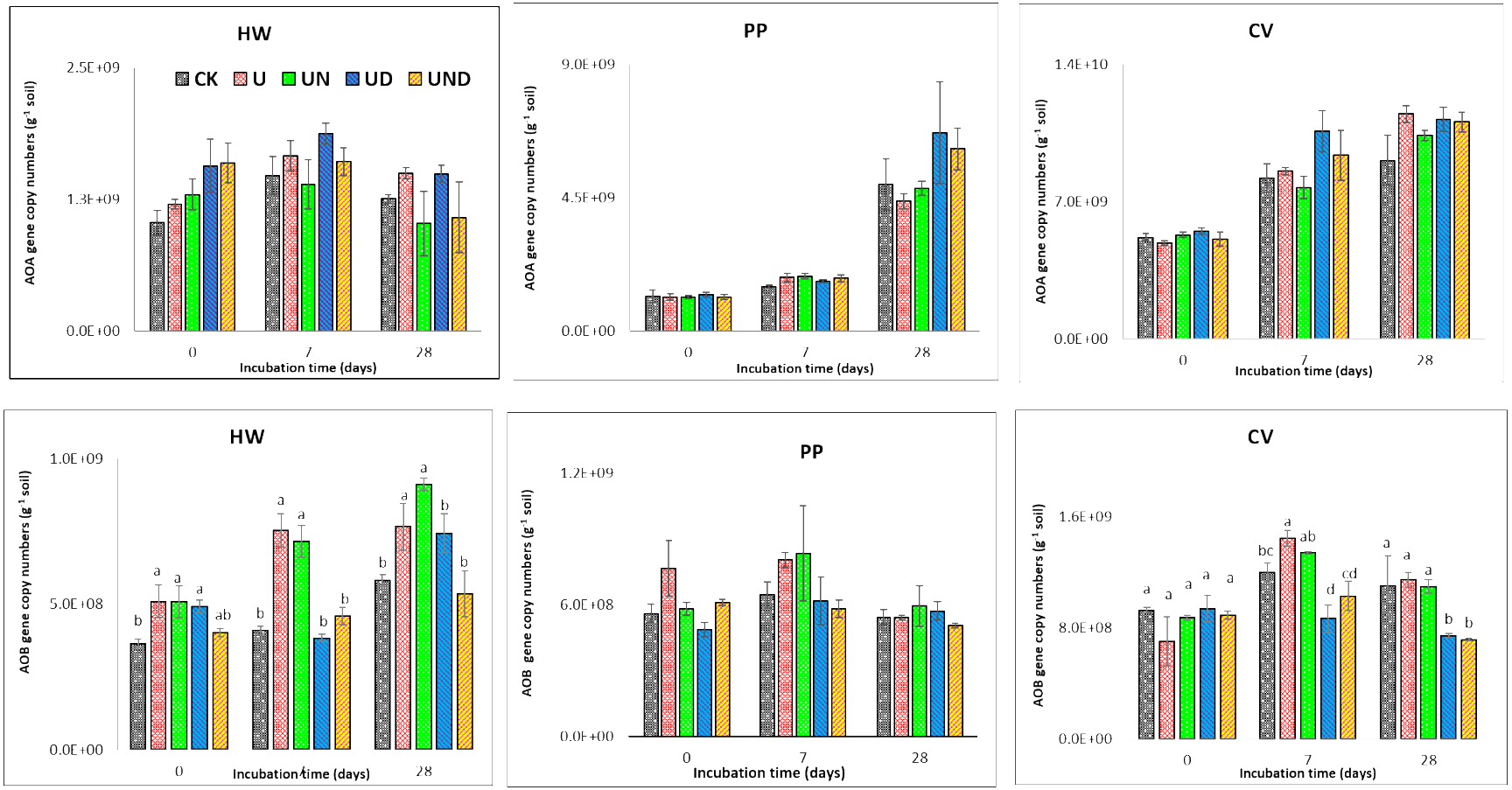

HW
PP
CV
CK
U
UN
UD
UND
AOA gene copy numbers (g-1 soil)
AOB gene copy numbers (g-1 soil)
2.5E+09
1.3E+09
0.0E+00
9.0E+09
4.5E+09
1.4E+10
7.0E+09
1.0E+09
5.0E+08
1.2E+09
6.0E+08
1.6E+09
8.0E+08
0
7
28
Incubation time (days)

Fig. S4.

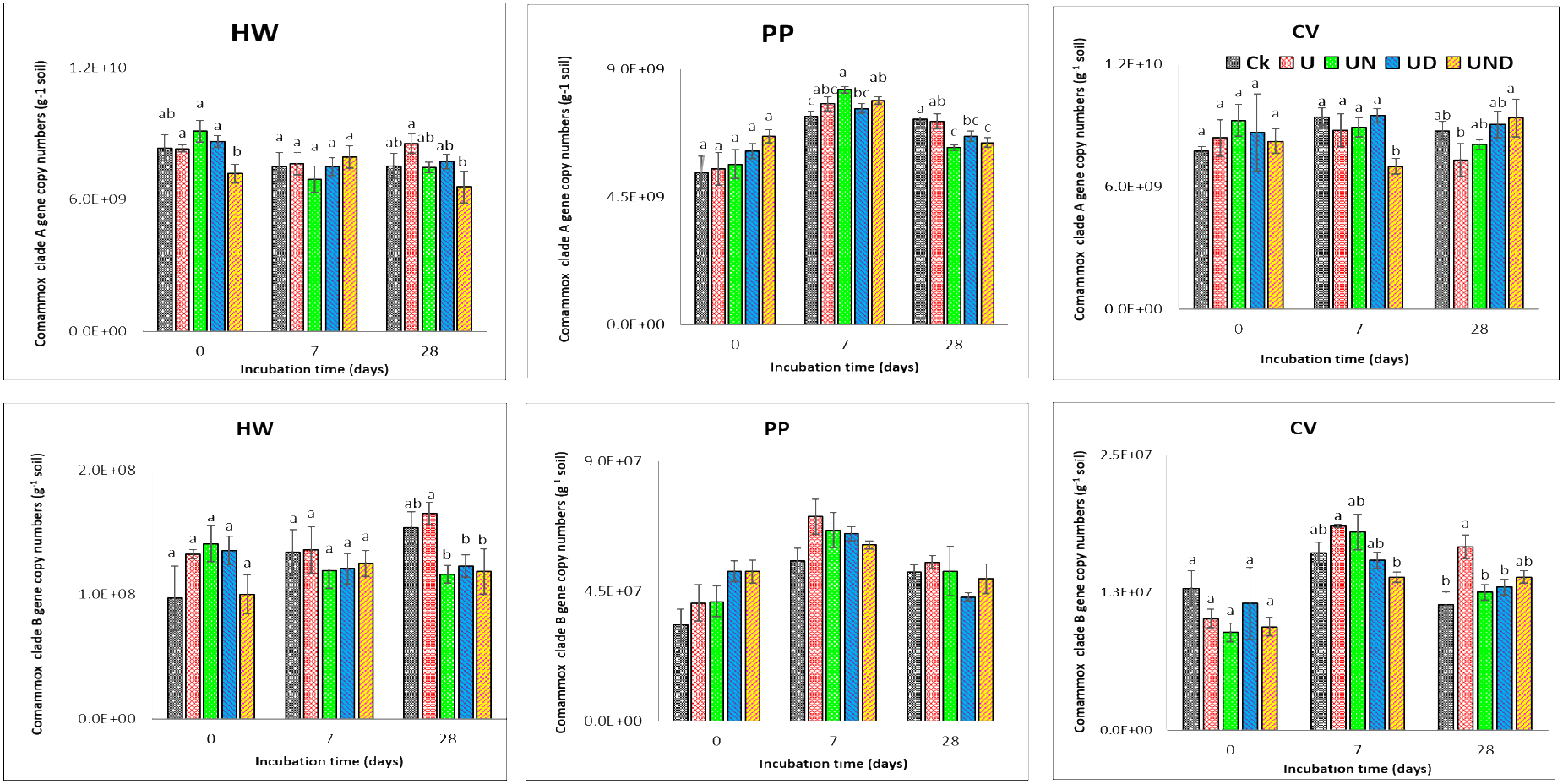

HW
PP
CV
Ck U UN UD UND
Comammox clade A gene copy numbers (g-1 soil)
Comammox clade B gene copy numbers (g-1 soil)
Incubation time (days)
0
7
28
1.2E+10
6.0E+09
0.0E+00
9.0E+09
4.5E+09
2.0E+08
1.0E+08
9.0E+07
4.5E+07
2.5E+07
1.3E+07

Fig. S5

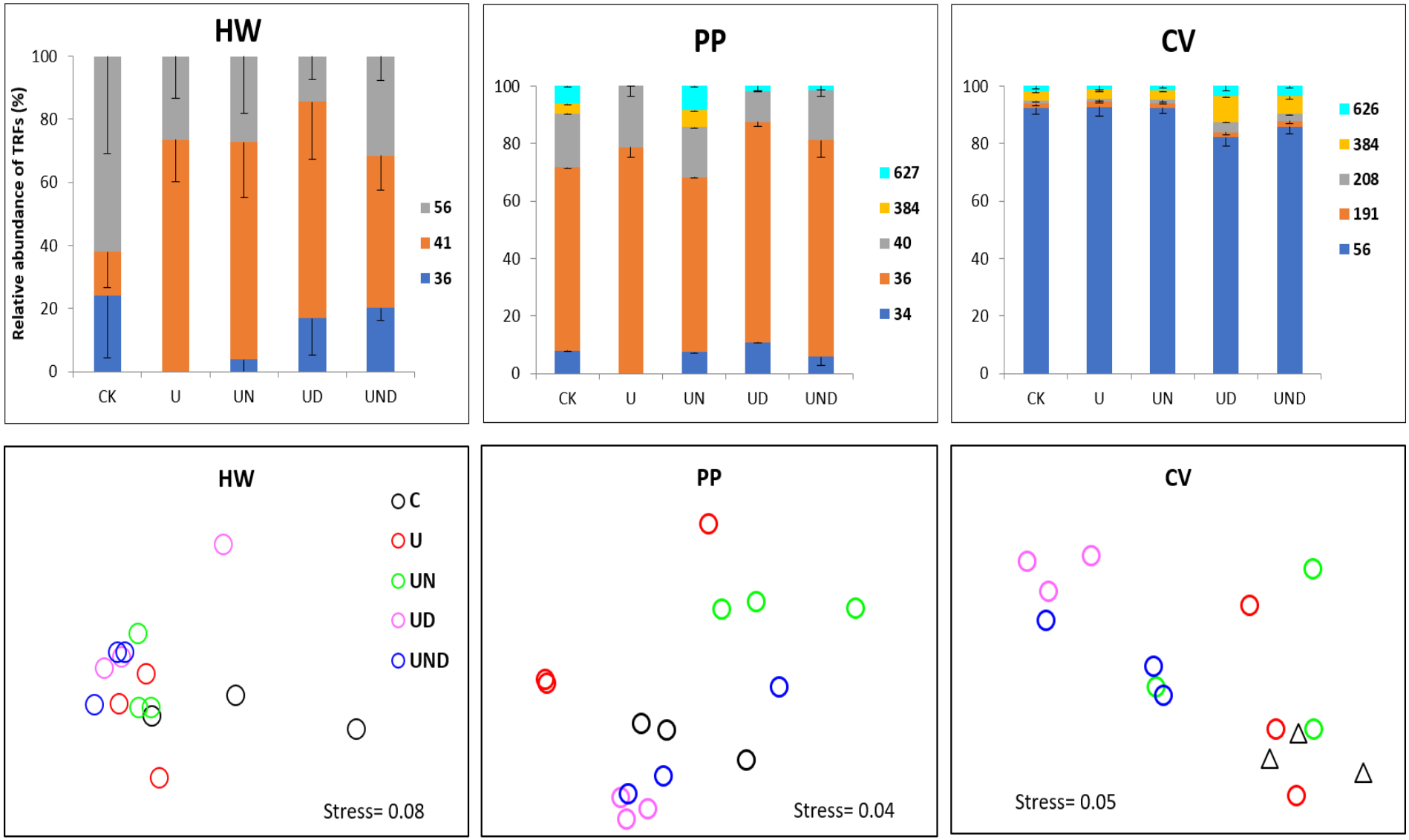
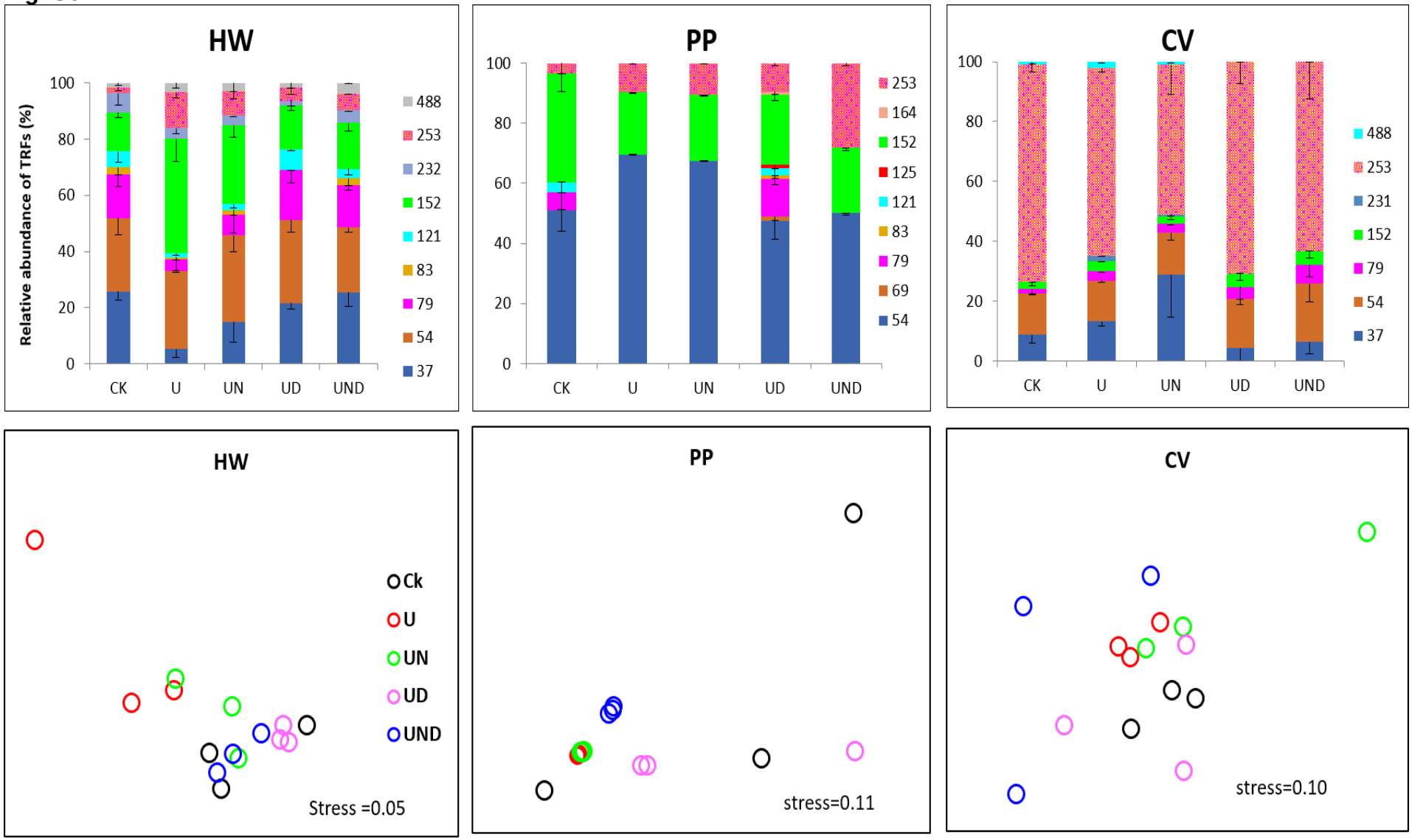

HW
Relative abundance of TRFs (%)
100
80
60
40
20
0
CK
U
UN
UD
UND
56
41
36
PP
627
384
40
36
34
CV
626
384
208
191
56
HW
C
U
UN
UD
UND
Stress= 0.08
PP
Stress= 0.04
CV
Stress= 0.05

Fig. S6


HW
PP
CV
Relative abundance of TRFs (%)
CK
U
UN
UD
UND
488
253
232
231
164
152
125
121
83
79
69
54
37
Ck
U
UN
UD
UND
Stress =0.05
stress=0.11
stress=0.10

Chapter 4. Effects of repeated applications of urea with DMPP on ammonia oxidizers, denitrifiers, and non-targeted microbial communities of an agricultural soil in Queensland, Australia

This chapter is based on an article published in Applied Soil Ecology 147: 103392

Applied Soil Ecology 147 (2020) 103392

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Effects of repeated applications of urea with DMPP on ammonia oxidizers, denitrifiers, and non-targeted microbial communities of an agricultural soil in Queensland, Australia

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ARTICLE INFO

Keywords:
3,4-dimethylpyrazole phosphate (DMPP)
Ammonia-oxidizing archaea
Ammonia-oxidizing bacteria
Comammox
Bacterial community

ABSTRACT

Nitrification inhibitors have been reported to reduce nitrous oxide emission and nitrate leaching in agricultural systems. The effects of repeated applications of urea alone or in combination with nitrification inhibitors on nitrogen (N) cycling microbes involved in nitrification and denitrification together with non-targeted microbes are not well understood. Therefore, the objective of this study was to investigate the effects of repeated application of urea and DMPP on soil physio-chemistry, ammonia oxidizers and total bacteria in the soil. We collected soil samples from a 4.5-year field experiment under crop rotation with repeated application of seven treatments, namely control (CK), Urea (U), Urea + DMPP (UE) applied at 40, 80 and 120 kg N ha^{-1}, each treatment with three replicates. Ammonia-oxidizing bacteria (AOB) gene copy numbers increased as the N application rate increased (from 0 to 120 kg N ha^{-1}). The use of DMPP significantly reduced AOB and *nirK* gene copy numbers compared to urea alone at an application rate of 120 kg N ha^{-1}. There was no treatment effect on the abundance of ammonia-oxidizing archaea (AOA), Comammox clade A and B, *nosZ* and bacterial *16S rRNA* genes. The community composition of AOB and AOA changed with N addition and use of DMPP but increasing N addition rate changed the composition of AOB only. Addition of N increased potential nitrification rates at 80 and 120 kg N ha^{-1}. There was no significant treatment effect on the relative abundance of bacteria at the phylum level. This experiment demonstrated that the application of N (with or without DMPP) at rates lower than 120 kg N ha^{-1} would not result in significant impacts on soil archaeal and bacterial ecology.

1. Introduction

Soil microorganisms are important players in nutrient transformations and maintenance of soil functions (Aislabie et al., 2013; Brevik et al., 2015; Bei et al., 2018). However, they are highly sensitive to agricultural management practices like applications of fertilizers (Eo and Park, 2016; Shen et al., 2016; Chen et al., 2019) and can be used as indicators for soil quality (Sharma et al., 2010).

High input of nitrogen (N) fertilizers has been used to increase crop yields (Duan et al., 2014). However, there is low crop nitrogen use efficiency because applied N fertilizer can be lost through ammonia (NH_3) volatilization, nitrous oxide (N_2O) emissions, nitrate (NO_3^-) leaching, erosion, and runoff processes (Chen et al., 2008; Castaldelli et al., 2019; Fuertes-Mendizábal et al., 2019). Nitrification has previously been thought to be a two-step process involving ammonia oxidation and nitrite (NO_2^-) oxidation. Ammonia oxidation to NO_2^- is the rate-limiting step of nitrification, regulated by the *amoA* gene encoding the alpha subunit of the NH_3 monooxygenase (AMO) within ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Gao et al., 2016; Fuertes-Mendizábal et al., 2019; Miao et al., 2019). Nitrite oxidation to NO_3^- is the second step of nitrification and is regulated by nitrite-oxidizing bacteria (NOB). Recently, a group of bacteria within the *Nitrospira* genus was discovered with the capacity to completely oxidize NH_3 to NO_3^- in a single organism (comammox *Nitrospira*) (Daims et al., 2015; van Kessel et al., 2015v. Denitrification involves the conversion of NO_3- back to N_2O and dinitrogen gas (N_2) via NO_2^-, nitric oxide (NO) (Kuypers et al., 2018; Castaldelli et al., 2019). Nitrous oxide can also be formed through nitrification by the chemical decomposition of hydroxylamine (NH_2OH) (Fuertes-Mendizábal et al., 2019). Denitrification is catalyzed by nitrate reductase encoded by the

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https://doi.org/10.1016/j.apsoil.2019.103392
Received 15 July 2019; Received in revised form 7 October 2019; Accepted 15 October 2019
Available online 05 November 2019

narG gene, nitrite reductase encoded by *nirS / nirK* gene, nitric oxide reductase encoded by *norB* and nitrous oxide reductase encoded by the *nosZ* gene (Braker and Tiedje, 2003; Shrewsbury et al., 2016).

Nitrification inhibitors are compounds that delay the oxidation of ammonium (NH_4^+) to NO_3^- thereby preventing N losses through nitrification and denitrification (Suter et al., 2016; Fuertes-Mendizábal et al., 2019). 3, 4-Dimethylpyrazole phosphate (DMPP) is one of the nitrification inhibitors that has gained commercial use (Zerulla et al., 2001).

Fertilizer application has been shown to cause short and long-term effects on soil physicochemical properties which in turn influence soil microbial communities (Shen et al., 2016; Dai et al., 2018). Although several studies have investigated the effects of repeated N fertilizer application on soil microbial communities (Geisseler and Scow, 2014; Zhou et al., 2015; Shen et al., 2016), little information is available on the response of soil nitrifying, denitrifying and non-targeted microbes (i.e., who are not supposed to involve in nitrogen cycling processes) following repeated application of N fertilizers with nitrification inhibitors (Shi et al., 2017).

We measured changes in soil chemical properties, and the structure and composition of soil bacterial and N-cycling microbial functional communities, following the repeated applications of urea (U), and Urea + DMPP (UE) at different N rates for 4.5 years. The objective of this study was to investigate the effects of repeated application of urea and DMPP on soil physio-chemistry, ammonia oxidizers and total bacteria in the soil. We tested the following hypotheses to achieve our objectives: a) Repeated applications of urea and DMPP will significantly reduce soil pH and increase soil total carbon (TC) and total nitrogen (TN); and b) The levels of soil TC, TN, and pH will decrease bacterial composition and increase AOB and *nirK* gene copy numbers in the soil. This study will contribute to our understanding of how agricultural N management practices influence soil microbial ecosystems and their biological functions.

2. Materials and methods

2.1. Site description and experimental design

Colonsay experimental site (27°28′S 151°23′E) is situated in the Formartin district of the Darling Downs, southern Queensland. Southern Queensland, Australia. The soil in the area was a grey vertisol (Soil Survey Staff, 2014). The soil was classified as clay with 30.1 % sand, 11.4% silt and 58.5 % clay content. The area receives an average annual rainfall of 530.8 mm (2013–2018, Bureau, 2019).

The experiment was established in 1985. Since 2013 when DMPP treated urea (ENTEC®) was introduced into this experiment, the site has been under rainfed production with a crop rotation of wheat (*Triticum aestivum* L.), cotton (*Gossypium hirsutum* L.), grain sorghum (*Sorghum bicolor* (L.) *Moench*), mungbean (*Vigna radiata*) and barley (Hordeum vulgare L.) in that order. The experiment had a completely randomized block design with three replicates that had 7 treatments including 3 nitrogen rates (40, 80, and 120 kg N ha^{-1}) with (as ENTEC®) or without DMPP, and the control (no nitrogen, no DMPP), established on 13*2 m^2 plots. Nitrogen was applied as urea. Urea + DMPP treatments were applied as fertilizer brand ENTEC® (Incitec Pivot), granulated urea containing < 0.15% DMPP (based on DMPP: urea N). Before each cropping season, treatments were applied in the pre-plant season, i.e., on 10th May 2013 prior to wheat sowing; on 22nd July 2014 prior to cotton sowing; 10th August 2015 prior to grain sorghum; 30th June 2016 prior to mungbean sowing; and on 7th June 2017 prior to the barley sowing. The plots also received 10 kg P ha^{-1} as triple superphosphate applied at the planting date of each season (in the seed trench in contact with seed). An estimated starting soil water of approximately 65 mm was recorded from the on-site rain gauge on 6th June 2017 before barley sowing. Barley was sown on 32 cm rows at a rate of 65 kg ha^{-1} to a depth of approximately 6.5 cm on 7th June 2017, using minimum tillage Janke parallelogram coulter disc, spear point tines, and press wheel assemblies on small plot equipment. Five rows of barley spaced 32 cm apart were sown between 2 m wheel centers across each plot. Post-emergence herbicides were applied on 14th July 2017. The barley crop was harvested on 23rd November 2017. The total growing season rainfall from planting to harvest was 153 mm

2.2. Soil sampling

Following harvesting, soil samples were collected on 30th November 2017 from the treatment plots at a depth of 0–10 cm with an auger of 7.6 cm internal diameter by taking 5 cores from the plant line or stubble row per plot and homogenized to form one composite sample for each plot. After sampling, the soil was transported on ice to the lab. A five (5) g subsample from each soil sample was taken for potential nitrification rate (PNR) measurement (extraction was done within 1 week of sampling), and 500 g of soil for physiochemical analysis. Subsamples stored at −20 °C immediately and used for soil DNA extraction and molecular analyses.

2.3. Analysis of soil chemical properties

Soil pH was determined at a ratio of 1:5 (weight/volume, soil: water) with a pH meter (Mettler Toledo Switzerland). Soil total carbon (TC) and total nitrogen (TN) were analyzed on the elemental analyzer (Leco Trumac CN) using the Dumas digestion method. Ammonium and NO_3^--N concentration were extracted at a ratio of 1:5 (w:v, 5 g fresh soil: 25 ml 2 M KCl). The extracts were filtered through Whatman paper (42), after shaking for one (1) hour at 175 rpm followed by calorimetric analysis using a segmented flow analyser (Skalar SAN + +).

2.4. Potential nitrification rates measurement

Soil potential nitrification rates (PNR) were determined according to the chlorate inhibition method (Hu et al., 2015). Briefly, fresh soil samples (5 g) were placed in 50-mL falcon tubes with 20 ml ammonium sulfate (1 mM). Potassium chlorate with a final concentration of 10 mM was added to the tubes to inhibit nitrite oxidation. The falcon tubes were covered with parafilm with small holes for aeration and incubated in the dark at 25 °C for 24 h, and then nitrite was extracted with 10 ml of 2 M KCl. The supernatant was measured by spectrophotometry at a wavelength of 540 nm with N-(1-naphthyl) ethylenediamine and sulfonic acid).

2.5. Soil DNA extraction

DNA was extracted from 0.25 g of each individual soil sample using the MoBio PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA USA) following the manufacturer's instructions with slight modifications where a fast prep beating system (Bio-101 Vista CA, USA) at a speed of 5.5 ms^{-1} for 30 s was used for the initial cell lysis step (Hu et al., 2015). The DNA concentration was assessed photometrically using the NanoDrop® ND-2000c Spectrophotometer UV–vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.6. Quantitative PCR analysis of N–cycling functional genes

Abundances of the nitrifying, denitrifying and *16S rRNA* genes were quantified on a Bio-Rad CFX384 optical real-time PCR detection system (Bio-Rad, Laboratories Inc., Hercules, CA, USA) using the primer sets and thermal conditions shown in Table 1. The 10-µl reaction mixture contained 5 µl of Sensimix (Bioline Sydney, NSW Australia), 0.25 µl of each primer (10 µM), and 1 µl of template DNA. Standard curves were generated using 10-fold serial dilutions of plasmids containing correct inserts of the target genes. Melting curve analysis was performed

Table 1
The primers and thermocycling programs used for quantification of the N- cycling Functional genes and *16S rRNA* gene.

Primers	Sequence		Length bp	References	Thermocycling conditions
Bacterial 16 S *rRNA*					10 min at 95 °C, 40 cycles of (30 s at 95 °C, 45 s at 55 °C, and 45 s at 72 °C), 10 min at 72 °C.
1369F	CGGTGAATACGTTCYCGG	100		Suzuki et al. (2000)	
1492R	CGGTGAATACGTTCYCGG				
AOA *amoA*				Tourna et al. (2008)	
CrenamoA-23f	ATGGTCTGGCTWAGACG	629			
CrenamoA-616r	GCCATC CATCTGTATGTCCA				
AOB *amoA*					10 min at 95 °C, 40 cycles of (30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C), 10 min at 72 °C.
amoA- 1 F	GGGGTTTCTACTGGTGGT		491	Rotthauwe et al. (1997)	
amoA-2R	CCCCTCKGSAAAGCCTTCTTC				
nirK					
nirK876	ATY GGC GGV CAY GGC GA		470	Bárta et al. (2010)	
nirK1040	GCC TCG ATC AGR TTR TGG TT				
Comammox clade A					10 min at 95 °C, 25 cycles of (30 s at 94 °C, 45 s at 42-52 °C, and 60 s at 72 °C), 10 min at 72 °C.
comaA-244F	TAYAAYTGGGTSAAYTA		415	Pjevac et al. (2017)	
comaA-659R	ARATCATSGTGCTRTG				
Comammox clade B					
comaB-244F	TAYTTCTGGACRTTYTA		415	Pjevac et al. (2017)	
comaB-659R	ARATCCARACDGTGTG				
NosZ 1					10 min at 95 °C, 40 cycles of (15 s at 95 °C, 15 s at 60 °C, 30 s at 72 °C, 15 s at 82 °C), 10 min at 72 °C.
nosZ1F,	WCSYTGTTCMTCGACAGCCAG		259	Henry et al. (2006)	
nosZ1R	ATGTCGATCARCTGVKCRTTYTC				

between 72 and 94.5 °C at the end of each amplification assay to evaluate the specificity of quantitative PCR (qPCR), and the amplification efficiencies for all qPCR runs ranged between 80 and 110% with R^2 of 0.99.

2.7. Terminal restriction fragment length polymorphism (T-RFLP) analysis

T-RFLP analysis of the ammonia-oxidizing microbes was performed on extracted DNA using targeted marker gene PCR amplification separately focusing on the *amoA* genes AOA, and AOB using the fluorescently labeled primers (Hu et al., 2015).

A 25 µl PCR reaction mixture contained 2 µl of diluted template DNA (1–10 ng), 0.5 µl of each primer (10 µM), 5 µl MyTaq buffer, 1.5 U of MyTaq polymerase (Bioline, Sydney, Australia). The PCR reaction was conducted using the primer sets and thermal cycling conditions shown in Table 1. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA, USA) and quantified using the NanoDrop ND-2000c Spectrophotometer. The restriction digestion was carried out in a 10 µl mixture containing 200 ng of purified PCR products, 0.1 µl of BSA, 1 µl of ×10 NE buffer, and 5U of the restriction enzymes MspI for AOB; RsaI (BioLabs, Sydney, Australia) for AOA. The digests were incubated at 37 °C for 3 h and denatured for 10 min at 95 °C. Terminal restriction fragments (TRFs) were size separated with an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, CA, USA) and analyzed using a local southern size calling method (peaks > 50 bp) and a peak amplitude threshold setting of 50, using GeneMapper version 4.0 (Applied Biosystems). TRFs with peak height comprising less than 1% of the total peak height were removed, and peaks that differed by less than 1bp were combined into the same TRF (Hu et al., 2015). The relative fluorescent abundances of all TRFs were exported for further analysis of the community composition.

2.8. Illumina sequencing and analysis of 16S rRNA gene

The V_4 region of bacterial *16S rRNA* was selected for amplification with primers 515 F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) (Caporaso et al., 2011) with Illumina adapter overhang sequences attached. The reaction was carried out in 25 µl mixtures containing 12.5 µl red mix, 0.5 µl each primer and 2 µl template. After the initial enzyme activation for 10 min at 95 °C, 30 cycles of the following program were used for amplification (20 s at 95 °C, 20 s at 55 °C, and 20 s at 72 °C), 5 min at 72 °C, on the Bio-Rad C1000 Touch thermal cycler (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The success of the PCR was confirmed by 2% gel electrophoresis and cleaned using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA, USA). A second PCR was conducted using a 50 µl reaction volume containing 10.5 µl red mix, 5 µl each of index 1 and 2, and 5 µl of the cleaned PCR product as template DNA. The second PCR was conducted under the conditions of 10 min at 95 °C, 8 cycles of (20 s at 95 °C, 20 s at 55 °C, and 20 s at 72 °C), 5 min at 72 °C. The PCR success was again confirmed on a 2% agarose gel and PCR products purified again using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA, USA). The final library was made by mixing all PCR products in equimolar ratios and quantified using the JetSeq library quantification Lo-ROX Kit (Bioline) then sequenced on an Illumina MiSeq sequencer. Samples were rarefied to a sequence depth of 4806 to ensure 3 replicates per sample and correction of the differences due to sequencing efforts before downstream analysis.

2.9. Statistical analysis

Data are represented as the means of three replicates. Sequence analysis was done using Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010). The gene copy numbers were calculated using the equation described in (Behrens et al., 2008). Data were analysed using analysis of variance (ANOVA) at $p < 0.05$ followed by the Fisher test to compare treatment means only if there was a significant effect as shown by ANOVA in Minitab 18 statistical software. Pearson's correlation was performed to assess the correlation between the soil microbial communities and soil physicochemical properties.

3. Results

3.1. Effects of repeated urea with DMPP applications on soil chemical properties and potential nitrification rates

Repeated applications of urea with or without DMPP changed soil pH, TN, TC, NH_4^+-N, NO_3^--N and PNR ($p < 0.05$) (Table 2). Soil pH ranged from 9.08 to 7.83. Addition of N significantly reduced soil pH relative to control. The use of DMPP did not affect pH change with N alone addition except when N was applied at higher rates (120 kg Nha^{-1}). Nitrogen addition significantly increased TN compared to Control. Increasing N application rate and use of DMPP had no effect

Table 2
Soil chemical properties and potential nitrification rates (PNR) across the seven treatments.

Treatment	CK	40U	40UE	80U	80UE	120U	120UE
pH	9.08 a†	8.59 b	8.44 b	8.21 c	8.24 c	7.83 d	8.21 c
Total N (g kg^{-1})	0.80 c	0.90 b	0.90 b	0.90 b	0.90 b	1.00 a	0.90 b
Total C (g kg^{-1})	12.10 c	14.10 a	14.20 a	13.50 ab	13.10 b	13.40 ab	12.80 bc
NH_4^+-N (mg kg^{-1})	1.19 b	2.31 a	2.89 a	2.89 a	3.00 a	2.98 a	3.01 a
NO_3^-N (mg kg^{-1})	11.42 c	12.99 c	18.79 bc	35.38 bc	32.55 bc	89.14 a	46.65 b
PNR (mg NO_2^--N $kg^{-1}hr^{-1}$)	0.61 c	0.96 c	1.46 bc	3.50 a	2.60 ab	3.69 a	3.18 a

Treatments: CK- control; U-Urea, and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}. Values are means (N = 3).
† Values within the same row followed by the same letter are not significantly different at $p < 0.05$ (Fisher Test).

($p < 0.05$) on TN except when N was applied at 120 kg N ha^{-1}. The addition of N significantly increased TC and NH_4^+-N levels compared to control but there was no significant effect of N application rate and use of DMPP on the levels of TC and NH_4^+-N. Nitrate concentration did not change between urea and urea + DMPP treatments except at the application rate of 120 kg N ha^{-1}. Addition of N did not change PNR except at the application rate of 80 and 120 kg N ha^{-1}. Use of DMPP did not change PNR at any application rate relative to urea applied alone.

Overall, repeated applications of N with DMPP significantly reduced soil pH and increased soil nutrient status. Higher application rates increased PNR.

3.2. Effects of repeated urea with DMPP applications on ammonia oxidizers, denitrifiers, and 16S rRNA gene copy numbers

AOB gene copy numbers did not change with N addition relative to control. Increasing N application rate to 120 kg N ha^{-1} significantly increased AOB gene copy numbers. The use of DMPP did not change AOB gene copy numbers compared to urea alone except at the application rate of 120 kg N ha^{-1} where DMPP significantly reduced AOB gene copy numbers compared to N alone (Fig. 1a). The addition of N did not change AOA, Comammox clade A and Comammox clade B genes copy numbers relative to control. Increasing the N application rate or use of DMPP did not change AOA (Fig. 1b), Comammox clade A (Fig. 1c), and Comammox clade B genes copy numbers (Fig. 1d).

AOB gene copy numbers were significantly negatively correlated with pH and positively correlated with TN, NH_4^+-N, NO_3^--N concentration, and PNR. No significant relationship was found between AOA, comammox clade A and B with soil chemical properties and PNR at the sampling time (data not shown).

nirK gene copy numbers did not change with N addition except at the application rate of 120 kg N ha^{-1}. Use of DMPP did not change *nirK* gene copy numbers compared to urea alone except at the application rate of 120 kg N ha^{-1} (Fig. 2a). The addition of N did not change *nosZ* and *16S rRNA* gene copy numbers relative to control (Fig. 2 b and 2c). Increasing the N application rate or use of DMPP did not change *nosZ* and *16S rRNA* gene copy numbers (Fig. 2 b and 2c).

These results indicated that only AOB and *nirK* genes were responsive to repeated applications or N with DMPP.

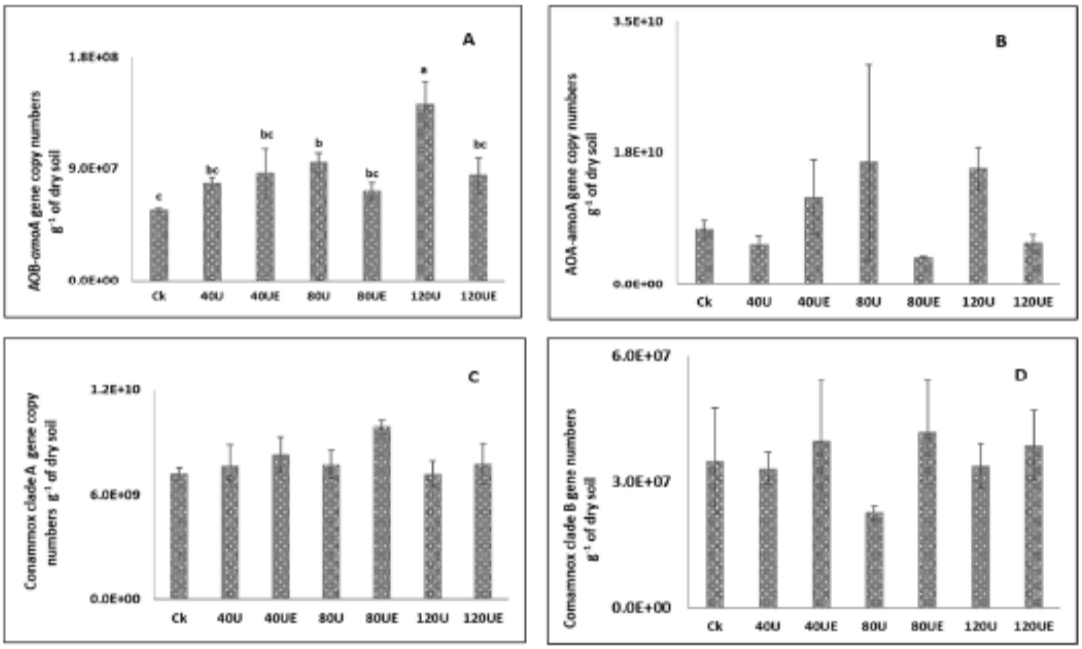

Fig. 1. The abundance of AOB (A), AOA (B), Comammox clade A (C) and Comammox clade B (D) genes across the seven treatments: CK, control; U, Urea; and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}, at Colonsay. Error bars represent standard error of three replicates. Means that do not share a letter are significantly different at $p < 0.05$ level (Fisher Test). Note that y-axes scales differ between charts. There were no significant differences across treatments on AOA (A), Comammox clade A (C) and clade B (D) gene copy numbers.

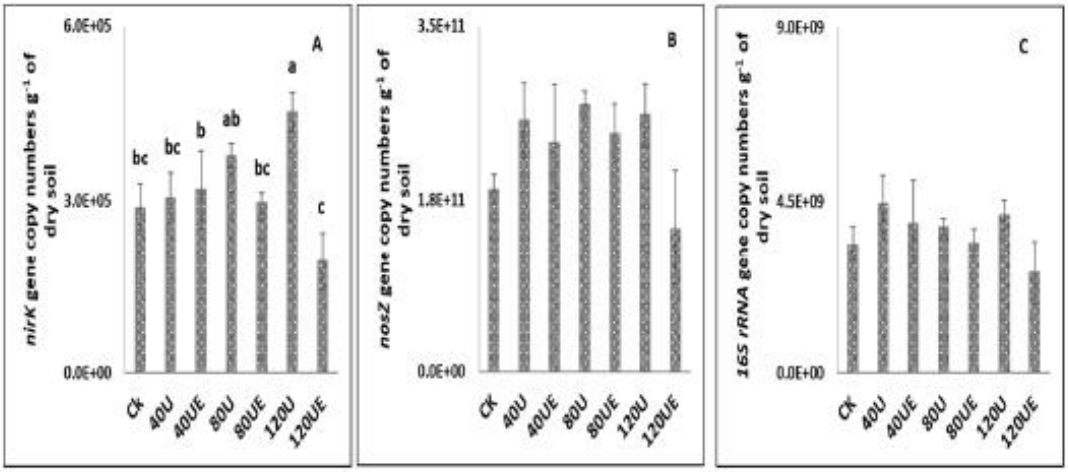

Fig. 2. The abundance of *nirK* (A), *nosZ* (B), and bacterial *16S rRNA* (C) genes across the seven treatments: CK, control; U, Urea; and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}, at Colonsay. Error bars represent standard error of three replicates. Means that do not share a letter are significantly different at $p < 0.05$ level (Fisher Test). Note that y-axes scales differ between charts. There were no significant differences across treatments on *nosZ*, *16S rRNA* gene copy numbers.

3.3. Effects of repeated Urea with DMPP application on community composition of AOA and AOB ammonia oxidizers

Digestion of the AOA *amoA* gene by the RsaI enzyme for TRFLP analysis produced 5 specific fragments of which TRFs 34 bp and 36 bp were the most abundant across all the treatments. The addition of N reduced and increased the relative abundance TRF 34 bp and TRF 36bp respectively compared to control. Increasing N application rate did not change the relative abundance of TRF 34 bp and 36 bp. The use of DMPP increased the relative abundance of TRF 34 bp and 36 bp at all application rates compared to N alone, and TRF 56 bp at an application rate of 40 and 80 kgN ha^{-1} respectively compared to N alone. (Fig. 3a).

Digestion of the AOB *amoA* by the MspI restriction enzyme for TRFLP analysis produced 6 fragments with TRFs 37 bp and 55 bp being the most abundant. The addition of N reduced the relative abundance of TRF 37 bp compared to Control. Increasing N application reduced the relative abundance of TRF 37 bp. The use of DMPP increased the relative abundance of TRF 37 bp compared to when N was applied alone at all application rates. The addition of N increased the relative abundance of TRF 55 bp compared to control. The use of DMPP reduced the relative abundance of TRF 55 bp compared to N alone at all application rates. High rates of N increased the number of fragments (Fig. 3b).

3.4. Effects of repeated urea with DMPP applications on soil bacterial community composition

The bacterial community within all treatments at Colonsay produced 189,692 sequences (5101–13627 sequences per sample). A total of 33 phyla, 379 families and 612 genera were shared among the 7

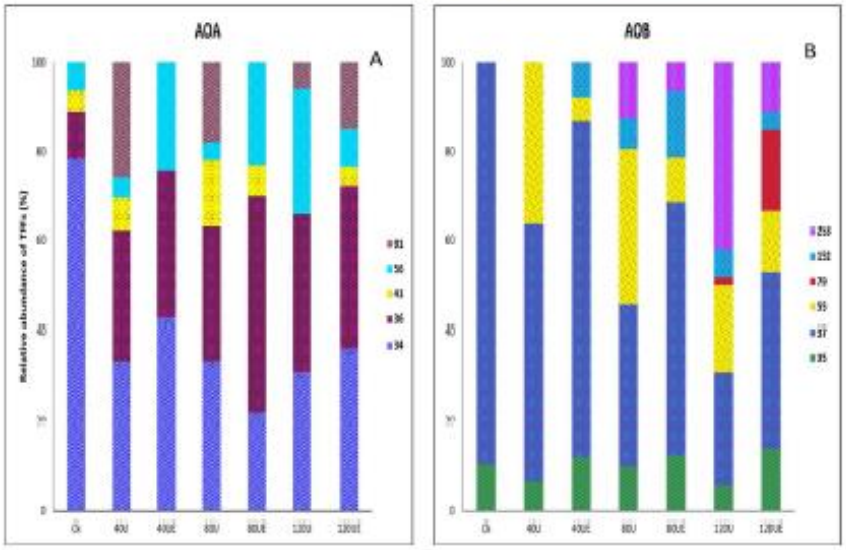

Fig. 3. Terminal restriction fragment length polymorphism (T-RFLP) fingerprints of the AOA *amoA* gene (A) digested using the RsaI enzyme and the AOB *amoA* gene (B) digested using the MspI enzyme across the seven treatments: CK, control; U, Urea; and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}, at Colonsay.

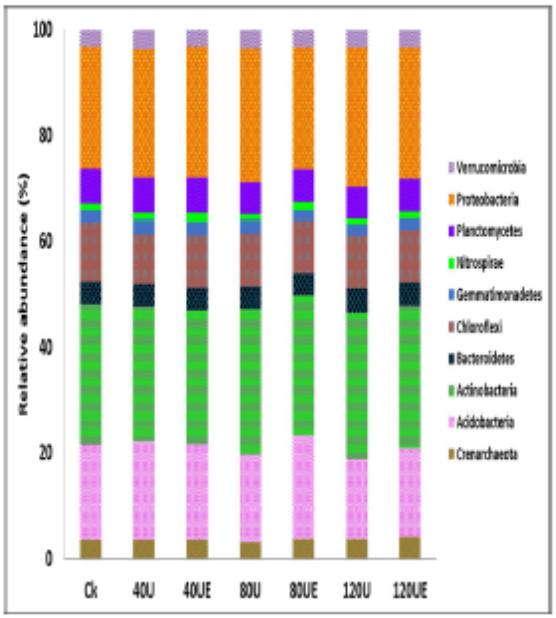

Fig. 4. Relative abundance of the dominant phyla (with abundance > 1% in at least one treatment, only identified sequences classified under a specific taxon were considered) across the seven treatments; CK, control; U, Urea and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}) at Colonsay.

treatments. At the phylum level, the microbial community was composed of 10 main phyla that were most abundant across the treatments (with > 1% mean in relative abundance in at least one treatment) (Fig. 4). The most abundant phyla included Actinobacteria which occupied about 25.2–27.6%, and *Proteobacteria* with 23.0–26.4% of the total bacterial sequences, followed by *Acidobacteria* (15.0–19.6%), *Chloroflexi* (9.3–11.2%) *Planctomycetes* (6.0–6.7%), *Crenarcheota* (3.2–4.1%), *Verrucomicrobia* (3.1–3.6%), *Bacteroidetes* (4.1–4.6%), *Gemmatimonadetes* (2.2–2.7%), *Nitrospirae* (1.1–1.7%) (Fig.4). There were no significant treatment effects on the relative abundance of soil microbial communities at the phylum level (Fig. 4). However, analysis at the class level in the abundant phyla revealed some significant treatment effects. At the class level, the relative abundance of *Actinobacteria and Thermoleophilia* classes of phylum *Actinobacteria* were significantly influenced by the N application compared to CK (Table 3). The addition of N did not change the relative abundance of *Actinobacteria* class except at the application rate of 80 and 120 kg N ha^{-1} relative to control (Table 3). Increasing the N application rate did not change the relative abundance of *Actinobacteria* class except at the application rate of 120 kg N ha^{-1}. Use of DMPP did not change the relative abundance of *Actinobacteria* class compared to N alone except at the application rate of 120 kg N ha^{-1} (Table 3). The relative abundance of *Thermoleophilia* class (of *Actinobacteria* Phylum) was significantly reduced by N addition compared to control (Table 3). Increasing N addition did not significantly change the relative abundance of *Thermoleophilia* class except at an application rate of 120 kg N ha^{-1}. Use of DMPP did not significantly change the relative abundance of *Thermoleophilia* class except at an application rate of 120 kg N ha^{-1} (Table 3). Class *TK10* (*Chloroflexi* phylum) was significantly reduced by the application of N compared to control (Table 3). There was no significant effect of increasing N application and use of DMPP on the relative abundance of *TK10* class members relative to urea (Table 3).

The relative abundance of *Proteobacteria* phylum was negatively correlated with pH ($p < 0.05$) and positively correlated with NO_3^--N concentration ($p < 0.05$) (Table 4). *Acidobacteria* phylum was negatively correlated to NO_3^--N concentration ($p < 0.05$). At the class level, the relative abundance *Actinobacteria* was negatively correlated to pH ($p < 0.01$), and positively correlated to TN ($p < 0.01$) and NO_3^--N concentration ($p < 0.01$). The relative abundance of *Thermoleophilia* class was positively correlated to pH ($p < 0.01$) and NH_4^+-N concentration ($p < 0.05$), but negatively correlated to TN ($p < 0.01$), NO_3^--N concentration and TC ($p < 0.05$). *TK10* class (of *Chloroflexi* phylum) was positively correlated with pH and NH_4^+-N concentration but negatively correlated with TN ($p < 0.01$) (Table 4). Overall, repeated applications of N and DMPP did not influence bacterial composition at the phylum level but resulted in such changes at higher resolution taxonomic levels within some members of *Actinobacteria* and *Chloflexi* phyla. These changes in bacterial composition were controlled by soil pH, TN, NH_4^+-N, and NO_3^--N concentration.

4. Discussion

4.1. Effects of repeated urea with DMPP on soil physicochemical properties

The reduction in soil pH with N addition increased N application rate, and the use of DMPP indicated that repeated application of N and DMPP could lead to soil acidification. A reduction in soil pH due to repeated fertilizer application (Guo et al., 2010; Schroder et al., 2011; Dai et al., 2018) or increasing N application rate in repeated fertilizer application experiments (Zhou et al., 2015; Shen et al., 2016; Chen

Table 3
The relative abundance of soil microbial communities across the seven treatments.

Phylum	Class	CK	40U	40UE	80U	80UE	120U	120UE
Actinobacteria		25.71 a†	24.73a	24.48a	26.67a	25.74a	27.02a	26.05 a
	Actinobacteria	12.12 c	13.70 bc	12.99 c	16.29 ab	14.05 bc	17.62 a	14.45bc
	Thermoleophilia	10.54 a	8.59 b	8.90 b	8.46 bc	9.37 b	7.44 c	9.15 b
Proteobacteria		22.32 a	23.62 a	24.13 a	24.45 a	22.37 a	25.66 a	24.15 a
Acidobacteria		17.96 a	18.56 a	17.98 a	16.46 a	19.57 a	15.04 a	16.76 a
Chloroflexi		10.88 a	9.04 a	9.72 a	9.71 a	9.60 a	9.61 a	9.59 a
	TK10	2.60 a	1.81 b	1.97 b	1.81 b	1.79 b	1.78 b	1.69 b
Planctomycetes		6.38 a	6.43 a	6.52 a	5.80 a	6.00 a	5.89 a	5.91 a
Crenarchaeota		3.45 a	3.46 a	3.56 a	lang="EN-AU" > 3.05 alang="EN-AU" >	lang="EN-AU" > 3.61 lang="EN-AU" > a	lang="EN-AU" > 3.57 a	4.01 a
Verrucomicrobia		3.11 a	3.52 a	3.03 a	3.44 a	3.24 a	3.16 a	3.24 a
Bacteroidetes		4.12a	4.24a	4.18a	4.07a	3.96a	4.42a	4.32a
Gemmatimonadetes		2.40a	2.60a	2.44a	2.43a	2.11a	2.15a	2.35a
Nitrospirae		1.24 a	1.42 a	1.69 a	1.28 a	1.40 a	1.09 a	1.21 a

Treatments: CK- control; U-Urea, and UE, Urea + DMPP applied at rates 40, 80 and 120 kg N ha^{-1}. Only for phyla with relative abundance > 1% shown. Only classes affected by treatment application within the abundant phyla were included. Values are means (N = 3).

† Values within the same row followed by the same letter are not significantly different at $p < 0.05$ (Fisher Test).

Table 4
Pearson's correlation between soil properties and the relative abundance of soil bacterial communities at different taxonomic levels.

Phylum	Class	Soil properties pH (1:S_{water})	Total N g kg^{-1}	Total C g kg^{-1}	NH_4^+-N mg kg^{-1}	NO_3^-N mg kg^{-1}
Actinobacteria		−0.30	0.22	−0.12	−0.29	0.22
	Actinobacteria	−0.63**	0.60**	0.12	−0.32	0.67**
	Thermoleophilia	0.70**	−0.81**	−0.48*	0.52*	−0.51*
Proteobacteria		−0.44*	0.40	0.16	−0.11	0.48*
Acidobacteria		0.41	−0.34	0.02	0.15	−0.50*
Chloroflexi		0.35	−0.28	−0.30	0.37	−0.15
	TK10	0.61**	−0.68**	−0.43	1**	−0.16
Planctomycetes		0.39	−0.19	0.14	0.24	−0.36
Crenarchaeota		−0.02	−0.14	−0.08	−0.15	0.02
Verrucomicrobia		−0.08	0.23	0.32	−0.23	−0.10
Bacteroidetes		−0.12	−0.04	−0.20	0.10	0.297
Gemmatimonadetes		0.19	−0.11	0.11	0.13	−0.13
Nitrospirae		0.18	−0.17	0.22	0.08	−0.24

**significant at the 0.01 probability level.
*Significant at the 0.05 probability level. Only classes affected by treatment application within the abundant phyla were included.

et al., 2019) has been reported. The significant increase in soil pH level by DMPP compared to N alone at a rate of 120 kg N ha^{-1}, indicated the ability of DMPP to counter the extent of pH reduction by N as reported by other authors (Shi et al., 2017).

Application of N alone or with DMPP increased TN, TC, NH_4^+-N, and NO_3^--N and concentration compared to CK. An increase in NO_3^--N, and NH_4^+-N concentration due to increasing N application rate in repeated experiments have been reported (Zhou et al., 2015; Shen et al., 2016; Chen et al., 2019). The significant increase in NO_3^--N at only 120 kg N ha^{-1} could imply that the N applied at the rate of 120 kg N ha^{-1} was in excess of the plant requirement as compared to other N rates therefore, there was N build up in the system in form of NO_3^--N.

The significant low NO_3^--N in DMPP treatment at 120 kg N ha^{-1} compared to N alone at the same rate indicated that although nitrification continued after the DMPP efficacy period, the NO_3^--N remained lower in DMPP treatment compared to urea alone. However, this may not be because of DMPP since sampling was done 5 months after treatment application, which was longer compared to the reported 100 days of conservation of mineral N by DMPP (Duncan et al., 2017). Future experiments should include quantifying of the inhibitor compounds to ascertain their longevity at field level as this was not done in the current experiment.

4.2. Effects of repeated urea with DMPP on the N-cycling functional groups

Ammonia oxidizers are key players to N cycling, involved in the first and rate-limiting step of nitrification (Carey et al., 2016). Our results generally indicated that AOB are more responsive to increased N addition, or changes in soil pH, TN, and NO_3^- accumulation compared to AOA, (O'Callaghan et al., 2010; Carey et al., 2016; Ouyang et al., 2018) and Comammox. Further, a significant correlation between NO_3^--N concentration and PNR with AOB gene copy numbers (data not shown) implied that AOB could have a greater influence on nitrification in repeated fertilizer applied soils (nutrient-rich soils) than AOA and Comammox. Therefore, the significant difference between AOB gene copy numbers in N alone and N + DMPP at a rate of 120 kg N ha^{-1} would indicate the ability of DMPP to inhibit nitrification by suppressing the growth of AOB genes under repeated application of N and N + DMPP. Other researchers reported similar findings and showed an increase in AOB gene copy numbers due to repeated application of urea alone or a decrease when urea was applied with DMPP (Shi et al., 2017). However, in our case, the results were seen only at a higher application rate of 120 kg N ha^{-1}, which could be linked to the fact that, at this application rate, the N was more than plant needs as explained above. This would imply that at this rate, there was more N than the plant needed resulting in more N build up in the system in the form of NO_3^--N. DMPP prevented NH_3 oxidation by inhibiting the activity of AOB.

nirK genes have been reported to be contained in AOB and are responsible for the nitrifier denitrification process, therefore, the *nirK* genes amplified in this study could be contained in AOB (Shaw et al., 2006; Cantera and Stein, 2007; Di et al., 2014). This finding was evidenced by the similar trends of AOB and *nirK* gene copy numbers at an application rate of 120 kg N ha^{-1} and the fact that only *nirK* abundance was influenced by treatment application but not *nosZ* genes.

4.3. The effect of repeated urea with DMPP application on soil bacterial communities

The lack of significant treatment effects of N addition, application rate and DMPP on the relative abundance of soil total bacteria at the phylum level would suggest that repeated application of N with or without DMPP was not detrimental to soil bacterial composition.

Although no significant treatment effect on the relative abundance of soil bacteria was reflected at the phylum level, correlation analysis revealed that soil NO_3-N concentration was significantly negatively correlated with the relative abundance of *Acidobacteria* phylum (Table 4). Our results are in line with the report of inhibition of *Acidobacteria* by N application from a recent review (Dai et al., 2018), which supports the theory that considers *Acidobacteria* to be oligotrophic (Fierer et al., 2007; Eilers et al., 2010; Fierer et al., 2012).

The significant negative and positive correlation of soil pH and NO_3-N concentration respectively with the relative abundance of *Proteobacteria* phylum was in line with reports by other researchers that *Proteobacteria* prefers nutrient-rich soils (Fierer et al., 2012; Zhou et al., 2015; Dai et al., 2018). This is because the increase in N addition in our experiment significantly reduced soil pH.

At lower taxonomic levels, we found significant treatment effects on the composition of some members of the major phyla (Table 3). The increase in the relative abundance of the members of *Actinobacteria* phyla with an increase in N application rates is in line with the previous reports that classified *Actinobacteria* as fast-growing Copiotrophs stimulated in high nutrient environments (Fierer et al., 2007, 2012). These results were also confirmed with the significant positive correlation of the relative abundance of *Actinobacteria* class with TN, and NO_3^--N (Table 4).

The response of *Thermoleophilia* class and *Actinobacteria* class was different although they are both members of *Actinobacteria* phylum. This indicated that the response to environmental disturbances by the same members of a given taxon can be different (Zeng, 2016). This could also be attributed to the specific responses of different subgroups to different soil chemical properties (Eo and Park, 2016). In this study, for example, correlation analysis showed that the relative abundance of

Thermoleophilia class correlated to soil pH, TN, TC, NH_4^+-N, and NO_3^-N in the opposite way to that of *Actinobacteria* class. Other researchers have reported such trends for different lower taxa of other phyla (Eo and Park, 2016; Zeng, 2016). Class *TK10* of *Chloroflexi* phylum decreased when N was added with or without DMPP application regardless of the application rate. The negative correlation of the relative abundance of this subgroup with TN concentration and NO_3^--N further indicated the effect of N addition on *Chloroflexi*. Our findings are in line with the reports of a decrease in the relative abundance of the members of the *Chloroflexi* phylum with the addition of N fertilizer by other researchers (Fierer et al., 2012; Zhou et al., 2015; Eo and Park, 2016). The reduction of *Chloroflexi* taxa has been speculated to be due to competition from the bacteria that have been stimulated under high N, or due to the changed soil chemical properties (Eo and Park, 2016).

It is expected that changes realized at lower taxonomic levels would be reflected at higher taxonomic levels (Zeng, 2016). However, the lack of reflection of these changes at higher levels, despite their presence at lower levels of some taxa, could be due to the lower relative abundance of the changed groups. For instance, at the phylum level, no significant changes were reflected in the *Chloroflexi* and *Actinobacteria* although some of their members at lower levels were significantly influenced by the treatments.

5. Conclusion

It can be concluded that the relatively long term between the treatment application and the sampling date was a key player affecting the soil microbial community composition and size, as NIs and N fertilizer effects on soil physio-chemistry and microbial communities are strongest shortly after application. Therefore care should be taken while interpreting our results as the focus and our findings differ from those of short-term experiments.

This study revealed that when applied at low N rates of 40 kg N ha-1, urea, and DMPP application did not pose negative effects to soil ecosystems. However, at higher application rates of 80 and 120 kg N ha-1, urea and DMPP altered soil bacterial composition at higher resolution taxonomic levels. The application rate of N was shown to be an important factor that contributed to shifting composition, size, and function of N cycling microbial communities (particularly AOB) in repeated applications. For sustainable long-term maintenance of soil microbial composition and function, lower fertilizer rates of 40 kg N ha-1 should be encouraged in repeated N application experiments. This study has improved our understanding that the application of urea and DMPP at lower rates can be used without negatively affecting microbial ecology.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgment

We acknowledge the financial support from the Australian Research Council (LP160101134). We thank the Trace Analysis for Chemical, Earth, and Environmental Sciences (TrACEES), The University of Melbourne, for analytical support.

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Chapter 5. Temporal response of ureolytic and ammonia-oxidizing microbes and pasture yield to urea and NBPT at Leigh Creek of Victoria in Australia

This chapter is based on an article that has been submitted to the Journal of Applied Soil Ecology for possible publication.

Title: **Temporal response of ureolytic and ammonia-oxidizing microbes, and pasture yield to urea and NBPT at Leigh Creek, Victoria, Australia**

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Highlights

- The repeated use of N and Green Urea NV® significantly reduced the abundance of ureolytic microbes relative to control.
- The addition of Green Urea NV® significantly reduced *ureC* gene copy numbers relative to N alone at application rate of 40 kg N ha^{-1} on sampling days 7 and 45.
- The addition of Green Urea NV® did not significantly affect the abundance of ammonia oxidizers.
- Green Urea NV® had no significant effect on pasture DM yield, N-uptake or NUE.

Abstract

The urease inhibitor *N*-(*n*-butyl) thiophosphoric triamide (NBPT) has been reported to effectively reduce nitrogen (N) losses by inhibiting urea hydrolysis. However, the effect of NBPT on soil ureolytic and ammonia-oxidizing microbes is not well understood, with inconsistent effects on crop yield and nitrogen use efficiency (NUE). A field experiment was conducted at Leigh Creek, Victoria at a site with a history of repeated application of urea alone (40U) or with NBPT (as Green Urea NV® (40GU)) at 40 kg N ha^{-1}, and urea applied at 80 kg N ha^{-1} (80U), to perennial ryegrass *(Lolium perenne* L.). We aimed to investigate the effects on temporal dynamics of ureolytic and ammonia-oxidizing microbes, pasture dry matter (DM) yield and NUE within a season following treatment applications. The abundance of ureolytic microbes was higher in control (CK) compared to all N treatments on all sampling days. The *ureC* gene copy numbers in 40 GU were significantly lower than that of 40U on sampling days 7 and 45. NBPT had no significant effect on the abundance of ammonia oxidizers, but increasing urea application rate significantly increased the abundance of ammonia-oxidizing bacteria (AOB) on days 7 and 45, and complete ammonia oxidizers (comammox *Nitrospira*) clade B on day 7 compared to 40U. NBPT had no significant effect on pasture DM yield, N-uptake, or NUE. Increasing N application rate significantly increased pasture DM yield and N- uptake but this did not influence the pasture NUE.

Keywords: Green Urea NV®, NBPT, Nitrogen use efficiency, comammox *Nitrospira*, Ammonia-oxidizing Archaea, Ammonia- oxidizing bacteria.

5.1. Introduction

By 2050, the world population is expected to increase by 2-3 billion (DESA, 2017; Foley, 2011). This implies an expected increase in demand for agricultural land and N fertilizers (Tilman et al., 2001). However, much of the applied fertilizer nitrogen (N) is lost from the plant-soil system through ammonia (NH_3) volatilization and nitrous oxide (N_2O) emissions (Castaldelli et al., 2019; Chen et al., 2008; Fuertes-Mendizábal et al., 2019), causing adverse environmental and economic consequences (Galloway et al., 2008; Stark and Richards, 2008).

The N cycling process is largely microbially mediated. For instance, once urea fertilizer is applied on soil, it quickly undergoes hydrolysis which is catalysed by the urease enzyme, producing ammonium (NH_4^+) or ammonia (NH_3) depending on pH (Singh et al., 2008). The *ureC* gene that encodes the alpha subunit of the urease enzyme has been used to identify and study the functional ureolytic microbial communities (Wang et al., 2018). The NH_3 then undergoes oxidation to nitrite (NO_2^-) by ammonia-oxidizing archaea (AOA) or ammonia-oxidizing bacteria (AOB) using the ammonia monooxygenase (AMO) enzyme encoded by the *amoA* gene. The NO_2^- is then oxidized to NO_3^- by nitrite-oxidizing bacteria (NOB) (Caceres et al., 2018; Hayden et al., 2014; Kits et al., 2017). Recently, comammox organisms in the NOB *Nitrospira* were discovered with the ability to undertake complete nitrification, oxidizing ammonia to NO_3^- (Daims et al., 2015; van Kessel et al., 2015). However, the contribution of comammox organisms to nitrification in many soils is not yet investigated.

Urease inhibitors, such as N-(n-butyl) thiophosphoric triamide (NBPT), are developed to minimize N loss through inhibiting the urease enzyme activity, delaying urea hydrolysis and preventing the elevated pH that drives NH_3 loss (Akiyama et al., 2010; Harty et al., 2016). By inhibiting urea

hydrolysis, NBPT slows the formation of NH_4^+-N, a precursor for nitrification, and therefore could indirectly influence nitrification and subsequent denitrification (Menendez et al., 2009).

Several studies at field and laboratory levels have reported the effect of NBPT on reducing hydrolysis rate and associated NH_3 loss, as well as its contribution to reducing the emissions of N_2O (Salazar et al., 2014; Silva et al., 2017). However, few studies have investigated the effect of NBPT on ureolytic, ammonia-oxidizing, or other N cycling microbes (Shi et al., 2017; Xi et al., 2017). Conflicting results exist regarding the impact of NBPT on yield and NUE, with no significant (Abalos et al., 2012; Dougherty, 2016) to positive effect (Dawar, 2011; Vistoso, 2012). The effects of repeated application of urea and NBPT on soil N cycling; microbial communities and yield is important as this has implications on soil health.

We investigated the effects of urea application rate and the use of NBPT on a) the abundance of soil N-cycling microbial communities involved in urea hydrolysis and nitrification, and b) pasture dry matter (DM) yield, N uptake, and nitrogen use efficiency (NUE). We hypothesized that: i) increasing the urea application rate would increase the abundance of ureolytic and nitrification organisms, ii) application of NBPT would reduce ureolytic and nitrification microbial abundance by inhibiting urea hydrolysis, and iii) use of NBPT would improve pasture DM yield, N uptake, and NUE by delaying the supply of plant-available forms of N and reducing the risk of N loss as NH_3.

5.2. Materials and methods

The experimental site, soil sampling, and analysis

The field experiment commenced in mid-February 2018 on an established long-term experimental site with perennial ryegrass (*Lolium perenne* L.) pasture at Leigh Creek, Victoria (37°55'S,

143°95'E). The area has a mean annual rainfall of 686.9 mm with a mean maximum and minimum annual temperatures of 17.4 ^{o}C and 7.1 ^{o}C respectively (BOM, 2019). The soil contains 27.1% clay, 26.7% silt, and 46.2 % sand in the topsoil (0-10 cm) and is classified as a Chromosol (Isbell, 2016). Prior to the commencement of the experiment, the soil properties in the 0-10 cm layer were measured and are as follows: pH_{water} of 6.0, organic carbon of 3.0 %, NH_4^+-N and NO_3^--N of 5.1 and 20 mg kg^{-1} soil respectively, cation exchange capacity of 12 cmol(+)/kg. The field experiment had a history of repeated surface application of urea and NBPT coated urea (Green Urea NV®) under the treatments of control (CK), granular urea (46% N) applied at 40 kg N ha^{-1} (40U) and 80 kg N ha^{-1} (80U), and urea coated with the urease inhibitor NBPT (as Green Urea NV® at 40 kg N ha^{-1} (Agrotain® @ 5 L t^{-1} urea)) (40GU). The proportion of NBPT in the GU was 0.14% N on a weight basis. The experiment was established using a completely randomized block design with each treatment replicated thrice and had the first application of treatments on 5th October 2014.

On 19th February 2018, the plots (4 x 1 m) received the treatments above after pasture had been harvested to a height of 5 cm. Irrigation was applied on day 0 (19th February 2018), day 2 and day 17 after treatments were applied. Soil samples (0-10 cm) were collected on days 0 (1-3 hours), 1, 2, 3, 4, 5, 6, 7, 10, 13, 17, 24, 31, 38, and 45 by taking 3 cores (7.6 cm-diam.) per plot to form a composite sample, and were transported to the laboratory on ice. The collected soil samples were analyzed for physicochemical properties and microbial composition (stored at -20^{o}C for DNA extraction). Gravimetric water content was determined by oven drying sub-samples at 105 °C for 24 h. Five grams of soil were used for soil pH determination on a ratio of 1:5 (weight/volume, soil: water) with a pH meter (Mettler Toledo Switzerland). Soil urea, NH_4^+-N, and NO_3^--N were extracted from 5 g of soil using a ratio of 1:5 (fresh soil: 2 M KCl- Phenyl Mercuric acetate (PMA),

w/v) by shaking at 175 rpm for 1 h. The solution was filtered through a Whatman 42 filter paper and measured by a Segmented Flow Analyzer (SAN++, Skalar).

Plant sampling and analysis

The pasture was harvested on day 45 (4th April 2018) from a 4 x 0.7m (2.8 m^2) area within each plot, leaving 5 cm of stubble on the ground. Pasture dry matter (DM) was determined by oven-drying of the subsamples at 60 °C until constant weight (Rayment and Higginson, 1992). The dried samples were then ground and passed through 2 mm sieves for analysis of total N to calculate pasture N uptake and NUE. Shoot total N was determined by the Dumas combustion method (LECO-Trumac). The shoot N uptake and NUE were calculated using the equations below:

Shoot N uptake (kg N ha^{-1}) = Shoot DM (Kg ha^{-1}) x N concentration in plant shoots (%)/100 [1]

$$NUE\ (\%) = (NU_f - NU_0) / N\ rate \times 100 \quad [2]$$

where NU_f and NU_0 are the shoot N uptake in the treatments with and without N fertilizer, respectively (Wang et al., 2019).

Soil DNA extraction

Soil DNA was extracted from 0.25 g of soil sample collected on days 0, 7, and 45, using the MoBio PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA USA) following the manufacturer's instructions with slight modifications where a fast prep beating system (Bio-101 Vista CA, USA) at a speed of 5.5 ms^{-1} for 30s was used for the initial cell lysis step (Hu et al., 2015). The DNA concentration was assessed photometrically using the NanoDrop® ND-2000c Spectrophotometer UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Quantitative PCR analysis of N–cycling functional genes

The abundance of the ureolytic and ammonia-oxidizing microbes was quantified on a Bio-Rad CFX384 optical real-time PCR detection system (Bio-Rad, Laboratories Inc., Hercules, CA, USA) using the primer sets and thermal conditions shown in Table 1. The 20-µl reaction mixture contained 10 µl of Sensimix (Bioline Sydney, NSW Australia), 0.5 µl of each primer (10 µM), and 1 µl of template DNA. Standard curves were generated using 10-fold serial dilutions of plasmids containing correct inserts of the target genes. Melting curve analysis was performed between 72 and 94.5°C at the end of each amplification assay to evaluate the specificity of quantitative PCR (qPCR), and the amplification efficiencies for all qPCR runs ranged between 80 and 110% with R^2 of 0.99.

Statistical analysis

Data are represented as the means of three replicates. The gene copy numbers were calculated using the equation described in (Behrens et al., 2008). Data were analyzed in Minitab18 using a one-way analysis of variance (ANOVA) at $p < 0.05$ followed by the Fisher test to compare treatment means only where there was a significant effect within each sampling day. Pearson's correlation was performed to assess the correlation between the soil microbial communities and soil physical and chemical properties.

5.3. Results

Temporal changes in soil pH and soil N content during the pasture growth period

Generally, soil pH for the whole period in all the treatments ranged from 5.1 to 6.5. The control plots had the highest pH which remained relatively unchanged over the 45 sampling days, followed by 40 U, 40 GU and 80 U (Fig. 1). pH increased in response to fertilizer application over the first

7 days. Despite the rise in pH, the pH of all the fertilizer treatment plots remained lower for most of the time during the experiment compared to control ($p < 0.05$). There was a drop in pH after some days for all the treatments with the highest pH reduction in 80 kg N ha^{-1} urea followed by Green urea NV® (40 kg N ha^{-1}) and lastly, 40 kg N ha^{-1} urea applied alone (Fig. 1).

Urea concentration remained high for all N containing treatments dropping to the control level by day 5 (Fig. 2 A). NBPT had higher urea concentration compared to 40U but it was only significantly higher on day 1. There was a significantly higher urea concentration in 80U compared to 40U for the first 3 days (Fig. 2 A).

The addition of urea significantly increased soil NH_4^+-N concentration up to sampling day 31 compared to the control. The addition of NBPT significantly reduced NH_4^+-N concentration compared to N alone during the sampling period from day 2 to day 4. The NH_4^+-N concentration remained lower for 40GU compared to 40U until day 38 when it became higher. Increasing the N application rate significantly increased soil NH_4^+-N concentration for the sampling period between day 1 and day 7 (Fig. 2 B).

Soil NO_3^--N concentration increased after sampling day 3 to a peak on day 17 before declining. The NO_3^--N concentration was lower than NH_4^+-N concentration. The addition of N significantly increased NO_3^--N concentration compared to the control from day 4 to 31. The addition of NBPT did not significantly alter NO_3^--N concentration compared to urea alone. Increasing N application rate significantly increased NO_3^--N concentration from sampling day 3 (Fig. 2 C).

Temporal changes in N-cycling microbial gene copy numbers

The addition of urea significantly reduced *ureC* gene copy numbers compared to control on all sampling days. Increasing the urea application rate did not significantly change *ureC* gene copy

numbers. The use of NBPT significantly reduced *ureC* gene copy numbers compared to urea alone (Fig.3). Pearson's correlation revealed that soil pH was positively correlated with the *ureC* gene copy numbers on day 7 ($p < 0.05$) and day 45 ($p < 0.01$) of sampling (Table 3).

AOA and comammox *Nitrospira* clade A were not significantly affected by the addition of urea, N application rate, or use of NBPT (Fig.4 A and C). AOB was not significantly affected by using urea alone or with NBPT at an application rate of 40 kg N ha^{-1} but increased with an increasing N application rate (Fig. 4 B).

The addition of N did not significantly increase comammox *Nitrospira* clade B gene copy numbers except for the application rate of 80kg N ha^{-1} on day 7. The use of NBPT did not influence comammox *Nitrospira* clade B gene copy numbers (Fig. 4 D).

Pearson's correlation analysis revealed that NO_3^--N concentration was positively correlated to AOB gene copy numbers on day 7 ($p < 0.01$). Soil pH was negatively correlated to the AOB gene copy numbers on sampling day 7 ($p < 0.05$) and day 45 ($p < 0.01$) (Table 3).

Pasture DM yield, N uptake, and NUE

The addition of N increased pasture DM yield significantly compared to the control. Increasing the N application rate significantly increased pasture DM yield. The use of NBPT did not significantly change pasture DM yield relative to N alone (Table 2). The addition of N increased N uptake significantly relative to control. Increasing the N application rate increased N uptake in the pasture. The use of NBPT did not change pasture N uptake relative to N alone. Increasing the N application rate or the use of NBPT did not significantly influence pasture NUE (Table 2).

5.4. Discussions

Temporal changes in soil pH and soil N content

The initial lower soil pH in the fertilizer treatments compared to the control indicated that repeated applications of urea since 2014 led to acidification, and this was higher with higher inputs of urea. pH reduction due to N-fertilizer addition results from the release of protons during nitrification, and when NH_4^+-N is taken up by crops (Geisseler and Scow, 2014; Tian and Niu, 2015).

The concentration of urea declined quickly in the fertilizer treatments and by day 5, it had reached background levels. This could be linked to NH_3 loss via volatilization due to localized pH increase as a result of hydrolysis. Under acidic conditions, urea can also be taken up preferentially by AOB (described below) (Allison et al., 1991; Marsh et al., 2005). Other factors that might have contributed to such a quick decline in urea concentration include to some extent direct urea uptake by the crop (Dawar, 2011). Although urease enzyme activity was not measured in this experiment, it may have contributed to the quick decline in urea concentration as pasture soils have been reported to have high urease enzyme activity (Suter et al., 2011). The unexpected quick drop of urea concentration in the NBPT treatment could be explained by the rapid degradation of NBPT in acidic conditions of our soil (Engel et al., 2015; Engel et al., 2013). The effect of NBPT has been reported to be low in pasture soils as they have high urease enzyme activity (Suter et al., 2011). However, other factors including the NBPT application rate, soil and environmental factors could also contribute to reduced NBPT efficacy.

The significantly lower NH_4^+-N concentration in NBPT compared to urea alone in the initial days could be attributed to the inhibiting effect of NBPT on urea hydrolysis thereby preventing the rise in pH in the initial days of treatment application that could otherwise lead to N loss via NH_3 volatilization.

The increase in soil NH_4^+-N concentration towards the end of the experiment in the NBPT treatment compared to urea alone could be due to the release of NH_4^+-N initially preserved due to the inhibition of the hydrolysis by NBPT as NBPT activity had been finished. It could also be linked to the lower pH in the NBPT treated soil compared to that of N alone which could influence the equilibrium between NH_3 and NH_4^+ in the soil preventing NH_3 loss as gas and increasing the NH_4^+-N concentration (Sigurdarson et al., 2018). This was confirmed by the negative correlation between soil pH and NH_4^+-N concentration on sampling days 7 and 45 ($p<0.05$, n=12 (Table 3)), which showed that treatments with lower pH had higher NH_4^+-N concentration.

Generally, soil NH_4^+ concentration was higher than NO_3^--N indicating that NO_3^--N was being preferentially taken up by the crops.

Treatment effect on N cycling microbes

The reduction of *ureC* gene copy numbers with N addition is contrary to our expectations but it could be linked to the soil pH effect as the site had repeated application of urea and NBPT which had decreased soil pH. This is supported by the lower *ureC* gene copy numbers in the fertilizer treatments on day 0, compared to the control (Fig. 3) which corresponded to the lower soil pH (Fig. 1) that had occurred due to repeated N fertilization on the site. This pH effect was further confirmed by the positive correlation of *ureC* gene copy numbers with soil pH on day 7 ($p <0.05$, n=12) and day 45 ($p <0.01$, n=12 (Table 3)). In line with our field results, *ureC* gene copy numbers have been shown to be higher in alkaline conditions (Fisher et al., 2017). Compared to urea alone, NBPT significantly reduced *ureC* gene copy numbers on both day 7 and day 45. The delay in urea hydrolysis by NBPT has been reported to last up to 15 days (Cantarella et al., 2018; Khan et al., 2013). Therefore, the reduction in *ureC* gene copy numbers reported in this experiment may not be solely due to the NBPT inhibition to urea hydrolysis as NBPT is not expected to still be active

in soil by day 45. The reduction in *ureC* gene copy numbers may be better explained by the decline in soil pH which was supported by the trend in *ureC* copy numbers of CK> 40U> 40GU (Fig.3), that matches the pH trend in these treatments on day 0, 7 and 45 (Fig.1). Our findings that increasing N rate did not affect *ureC* gene copy numbers contradict previous work where an increase in *ureC* gene copy numbers was observed with increasing N (Zhang et al., 2019). However, the study of Zhang et al. (2019) was undertaken in alkaline soil (pH 7.2 to 8.4) whereas our soil was acidic (pH 5.2 to 6.3), indicating that pH had a much greater effect on *ureC* than N inputs. Additionally, soil microbial communities could respond differently in different soils, accounting for different findings. The reduction in *ureC* gene copy numbers with the addition of urea compared to control or urea + NBPT compared urea alone could imply that repeated application of these treatments may reduce ureolytic activity in this soil as *ureC* gene copy numbers are positively correlated with ureolytic activity in soils (Fisher et al., 2017; Sun et al., 2019).

The increase in AOB and comammox *Nitrospira* clade B gene copy numbers with increasing application rate (Fig. 4 B and D) indicated that these microbes were more responsive to increased nutrient availability in this soil environment compared to AOA and comammox *Nitrospira* clade A (Fig. 4 A and C). In line with our findings, other researchers reported an increase in AOB abundance with increased N input (Jia and Conrad, 2009).

The significant positive correlation between soil NO_3^--N concentration and the AOB gene copy numbers and not AOA/comammox *Nitrospira* implied that AOB were the key players to nitrification in this soil as opposed to comammox *Nitrospira* and AOA. Soil pH has been shown to be a key player in shaping ammonia-oxidizing communities in different environments (Gubry-Rangin et al., 2010; Li et al., 2018). In this study, soil pH was negatively and significantly

correlated to the abundance of AOB but not other ammonia oxidizers (Table 3), implying that soil pH controlled the abundance of AOB and not other ammonia oxidizers. This could be linked to the preferential uptake of urea under acidic conditions by ureolytic AOB leading to NO_3^--N accumulation (Marsh et al., 2005). This phenomenon could explain the increased AOB gene copy numbers in the treatment with an increased N application rate due to increased substrate availability. This indicated that AOB could be favoured by high N inputs in soils that have become acidic due to repeated application of urea N and could be key players to nitrification.

NBPT had no effect on the abundance of ammonia oxidizers which is in line with the reports by other authors (Xi et al., 2017). However, our results contradict the report by Luchibia et al. (2020) from their incubation experiment that NBPT significantly reduced AOB and comammox *Nitrospira*, findings which the authors associated to the inhibition of hydrolysis by NBPT influencing substrate availability for these ammonia oxidizers or the direct effect of NBPT to the intracellular urease within these ammonia oxidizers, influencing their growth. The explanation for the lack of response of ammonia oxidizers to NBPT application on a repeated basis in our study is unclear but it could be due to the lack of susceptibility of ammonia oxidizers under these conditions which could be influenced by other soil or environmental conditions.

Effects on pasture DM yield, N uptake, and NUE

Pasture DM yield and N uptake increased with increased addition of N as expected. The use of NBPT did not increase pasture DM yield, N uptake or NUE compared to N alone, which has previously been reported (Dougherty, 2016; Suter et al., 2013). A previous study reported an increase in pasture DM yield, NUE, and N-uptake with the use of NBPT compared to urea applied alone at two application rates in a pastoral system (Zaman et al., 2013). These authors attributed their findings to reduced hydrolysis and nitrification by NBPT, but we did not observe any

significant contribution of NBPT to nitrification despite it having reduced NH_4^+-N concentration. NBPT was also reported to increase ryegrass NUE, N uptake and yield compared to urea alone and this was attributed to the direct absorption of urea and delayed hydrolysis (Dawar, 2010; Dawar et al., 2012). Some possible explanations for the lack of pasture yield response to NBPT in our study include: first, the initial soil mineral N level could be high such that the conserved N due to NBPT was not sufficient to improve crop yield (Cantarella et al., 2018; Li et al., 2015; Suter et al., 2013). However, the soil N level was unlikely excessive for pasture growth as there was an increase in DM yield, N uptake and NUE with increasing N application rate. Secondly, the N loss in the absence of NBPT could have been small, such that NBPT induced reduction in this loss was not beneficial to pasture yield. Thirdly, other soil factors like pH, texture, moisture and other nutrients or environmental factors like temperature may have limited the response of the pasture growth and yield to NBPT (Lam et al., 2018).

The significant increase in pasture DM yield and N uptake with increasing N application rate was an indication of increased N supply. Our results are in line with those of other authors who reported an increased DM yield and N uptake in ryegrass with increasing N application rate of up to 300 kg Nha^{-1} per year (Mora et al., 2007).

5.5. Conclusion

Our results demonstrated that the application of urea with NBPT reduced the rate of urea hydrolysis and mineral N production but had minimal effect on yield, N-uptake, and NUE. Increasing the urea application rate increased DM yield and N uptake but not NUE. The study also reported that AOB were important players to nitrification in acidic soils with a history of repeated fertilizer and GU application. The recommendation to use urea with NBPT needs further experimentation under different cropping, environmental and soil conditions to understand the

possible contribution of NBPT to crop production in the long-term. It is important for future studies to quantify the effects of repeated /long-term applications of urea applied with NBPT on enzyme activities.

Acknowledgment

We acknowledge the financial support from the Australian Research Council (LP160101134). We thank the Trace Analysis for Chemical, Earth, and Environmental Sciences (TrACEES), The University of Melbourne, for analytical support. We thank the staff of Incitec Pivot, also Mr Robert Impraim, Mr Jose Valentin Palacios Zevallos and Dr Xiufang Gao for their assistance during field sampling.

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Figure Legends

Figure 1. soil pH during the 45-day sampling period across the four treatments: CK, control; 40U, N applied at 40 kg N ha^{-1} as urea; 40GU, NBPT treated urea applied at 40 kg N ha^{-1} as Green Urea NV®; 80U, N applied at 80 kg N ha^{-1} as urea. Error bars represent standard errors from three replicates.

Figure 2. Soil urea (A), NH_4^+-N (B), and NO_3^--N (C), concentration during the 45-day sampling period across the four treatments: CK, control; 40U, N applied at 40 kg N ha^{-1} as urea; 40GU, NBPT treated urea applied at 40 kg N ha^{-1} as Green Urea NV®; 80U, N applied at 80 kg N ha^{-1} as urea. Error bars represent standard errors from three replicates. Note that y-axes scales differ between charts.

Figure 3. The abundance of *ureC* genes across the four treatments: CK, control; 40U, N applied at 40 kg N ha^{-1} as urea; 40GU, NBPT treated urea applied at 40 kg N ha^{-1} as Green Urea NV®; 80U, N applied at 80 kg N ha^{-1} as urea, at three sampling days. Error bars represent standard error of three replicates. Means that do not share a letter within a sampling day are significantly different at $p < 0.05$ level.

Figure 4. The abundance of AOA (A), AOB (B), comammox *Nitrospira* clade A (C), and comammox *Nitrospira* clade B (D) genes across the four treatments: CK, control; U, Urea; and Green Urea NV®, applied at rate 40 kg N ha^{-1}, and urea applied at 80 kg N ha^{-1}, at three sampling days. Error bars represent standard error of three replicates. Means that do not share a letter within a sampling day are significantly different at $p< 0.05$ level.

Figure 1.

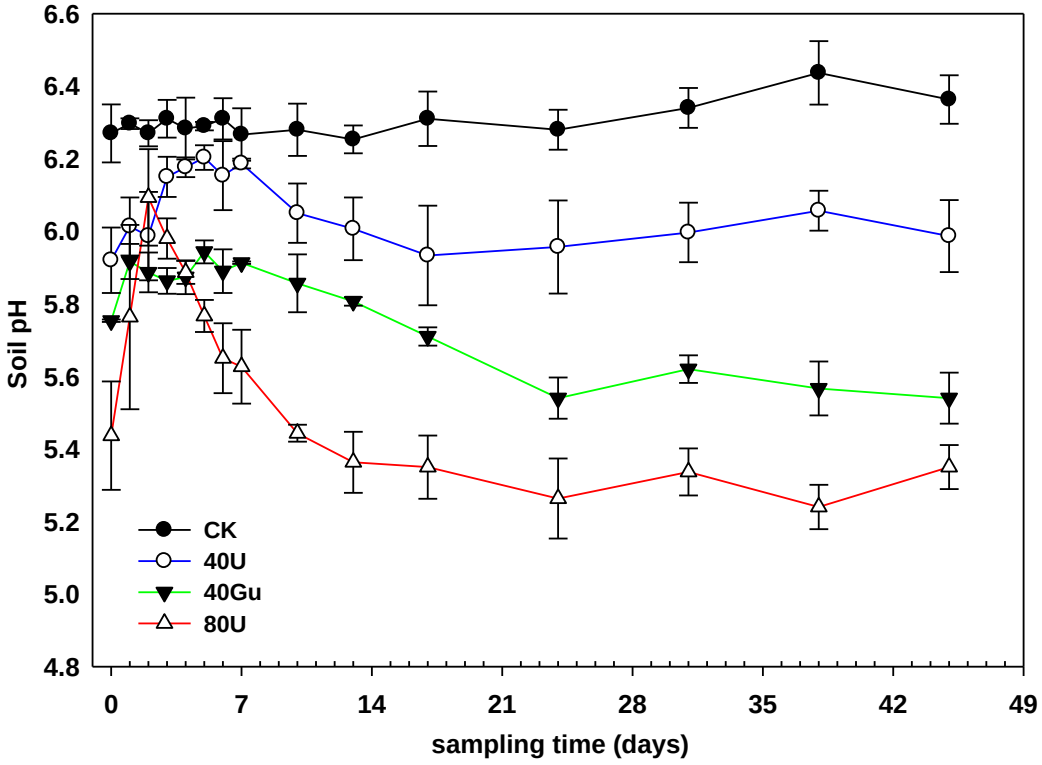
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
Soil pH
0
7
14
21
28
35
42
49
sampling time (days)
CK
40U
40Gu
80U

Figure 2.

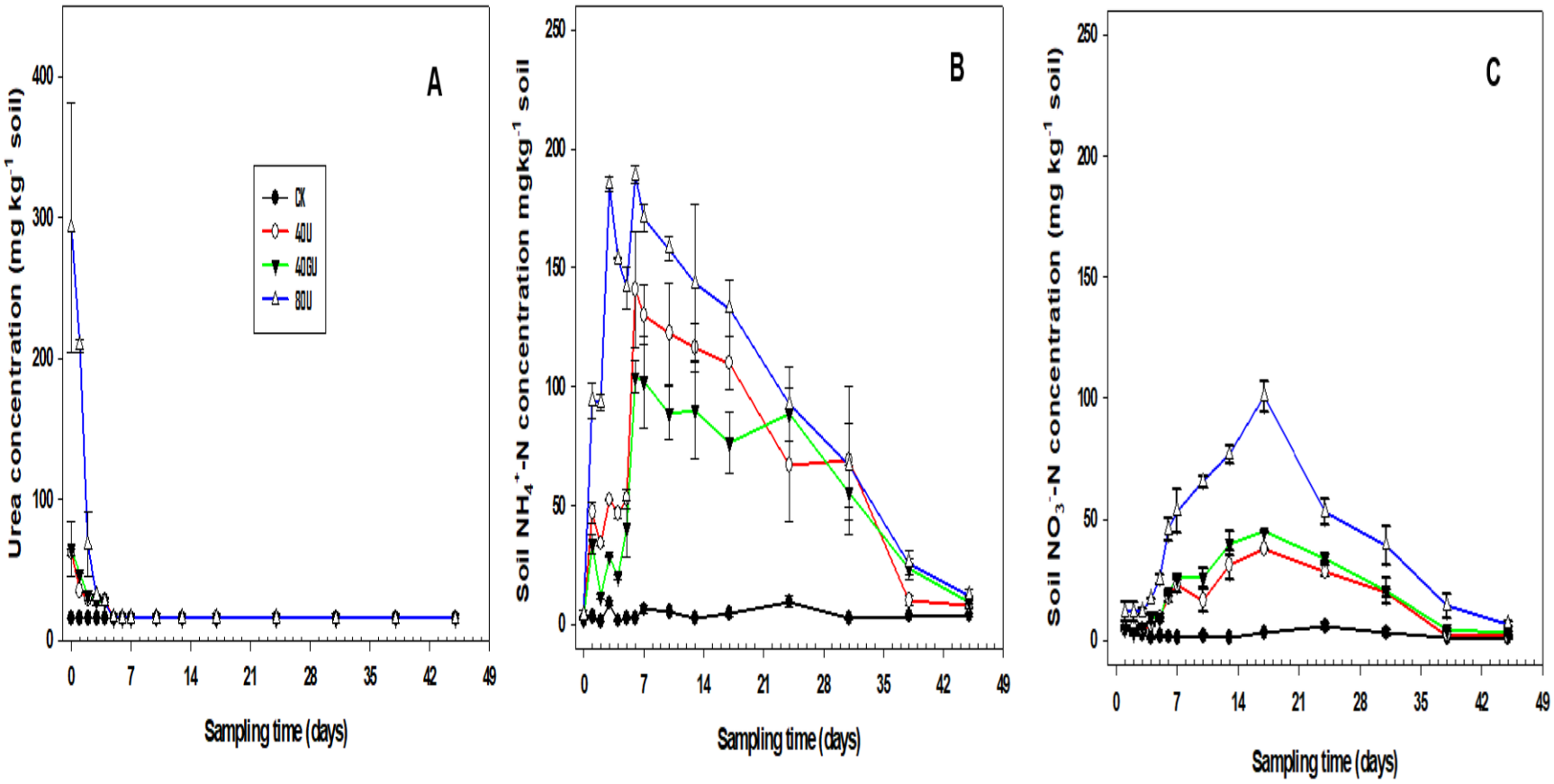
A
B
C
CK
40U
40GU
80U
Urea concentration (mg kg-1 soil)
Soil NH4+-N concentration mgkg-1 soil
Soil NO3--N concentration (mg kg-1 soil)
Sampling time (days)
Sampling time (days)
Sampling time (days)

Figure 3.

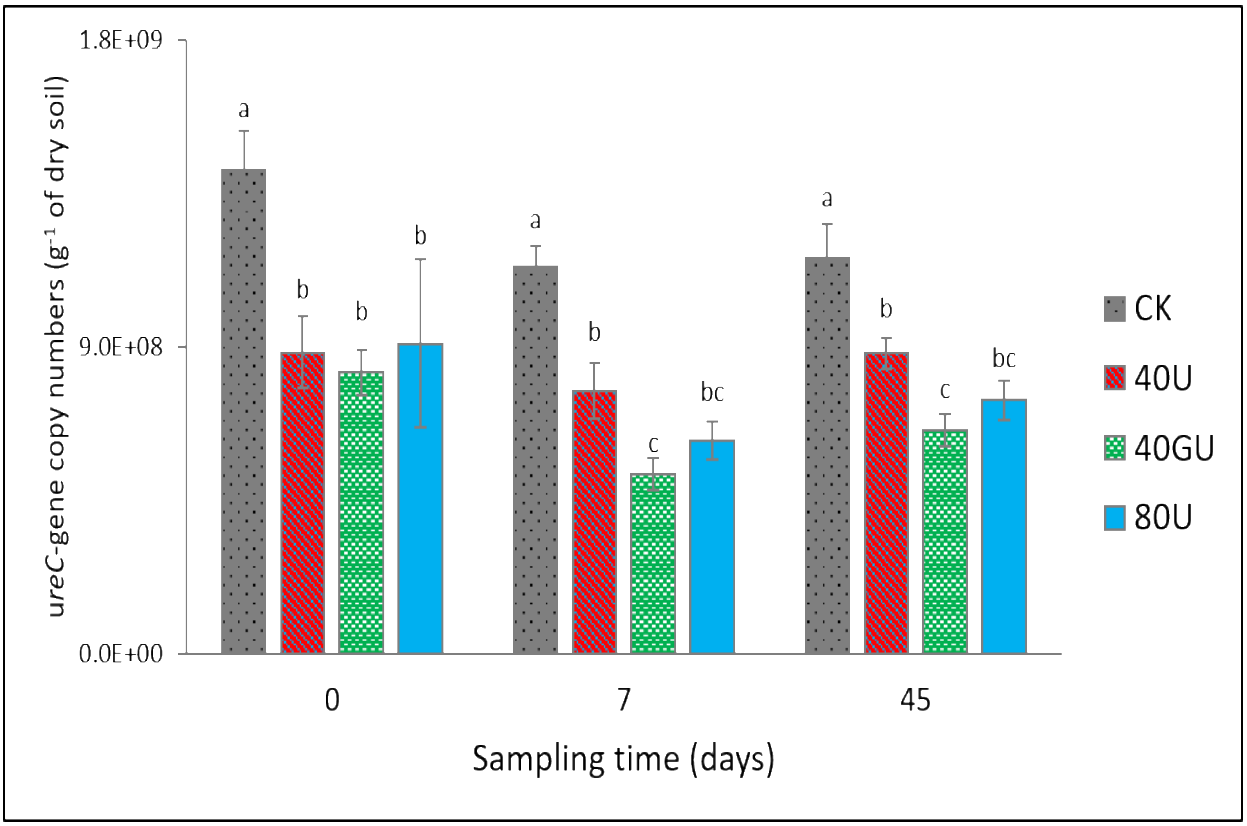
1.8E+09
9.0E+08
0.0E+00
ureC-gene copy numbers (g-1 of dry soil)
a
b
b
b
a
b
c
bc
a
b
c
bc
0
7
45
Sampling time (days)
CK
40U
40GU
80U

Figure 4.

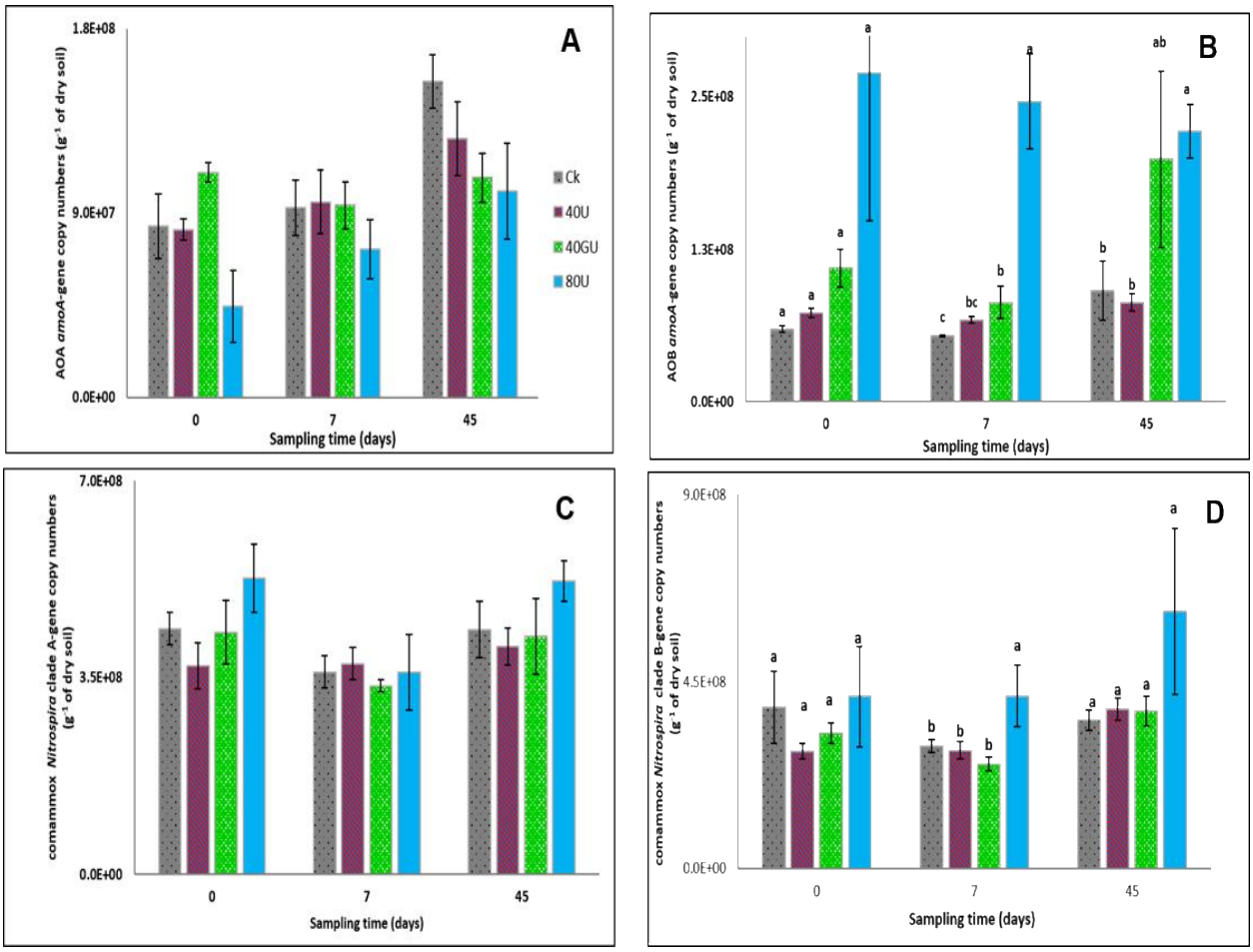
A
AOA amoA-gene copy numbers (g⁻¹ of dry soil)
1.8E+08
9.0E+07
0.0E+00
0
7
45
Sampling time (days)
Ck
40U
40GU
80U
B
AOB amoA-gene copy numbers (g⁻¹ of dry soil)
2.5E+08
1.3E+08
0.0E+00
Sampling time (days)
C
comammox Nitrospira clade A-gene copy numbers (g⁻¹ of dry soil)
7.0E+08
3.5E+08
0.0E+00
Sampling time (days)
D
comammox Nitrospira clade B-gene copy numbers (g⁻¹ of dry soil)
9.0E+08
4.5E+08
0.0E+00
Sampling time (days)

Table Legends

Table 1. The primers and thermocycling used for different gene qPCR amplification

Table 2. Pasture dry matter yield, N uptake, and nitrogen use efficiency (NUE)

Table 3. Pearson's correlation between soil pH, soil mineral N and soil microbial gene abundance

Table 1**.**

Gene	Primer	Sequence	Length (bp)	Thermocycling conditions	References
ureC	L2F	ATHGGYAARGCNGGNAAYCC	390	10 min at 95 °C, 40 cycles of (30 s at 95 °C, 45 s at 55 °C,	(Gresham et al., 2007)
	L2R	GTBSHNCCCCARTCYTCRTG		and 45 s at 72 °C), 10 min at 72°C.	
AOA *amoA*	CrenamoA-23f	ATGGTCTGGCTWAGACG	629	10 min at 95 °C, 40 cycles of (30 s at 95 °C, 45 s at 55 °C,	(Tourna et al., 2008)
	CrenamoA-616r	GCCATC CATCTGTATGTCCA		and 45 s at 72 °C), 10 min at 72°C.	
AOB *amoA*	amoA- 1F	GGGGTTTCTACTGGTGGT	491	10 min at 95 °C, 40 cycles of (30 s at 95 °C, 30 s at 56 °C,	(Rotthauwe et al., 1997)
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC		and 30 s at 72 °C), 10 min at 72°C.	
Comammox *Nitrospira* clade A	comaA-244F	TAYAAYTGGGTSAAYTA	415	10 min at 95°C, 25 cycles of (30 s at 94 °C, 45s at 42-52	(Pjevac et al., 2017)
	comaA-659R	ARATCATSGTGCTRTG		°C, and 60s at 72 °C), 10 min at 72°C.	
Comammox *Nitrospira* clade B	comaA-244F	TAYAAYTGGGTSAAYTA	415	10 min at 95°C, 25 cycles of (30 s at 94 °C, 45s at 42-52	(Pjevac et al., 2017)
	comaA-659R	ARATCATSGTGCTRTG		°C, and 60s at 72 °C), 10 min at 72°C.	

Table 2.

Treatment	Pasture dry matter yield (kg ha^{-1})	Shoot N uptake (kg ha^{-1})	NUE (%)
CK	1696.6±136.5 c	32.6±2.7 c	-
40U	2333.1±112.7 b	53.1±5.8 b	51.8±16.4a
40GU	2661.5±196.5 b	60.8±6.7 b	71.2±10.9a
80U	3564.5±51.8 a	88.4±1.4 a	70.0±5.1a

Treatments: CK, control; 40U, N applied at 40 kg N ha^{-1} as urea; 40GU, NBPT treated urea applied at 40 kg N ha^{-1} as Green Urea; 80U, N applied at 80 kg N ha^{-1} as urea. Values are mean (± standard error) (N = 3). Values within the same column followed by different letters are significantly different at $p< 0.05$ level.

Table 3.

	Soil pH			NO_3^--N		
	day 0	day7	day 45	day 0	day7	day 45
NH_4^+-N	0.44	-0.67*	-0.62*			
NO_3^-N	-0.64**	-0.91**	-0.67**			
ureC	0.4	0.66**	0.75**			
AOB	-0.45	-0.77**	-0.73**	-0.19	0.76**	0.51
AOA	0.47	0.27	0.4	-0.30	-0.17	-0.53
comammox *Nitrospira* clade A	-0.29	-0.13	-0.22	-0.27	0.24	0.22
comammox *Nitrospira* clade B	-0.22	-0.32	-0.47	0.05	0.30	0.56

**significant at the 0.01 probability level.

*Significant at the 0.05 probability level.

Chapter 6. General discussion and conclusions

6.1. Major findings and general discussion

Most of the studies have not investigated the molecular mechanism of the UI influence on hydrolysis and ammonia oxidation. More so, not so much information exists on the abundance and contribution of the newly discovered comammox *Nitrospira* microbes and their inhibition. The current study reported some reductions in *ureC* gene abundance due to NBPT inhibition but only towards the end of the incubation experiment which was linked to the fact that extracellular urease could absorb NBPT making it stable in cells for long. This was because the reduction took longer than the reported NBPT efficacy period of about 14 days (chapter 3). In the field experiment, the same trend of the reduction of *ureC* gene copy numbers due to NBPT inhibition to urea hydrolysis was also observed to be longer than 14 days (chapter 5) and this was speculated to be due to the reduced soil pH in the green urea treatment compared to urea alone. This was confirmed by the fact that the *ureC* gene copy numbers in all the N treatments were lower than control, a trend that was similar to that of effects of all the N treatments on soil pH, proving the role played by soil pH on *ureC* gene abundance in soil. *UreC* gene copy numbers have been reported to correlate positively with soil pH. Therefore, from these two studies, the efficacy of NBPT in soil could seem to last longer depending on the proportion of the soil microbes controlling the ureolytic activity e.g. the ratio between intracellular and extracellular urease enzyme; and on the effects of NBPT on soil pH especially under repeated applications. Therefore, in repeated chemical fertilizer applications, urea fertilizers used alone are likely to be more efficient and the use of urease inhibitors may not be very beneficial. This is because low soil pH could reduce the size and activity of ureolytic microbes. However, lower application rates of urea would be encouraged to avoid

acidification of soil as a result of high N application. This is because the soil pH reduction levels dependent on the rate of N application (Chapters 4 and 5).

Comammox *Nitrospira* and AOB gene abundances were reduced by NBPT (Chapter 3) which was explained by NBPT inhibition of urea hydrolysis, therefore, avoiding the accumulation of NH_3 as a substrate for the subsequent nitrification. Most ammonia oxidizers including AOB and comammox *Nitrospira* could have the ability to utilize urea as they possess some ureolytic genes and activities, enabling their dependence on urea as a substrate for hydrolysis. Therefore, the reported reduction in gene copies of these ammonia oxidizers could be linked to the direct effect of NBPT inhibition on the intracellular urease in the ammonia oxidizers, blocking hydrolysis and subsequent nitrification due to limited NH_3 availability (Chapter 3). Only AOB (Chapter 5) or AOB and comammox *Nitrospira* clade B (Chapters 4 and 5) increased with increased N addition in the field experiments with only AOB being reduced by DMPP (Chapter 5) at a higher application rate of 120 kg N ha^{-1}. This was an indication that these ammonia oxidizers were responsive to nutrient addition compared to AOA and comammox *Nitrospira* clade A. The soils in the three studies (Chapters 4, 5, and 6) had different soil pH and this indicated the ability of AOB to survive well at both alkaline and acidic conditions.

The effects of repeated applications of UI and NI were reported to significantly reduce soil pH and the reduction in soil pH was more with high application rates (Chapters 4 and 5). However, the results of Chapter 5, demonstrated that repeated applications of urea with Entec at different rates did not affect the relative abundance of total bacteria in the soil at the phylum level. This proved that such applications could not negatively influence non-targeted soil microbes. However, changes were apparent in some lower taxa of more abundant phyla which varied with the application rates, clearly highlighting the effect of application rate of urea and Entec on the non-

targeted microbes in the soil. Such repeated applications of N treatments could, therefore, change the composition of soil microbial communities in the long-term, promoting only the microbes that thrive under high nutrient conditions and eliminating those that cannot survive in such conditions. Therefore, in such applications, lower application rates of 40 kg Nha^{-1} would be encouraged to promote sustainable maintenance of soil microbial communities and soil health.

Generally, the reduction of soil NH_4^+-N by NBPT was higher than that of NBPT combined with DMPP. Likewise, DMPP was more efficient in reducing the nitrates concentration and nitrification compared to when it was combined with NBPT. The better performance of either NBPT or DMPP individually in reducing soil NH_4^+-N and NO_3^--N concentration, respectively, compared to their combination could be due to the negative interaction between the two when combined.

6.2. Conclusions and recommendations

Concerns of repeated/long-term application of fertilizers on soil have been raised. The repeated use of higher fertilizer rates led to more pH reduction in both field experiments (Chapters 4 and 5). Therefore, repeated application of urea with NBPT or DMPP led to soil acidification and changed other soil properties which influenced the response of soil microbial communities. Apart from influencing how the soil microbial organisms respond to the application of fertilizers + UI and NIs, the reduced pH and altered soil physiochemical conditions would also change the soil microbial communities. For repeated applications, lower application rates should be encouraged to minimize acidification which increases with increasing application rate.

This study has improved our understanding that nutrient application at lower rates can be used without negatively affecting soil physicochemical properties or microbial ecology.

In Chapter 3, the use of combined NBPT or DMPP alone was better than when they were combined in either reducing NH_3 or NO_3^- accumulation respectively, therefore, combining them may not be able to give better results. Future research should focus on how to develop a dual inhibitor compound whose individual compounds work together effectively.

6.3. Suggestions for future research

Several aspects of the comammox *Nitrospira* are yet to be tested concerning the urease and nitrification inhibitors, which should be considered in future studies e.g. how they respond to different UI and NIs or their combinations, the suitable rates for the inhibition of comammox *Nitrospira* as compared to other ammonia oxidizers that have been studied previously using these inhibitor compounds. This will contribute to the knowledge of manipulating N cycling microbes in the soil to reduce N losses associated with the N transformation.

Future studies on the effects of UIs and NIs on soil microbial organisms should incorporate the measurement of the changes of these inhibitor compounds and enzyme activities in different soils following the application of these inhibitor compounds. This will improve the performance of UIs and NIs in inhibiting various enzymes in different soils. Such a study will not only quantify the influence of these inhibitors on soil microbial community sizes, structure, and function but will also contribute to the understanding of the biological factors controlling the dynamics of the UIs and NIs once applied to the soil.

Several studies on the use of UI and NI have only considered the soil environment in determining their efficacy. However, the interaction between plants and soil microbial communities especially around the rhizosphere and how these may influence the efficacy of UI and NI needs to be understood better especially where the quantification of the UI and NI is involved.

Overall, this thesis provides new insights into the effects of repeated applications of UIs and NIs on soil N-cycling and non-targeted microbes with implications on soil health.