DOCTOR OF PHILOSOPHY

The potential of biological nitrification inhibitors to suppress soil nitrification and reduce greenhouse gas emissions

Ma, Yan

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PRIFYSGOL
BANGOR
UNIVERSITY

Yan Ma

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Supervisors: Prof. Dave Chadwick & Prof. Davey Jones (Bangor University)

Dr Laura Cardenas (Rothamsted Research)

Funding: Bangor-CSC Scholarship

School of Natural Sciences,

Bangor University,

Bangor, Gwynedd, UK

LL57 2UW

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Abstract

Biological nitrification inhibition (BNI) is a plant-mediated rhizosphere process where natural nitrification inhibitors (NIs) can be produced and released by roots to suppress nitrifier activity in soil. Several agricultural crops, such as rice, wheat, sorghum, and grasses, *Brachiaria humidicola*, have been found to have the ability to produce and release biological NIs from their roots. A few studies explored the effects of root exudates from grasses and crops (containing BNI activity) and specific BNI compounds on the transformation soil NH_4^+-N to NO_3^--N. However, less is known about the effects of biological NIs on soil emission of carbon dioxide (CO_2), N gaseous emissions other than nitrous oxide (N_2O), e.g. nitric oxide (NO) and dinitrogen (N_2). Less is known about what soil, environmental and inhibitor properties such as temperature, pH, moisture, organic matter, NH_4^+-N content in soil, biological NI concentration and stability, affect their efficacy. Moreover, there is only a limited understanding of the effects of biological NIs on microbial populations and enzymes responsible for promoting nitrification, especially the mechanism through which biological NIs inhibit N_2O emission. Hence, the study was to determine the potential of biological NIs to reduce soil nitrogen (N) losses and improve nitrogen use efficiency (NUE) through improved understanding of the factors that control their efficacy in soil, and clarify the mechanisms of action of BNI. Effects of 1,9-decanediol (identified biological NI from rice), linoleic acid (LA, identified from tropical pasture grass, *Brachiaria humidicola*) and proven NI DCD, applied at two different rates (12.7 and 127 mg NI kg^{-1} dry soil) on soil nitrification rates, greenhouse gas (GHG) (N_2O and CO_2) emissions, and also the ammonia oxidiser archaea (AOA) and bacteria (AOB) following NH_4^+-N application, were compared in Chapter 3. Results showed that LA and 1,9-decanediol are ineffective to inhibit soil nitrification at relatively lower concentrations. However, DCD was effective in inhibiting soil NH_4^+ transformation to NO_3^- and N_2O emissions under the same concentration. Thus, two higher concentration of LA and linolenic acid (LN) was added (635 and 1270 mg kg^{-1} dry soil) to determine their effects on soil nitrification in Chapter 4. In addition, the stability, and direct or indirect nitrification inhibition of LA, LN and DCD are explored using ^{14}C-labelling method, in a parallel incubation experiment. Results suggest that the apparent effect of LA and LN on soil NO_3^- concentration ($\geq$635 mg kg^{-1} dry soil) could be indirect under low-N conditions

(no addition of fertiliser NH_4^+) due to the addition of sufficient labile C in the biological NIs stimulating either i) microbial immobilisation of soil NH_4^+ or NO_3^- (under high C/N ratios), and/or ii) denitrification losses, such as N_2O. We also demonstrated that LA and LN were much more rapidly mineralised than DCD in soil. The residual inhibitory effects of *Brachiaria humidicola* (Bh, containing BNI capacity) and *Brachiaria ruziziensis* (Br, not be able to release biological NIs) after sheep urine application are explored in Chapter 5. *Brachiaria humidicola* inhibited N_2O emissions during the first peak compared with Br, which indicates the potential strategy for using Bh grass in sheep-grazed pastures to reduce nitrification rates and mitigate N_2O emissions. Based on the possible indirect inhibition by easily mineralised biological NIs to stimulate soil denitrification, Chapter 6 evaluated the effect of different C compounds (identified from cattle slurry; glucose, vanillin, cellulose, glucosamine and butyric acid), fresh and aged cattle slurry on soil NO_3^- consumption, N_2O and N_2 emissions during denitrification. Results showed that the liable C compounds (glucose, glucosamine and butyric acid) significantly stimulated soil N_2O emissions via denitrification than complex C compound (e.g. cellulose) and fresh or aged cattle slurry. We conclude that the required doses of LA, LN and 1,9-decanediol to inhibit soil nitrification were significantly higher than the application rates of the proven synthetic NI, DCD. The efficacy of biological NIs were largely related to the initial biological NI concentration and stability in soil, which increased as the increasing of BNI concentration and decreasing mineralisation rates. The apparent reduction of soil NO_3^- concentration after the application of biological NIs may result from biological NIs 1) directly inhibiting the nitrification process; 2) providing a C source to stimulate soil NH_4^+ and/or NO_3^- immobilisation; 3) providing a C source to promote soil denitrification. The synthetic NI, DCD, was confirmed to suppress the transformation of soil NH_4^+ to NO_3^-, and reduce soil N_2O emissions by impeding AOB but not AOA directly in a highly nitrifying soil. Further studies are necessary to measure the effects of biological NIs on direct soil microbial immobilisation and denitrification to provide more evidence for the mechanism of biological NIs on soil nitrification.

Contents

Abbreviations

AMO: Ammonia monooxygenases

ANOVA: Analysis of variance

AOA: Ammonia-oxidising archaea

AOB: Ammonia-oxidising bacteria

AT: Allylthiourea

Bh: *Brachiaria humidicola*

BNI: Biological nitrification inhibition

Br: *Brachiaria ruziziensis*

C: Carbon

Ca: Calcium

CH_4: Methane

C_2H_2: Acetylene

CO_2: Carbon dioxide

CS_2: Carbon disulphide

CSC: China Scholarship Council

DCD: Dicyandiamide

DEA: Denitrifying enzyme activity

DENIS: Denitrification system

Defra: Department of Environment & Rural Affairs

DMPP: 3,4-dimethylpyrazol-phosphate

DMPSA: 3,4-dimethylpyrazole succinic

EC: Electrical conductivity

ECD: Electron capture detector

ECW: Environment Centre Wales

EFs: N_2O emissions factors

FAO: Food and Agriculture Organization

FID: Flame ionization detector

GC: Gas chromatography

GHG: Greenhouse gas

HAO: Hydroxylamine oxidoreductase

HID: He Ionisation Detection

K: Potassium

LA: Linoleic acid

LA-EE: Ethyl linoleate

LA-ME: Methyl linoleate

LN: Linolenic acid

LN-ME: Methyl Linolenic

Mg: Magnesium

MHPP: Methyl 3-(4-hydroxyphenyl) propionate

N: Nitrogen

N_2: Dinitrogen

NH_3: Ammonia

NH_4^+: Ammonium

NI: Nitrification inhibitor

Nitrapyrin: 2-chloro-6-(trichloromethyl)-pyridine

NO: Nitric oxide

N_2O: Nitrous oxide

NO_2^-: Nitrite

NO_3^-: Nitrate

NUE: Nitrogen use efficiency

QPCR: Quantitative polymerase chain reaction

RMANOVA: Repeated measurement analysis of variance

SOC: Soil organic carbon

UNFCCC: United Nations Framework Convention on Climate Change

WFPS: Water filled pore space

WHC: Water holding capacity

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Chapter 1: Introduction

1.1. Background

Nitrogen (N) fertiliser input is the most important way to increase crop production and economic efficiency. Nevertheless, excessive application of N fertilisers and low nitrogen use efficiency (NUE) result in eutrophication and underground water pollution from nitrate (NO_3^-) leaching, and increasing gaseous N and carbon (C) losses mainly in the forms of nitrous oxide (N_2O), carbon dioxide (CO_2) or methane (CH_4) emission that cause global warming (Groenestein et al., 2019; Smolders et al., 2010). Nitrogen fixation, mineralisation, immobilisation, nitrification, denitrification, volatilization are major processes involved in the N cycle (Subbarao et al., 2015). Nitrification is a key soil N cycling process as it is responsible for the transformation of ammonium (NH_4^+) to NO_3^- (Firestone and Davidson, 1989), which is an important form of N used by agricultural crops (Mokhele et al., 2012), and a form of N that is readily lost to the environment via leaching (Cui et al., 2011). Nitrification also results in the production of N gases, e.g. N_2O with a global warming potential 310 times greater than that of CO_2 on a 100-year time horizon (UNFCCC, 2020), and nitric oxide (NO) (He et al., 2020). The subsequent denitrification process uses NO_3^- (the product of nitrification) in the production of N_2O, NO (Loick et al., 2016) and dinitrogen (N_2) gases. Thus, controlling soil nitrification is seen as an effective strategy to improve soil nitrogen use efficiency (NUE) and reduce greenhouse gases (GHG) emissions and NO_3^- leaching.

In the past decades, synthetic nitrification inhibitors (NIs), e.g. dicyandiamide (DCD) and 3,4-dimethylpyrazol-phosphate (DMPP) have been widely researched (Kou et al., 2015; Monaghan et al., 2013; Xu et al., 2019). The application of NIs retains higher soil NH_4^+ and lower NO_3^- concentrations (Yang et al., 2016), reduces soil N_2O emissions (Kou et al., 2015) and NO_3^- leaching (WU et al., 2007), and can increase crop yields (Abalos et al., 2014). But the efficacy of NIs can be variable, depending on: soil factors, e.g. soil properties (Gilsanz et al., 2016; McGeough et al., 2016), including pH (Robinson et al., 2014); environmental factors, e.g. temperature (Guardia et al., 2018), moisture content and aeration (Menéndez et al., 2012); management factors, e.g. N-fertiliser application rates and

methods (Xu et al., 2019); and crops factors, e.g. crop types (Abalos et al., 2014). However, several disadvantages of synthetic NIs have been reported, including the lack of chemical stability (Guardia et al., 2018; Marsden et al., 2016b), variable responses to soil types and moisture/temperature regimes (Marsden et al., 2016b; McGeough et al., 2016; Menéndez et al., 2012), high cost for spatially-targeting NI application in the field (Luo et al., 2015; Minet et al., 2018; Welten et al., 2014), and potential for food chain contamination (Lucas, 2013; Marsden et al., 2015). Because of these disadvantages, biological NIs are being researched as an alternative strategy to improve NUE from a range of N sources, e.g. chemical fertilisers, liquid manures and urine deposition by grazing livestock.

Biological nitrification inhibition (BNI) is a plant-mediated rhizosphere process where biological NIs are produced in plant roots and shoots and released from roots to suppress nitrifier activity in soil (Subbarao et al., 2006a). Several agricultural crops, such as rice (Sun et al., 2016), wheat (O'Sullivan et al., 2016; Subbarao et al., 2007b), sorghum (Subbarao et al., 2013; Zakir et al., 2008), and grasses, *Brachiaria humidicola* (Gopalakrishnan et al., 2007; Subbarao et al., 2009, 2008), have been found to have the ability to produce and release biological NIs from their roots, which decrease soil N_2O emissions and NO_3^- leaching losses, maintain higher soil NH_4^+ contents, and thus improve NUE. Most biological NIs inhibit both the ammonia monooxygenases (AMO) and hydroxylamine oxidoreductase (HAO) enzyme pathways (Subbarao et al., 2013, 2008), but the synthetic NIs only suppress the AMO pathway (Benckiser et al., 2013; Subbarao et al., 2013; Zakir et al., 2008) (Table 2.1). Several methods have been developed to detect and quantify BNI capacity (O'Sullivan et al., 2017; Subbarao et al., 2006a). A few studies have explored the effects of root exudates and specific BNI compounds from grasses and crops (containing BNI activity) (Souri and Neumann, 2010; Tesfamariam et al., 2014) on the transformation of soil NH_4^+-N to NO_3^--N (Lu et al., 2019; Nardi et al., 2013; Subbarao et al., 2008). However, less is known about the effects of biological NIs on emissions of CO_2, N gaseous emissions other than N_2O, e.g. NO, N_2. There is also a lack of understanding about what soil, environmental and inhibitor properties such as temperature, pH, moisture, organic matter, NH_4^+-N content in soil, biological NI concentration and stability, affect their efficacy. Moreover, there is only a limited understanding of the effects of biological NIs on microbial populations and enzymes responsible for promoting nitrification, especially the mechanism through which biological NIs inhibit N_2O emission.

Hence, the main aim of this thesis is to explore the factors influencing the efficacy of biological NIs and clarify the mechanism of BNI through studying the microbial populations and use of ^{14}C-labelling.

1.2. Thesis aims and objectives

1.2.1. Thesis aims

The overall aim of this PhD project was to determine the potential of biological NIs to reduce soil N losses and improve NUE through improved understanding of the factors that control their efficacy in soil, and clarify the mechanisms of action of BNI.

1.2.2. Thesis objectives and hypotheses

1) To determine the effects of biological NIs identified from tropical pasture grasses (*Brachiaria* species) and crops (e.g. rice) on soil nitrification, GHG emissions and other forms of N gases, such as NO and N_2. We hypothesised that soil applied with biological NIs, or grown with grasses and crops containing BNI capacity, and DCD, would retain higher soil NH_4^+ and lower NO_3^- concentrations, and reduce soil N_2O emissions, but the required effective dose of biological NIs may be higher than DCD.
2) To explore the inhibitor characteristics (e.g. concentration and stability) controlling the efficacy of biological NIs in soil. We hypothesised the rate of nitrification inhibition would increase with increasing rate of BNI addition, and decrease if the BNI was rapidly mineralised.
3) To clarify the influence of biological NIs on soil nitrification, via direct and indirect inhibition. We hypothesised that biological NIs directly inhibit the nitrification process at a range of concentrations, but at high rates of biological NI addition, the availability of added C stimulates microbial immobilisation of soil N, or N loss from the soil via denitrification, indirectly reducing nitrate concentrations in soil.

1.3. Thesis structure and chapter details

The thesis comprises 7 chapters, the details of each chapter and links between each chapter are described briefly below and shown in Fig. 1.1. Chapters 3-6 are presented in the forms of journal article manuscripts, with the authorship and progress (prepared to submit, already submitted or accepted) provided on the title page of each chapter.

Chapter 2: 'Literature review'. This chapter gives a review of current research regarding: 1) soil processes responsible for the gaseous N emissions; 2) synthetic NIs widely used in the past and their efficacy in inhibiting nitrification and disadvantages; 3) newly identified biological NIs from pasture grasses and crops; 4) factors that control the release of biological NIs; 5) the efficacy of biological NIs in suppressing soil nitrification; 6) knowledge gaps in our current understanding.

Chapter 3: 'Biological nitrification inhibitors linoleic acid and 1,9-decanediol are ineffective at inhibiting nitrification and ammonia oxidisers in a highly nitrifying soil'. This Chapter compares the effects of 1,9-decanediol (identified biological NI from rice), linoleic acid (LA, identified from tropical pasture grass, *Brachiaria humidicola*), and proven NI DCD, applied at two different rates (12.7 and 127 mg NI kg^{-1} dry soil) on soil nitrification rates, GHG (N_2O and CO_2) emissions, and also the ammonia oxidiser archaea (AOA) and bacteria (AOB) following NH_4^+-N application.

Chapter 4: 'Relative efficacy and stability of biological and synthetic nitrification inhibitors in a highly nitrifying soil: evidence of indirect nitrification inhibition by linoleic acid and linolenic acid'. Since the results in Chapter 3 show that LA and 1,9-decanediol are ineffective to inhibit soil nitrification at relatively lower concentrations. This chapter explores the efficacy of biological NIs identified from *Brachiaria humidicola*, LA and linolenic acid (LN), applied at a concentration of 12.7 127, 635 and 1270 mg NI kg^{-1} dry soil on the transformation of residual soil NH_4^+-N to NO_3^--N and GHG (N_2O and CO_2) emissions in a highly nitrifying soil, compared with the proven highly efficient NI, DCD. In addition, the stability, and direct or indirect nitrification inhibition of LA, LN and DCD are explored using ^{14}C-labelling method, in a parallel incubation experiment.

Chapter 5: 'Potential of biological nitrification inhibition by *Brachiaria humidicola* to mitigate nitrous oxide emissions following sheep urine application'. Since synthetic active compounds which

have been identified from the *Brachiaria grasses* and rice were used in Chapters 3 and 4, the residual inhibitory effects of *Brachiaria humidicola* (containing BNI capacity) and *Brachiaria ruziziensis* (not be able to release biological NIs) are explored in this Chapter. These C4 grasses were grown in soil and sheep urine was applied. The incubation system facilitated the measurements of N gases (N_2O, NO and N_2) and CO_2 emissions. Soil NH_4^+-N and NO_3^--N concentrations, and nitrifier and denitrifier gene abundance are also measured in Chapter 5.

Chapter 6*: 'Labile carbon sources stimulate soil nitrous oxide emissions during denitrification'. Results from Chapter 4 showed that the addition of specific biological NIs at higher doses increased N_2O (and CO_2) emission, indicating that denitrification (stimulated by the addition of available C in these biological NIs) may be responsible for some of the apparent nitrification inhibition observed in previous studies. This chapter evaluates the effect of different C compounds (identified from cattle slurry; glucose, vanillin, cellulose, glucosamine and butyric acid), fresh and aged cattle slurry on soil NO_3^- consumption, N_2O and N_2 emissions during denitrification.

Chapter 7: 'Discussion and outlook'. In this chapter, I present a discussion around the three objectives of the thesis (section 1.2.2), and summarise this study giving recommendations for future research. Discussion points arising from the thesis are as follow:

1) Efficacy of biological NIs compared with proven synthetic NIs.
2) Factors controlling the efficacy of biological NIs (e.g. degradation rates and concentrations)
3) Mechanism of biological NIs on soil nitrification and N_2O emissions: biological NIs act as:
    a) direct inhibitors of nitrification pathways
    b) a carbon source that stimulates N immobilisation
    c) a carbon source that stimulates denitrification

*Chapter 6 comprises 6 incubations using the denitrification system (DENIS) at Rothamsted Research (North Wyke). These incubations were performed in 2001-2002 by research staff at North Wyke. Because of the relevance of the relationship between lability of C compounds and subsequent denitrification fluxes (as N_2O and N_2) to my previous PhD Chapters, I was provided with the raw data (gas peak intensities) by my co-supervisors, Dr Laura Cardenas. I was responsible for calculating fluxes from the raw data, subsequent processing of the data, statistical analyses and interpretation of the results.

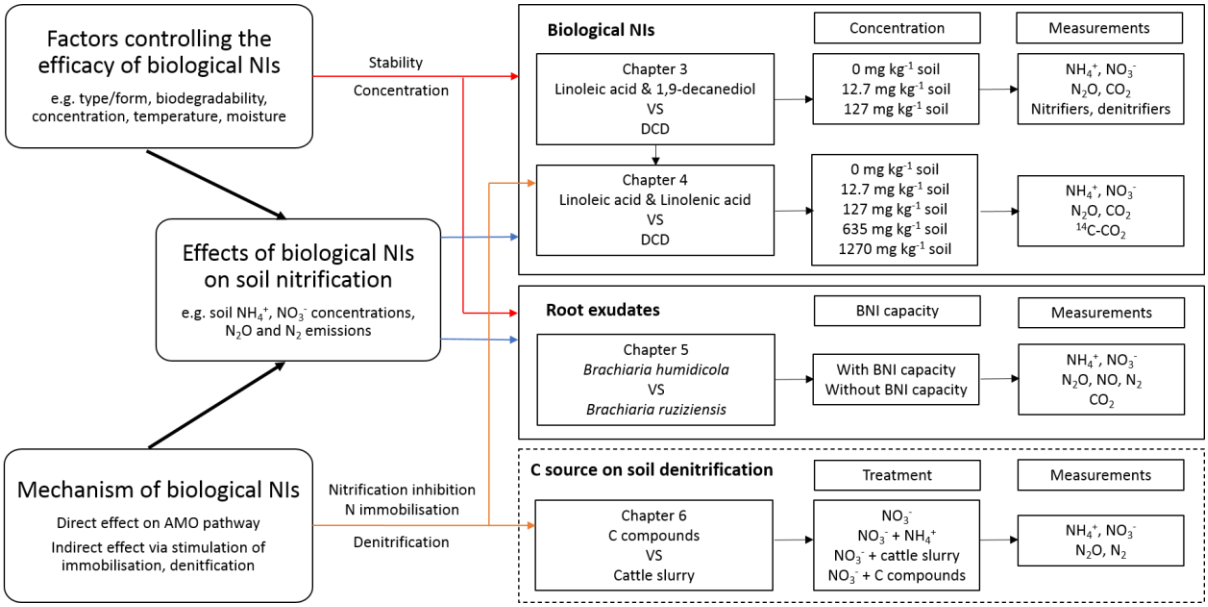

Fig. 1.1 Schematic diagram of the thesis

1.4. References

Abalos, D., Jeffery, S., Sanz-Cobena, A., Guardia, G., Vallejo, A., 2014. Meta-analysis of the effect of urease and nitrification inhibitors on crop productivity and nitrogen use efficiency. Agriculture, Ecosystems and Environment 189, 136–144. doi:10.1016/j.agee.2014.03.036

Benckiser, G., Christ, E., Herbert, T., Weiske, A., Blome, J., Hardt, M., 2013. The nitrification inhibitor 3,4-dimethylpyrazole-phosphat (DMPP) - quantification and effects on soil metabolism. Plant and Soil 371, 257–266. doi:10.1007/s11104-013-1664-6

Cui, M., Sun, X., Hu, C., Di, H.J., Tan, Q., Zhao, C., 2011. Effective mitigation of nitrate leaching and nitrous oxide emissions in intensive vegetable production systems using a nitrification inhibitor, dicyandiamide. Journal of Soils and Sediments 11, 722–730. doi:10.1007/s11368-011-0357-0

Firestone, M.K., Davidson, E.A., 1989. Microbiologial Basis of NO and N2O production and consumption in soil. Exchange of Trace Gases between Terrestrial Ecosystems and the Atmosphere 7–21. doi:10.1017/CBO9781107415324.004

Gilsanz, C., Báez, D., Misselbrook, T.H., Dhanoa, M.S., Cárdenas, L.M., 2016. Development of emission factors and efficiency of two nitrification inhibitors, DCD and DMPP. Agriculture, Ecosystems and Environment 216, 1–8. doi:10.1016/j.agee.2015.09.030

Gopalakrishnan, S., Subbarao, G. V., Nakahara, K., Yoshihashi, T., Ito, O., Maeda, I., Ono, H., Yoshida, M., 2007. Nitrification inhibitors from the root tissues of Brachiaria humidicola, a tropical grass. Journal of Agricultural and Food Chemistry 55, 1385–1388. doi:10.1021/jf062593o

Groenestein, C.M., Hutchings, N.J., Haenel, H.D., Amon, B., Menzi, H., Mikkelsen, M.H., Misselbrook, T.H., Bruggen, C. Van, Kupper, T., Webb, J., 2019. Comparison of ammonia emissions related to nitrogen use ef fi ciency of livestock production in Europe. Journal of Cleaner Production 211, 1162–1170. doi:10.1016/j.jclepro.2018.11.143

Guardia, G., Marsden, K.A., Vallejo, A., Jones, D.L., Chadwick, D.R., 2018. Determining the influence of environmental and edaphic factors on the fate of the nitrification inhibitors DCD and DMPP in soil. Science of the Total Environment 624, 1202–1212. doi:10.1016/j.scitotenv.2017.12.250

He, T., Xie, D., Ni, J., Li, Zhu, Li, Zhenlun, 2020. Nitrous oxide produced directly from ammonium,

nitrate and nitrite during nitrification and denitrification. Journal of Hazardous Materials 388, 122114. doi:10.1016/j.jhazmat.2020.122114

Kou, Y.P., Wei, K., Chen, G.X., Wang, Z.Y., Xu, H., 2015. Effects of 3,4-dimethylpyrazole phosphate and dicyandiamide on nitrous oxide emission in a greenhouse vegetable soil. Plant, Soil and Environment 61, 29–35. doi:10.17221/762/2014-PSE

Loick, N., Dixon, E.R., Abalos, D., Vallejo, A., Matthews, G.P., J.McGeough, K., Well, R., Watson, C., L.Laughlin, R.J., Cardenas, L.M., 2016. Denitrification as a source of nitric oxide emissions from incubated soil cores from a UK grassland soil. Soil Biology and Biochemistry 95, 1–7. doi:10.1016/j.soilbio.2015.12.009

Lu, Y., Zhang, X., Jiang, J., Kronzucker, H.J., Shen, W., Shi, W., 2019. Effects of the biological nitrification inhibitor 1,9-decanediol on nitrification and ammonia oxidizers in three agricultural soils. Soil Biology and Biochemistry 129, 48–59. doi:10.1016/j.soilbio.2018.11.008

Lucas, G.N., 2013. Dicyandiamide contamination of milk powders. Sri Lanka Journalof Child Health 42, 63–64. doi:10.4038/sljch.v42i2.5624

Luo, J., Ledgard, S., Wise, B., Welten, B., Lindsey, S., Judge, A., Sprosen, M., 2015. Effect of dicyandiamide (DCD) delivery method, application rate, and season on pasture urine patch nitrous oxide emissions. Biology and Fertility of Soils 51, 453–464. doi:10.1007/s00374-015-0993-4

Marsden, K.A., Marín-Martínez, A.J., Vallejo, A., Hill, P.W., Jones, D.L., Chadwick, D.R., 2016. The mobility of nitrification inhibitors under simulated ruminant urine deposition and rainfall: a comparison between DCD and DMPP. Biology and Fertility of Soils 52, 491–503. doi:10.1007/s00374-016-1092-x

Marsden, K.A., Scowen, M., Hill, P.W., Jones, D.L., Chadwick, D.R., 2015. Plant acquisition and metabolism of the synthetic nitrification inhibitor dicyandiamide and naturally-occurring guanidine from agricultural soils. Plant and Soil 395, 201–214. doi:10.1007/s11104-015-2549-7

McGeough, K.L., Watson, C.J., Müller, C., Laughlin, R.J., Chadwick, D.R., 2016. Evidence that the efficacy of the nitrification inhibitor dicyandiamide (DCD) is affected by soil properties in UK soils. Soil Biology and Biochemistry 94, 222–232. doi:10.1016/j.soilbio.2015.11.017

Menéndez, S., Barrena, I., Setien, I., González-Murua, C., Estavillo, J.M., 2012. Efficiency of nitrification inhibitor DMPP to reduce nitrous oxide emissions under different temperature and moisture conditions. Soil Biology and Biochemistry 53, 82–89. doi:10.1016/j.soilbio.2012.04.026

Minet, E.P., Ledgard, S.F., Grant, J., Murphy, J.B., Krol, D.J., Lanigan, G.J., Luo, J., Richards, K.G., 2018. Feeding dicyandiamide (DCD) to cattle: An effective method to reduce N2O emissions from urine patches in a heavy-textured soil under temperate climatic conditions. Science of the Total Environment 615, 1319–1331. doi:10.1016/j.scitotenv.2017.09.313

Mokhele, B., Zhan, X., Yang, G., Zhang, X., 2012. Review: Nitrogen assimilation in crop plants and its affecting factors. Canadian Journal of Plant Science 92, 399–405. doi:10.4141/CJPS2011-135

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

Nardi, P., Akutsu, M., Pariasca-Tanaka, J., Wissuwa, M., 2013. Effect of methyl 3-4-hydroxyphenyl propionate, a Sorghum root exudate, on N dynamic, potential nitrification activity and abundance of ammonia-oxidizing bacteria and archaea. Plant and Soil 367, 627–637. doi:10.1007/s11104-012-1494-y

O'Sullivan, C.A., Duncan, E.G., Whisson, K., Treble, K., Ward, P.R., Roper, M.M., 2017. A colourimetric microplate assay for simple, high throughput assessment of synthetic and biological nitrification inhibitors. Plant and Soil 413, 275–287. doi:10.1007/s11104-016-3100-1

O'Sullivan, C.A., Fillery, I.R.P., Roper, M.M., Richards, R.A., 2016. Identification of several wheat

landraces with biological nitrification inhibition capacity. Plant and Soil 404, 61–74. doi:10.1007/s11104-016-2822-4

Robinson, A., Di, H.J., Cameron, K.C., Podolyan, A., He, J., 2014. The effect of soil pH and dicyandiamide (DCD) on N2O emissions and ammonia oxidiser abundance in a stimulated grazed pasture soil. Journal of Soils and Sediments 14, 1434–1444. doi:10.1007/s11368-014-0888-2

Smolders, A.J.P., Lucassen, E.C.H.E.T., Bobbink, R., Roelofs, J.G.M., Lamers, L.P.M., 2010. How nitrate leaching from agricultural lands provokes phosphate eutrophication in groundwater fed wetlands: the sulphur bridge. Biogeochemistry 98, 1–7. doi:10.1007/s10533-009-9387-8

Souri, M.K., Neumann, G., 2010. Nitrification Inhibition Properties in Root Exudates of Brachiaria humidicola Plants 5, 13–25.

Subbarao, G.V., Yoshihashi, T., Worthington, M., Nakahara, K., Ando, Y., Sahrawat, K.L., Rao, I.M., Lata, J.C., Kishii, M., Braun, H.J., 2015. Suppression of soil nitrification by plants. Plant Science. doi:10.1016/j.plantsci.2015.01.012

Subbarao, G. V., Ishikawa, T., Ito, O., Nakahara, K., Wang, H.Y., Berry, W.L., 2006. A bioluminescence assay to detect nitrification inhibitors released from plant roots: A case study with Brachiaria humidicola. Plant and Soil. doi:10.1007/s11104-006-9094-3

Subbarao, G. V., Nakahara, K., Hurtado, M.P., Ono, H., Moreta, D.E., Salcedo, A.F., Yoshihashi, A.T., Ishikawa, T., Ishitani, M., Ohnishi-Kameyama, M., Yoshida, M., Rondon, M., Rao, I.M., Lascano, C.E., Berry, W.L., Ito, O., 2009. Evidence for biological nitrification inhibition in Brachiaria pastures. Proceedings of the National Academy of Sciences 106, 17302–17307. doi:10.1073/pnas.0903694106

Subbarao, G. V., Tomohiro, B., Masahiro, K., Osamu, I., Samejima, H., Wang, H.Y., Pearse, S.J., Gopalakrishnan, S., Nakahara, K., Zakir Hossain, A.K.M., Tsujimoto, H., Berry, W.L., 2007. Can biological nitrification inhibition (BNI) genes from perennial Leymus racemosus (Triticeae) combat nitrification in wheat farming? Plant and Soil. doi:10.1007/s11104-007-9360-z

Subbarao, G. V, Nakahara, K., Ishikawa, T., 2008. Free fatty acids from the pasture grass Brachiaria humidicola and one of their methyl esters as inhibitors of nitrification 89–99. doi:10.1007/s11104-008-9682-5

Subbarao, G. V, Nakahara, K., Ishikawa, T., Ono, H., Yoshida, M., Yoshihashi, T., Zhu, Y., Zakir, H.A.K.M., Deshpande, S.P., Hash, C.T., Sahrawat, K.L., 2013. Biological nitrification inhibition (BNI) activity in sorghum and its characterization. Plant Soil 366, 243–259. doi:10.1007/s11104-012-1419-9

Sun, L., Lu, Y., Yu, F., Kronzucker, H.J., Shi, W., 2016. Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency. New Phytologist 212, 646–656. doi:10.1111/nph.14057

Tesfamariam, T., Yoshinaga, H., Deshpande, S.P., Srinivasa Rao, P., Sahrawat, K.L., Ando, Y., Nakahara, K., Hash, C.T., Subbarao, G. V., 2014. Biological nitrification inhibition in sorghum: The role of sorgoleone production. Plant and Soil 379, 325–335. doi:10.1007/s11104-014-2075-z

United Nations Framework Convention on Climate Change (UNFCCC), 2020. Global Warming Potentials (IPCC Second Assessment Report).

Welten, B.G., Ledgard, S.F., Luo, J., 2014. Administration of dicyandiamide to dairy cows via drinking water reduces nitrogen losses from grazed pastures, in: Journal of Agricultural Science. Cambridge University Press, pp. S150–S158. doi:10.1017/S0021859613000634

Wu, S. fu, Wu, L. huan, Shi, Q. wei, Wang, Z. qiang, Chen, X. you, Li, Y. shan, 2007. Effects of a new nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on nitrate and potassium leaching in two soils. Journal of Environmental Sciences 19, 841–847. doi:10.1016/S1001-0742(07)60140-5

Xu, J., Zhu, T., Xue, W., Ni, D., Sun, Y., Yang, J., Xu, L., Chen, X., Li, H., Liu, M., 2019. Influences

of nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) and application method on nitrogen dynamics at the centimeter-scale. European Journal of Soil Biology 90, 44–50. doi:10.1016/j.ejsobi.2018.12.004

Yang, M., Fang, Y., Sun, D., Shi, Y., 2016. Efficiency of two nitrification inhibitors (dicyandiamide and 3, 4-dimethypyrazole phosphate) on soil nitrogen transformations and plant productivity: A meta-analysis. Scientific Reports 6, 1–10. doi:10.1038/srep22075

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

Chapter 2: Literature review

2.1. Introduction

In the past decades, global ammonia (NH_3) supply has increased significantly, reaching *ca.* 153.6 million tonnes in 2016, and is forecasted to reach 163.2 million tonnes in 2022 (FAO, 2019). Chemical fertilisers, manure application, symbiotic nitrogen (N) fixation and atmospheric N deposition (contributing a small share) are the main forms of global N supply to soil, accounting for 61%, 16%, 18% and 5% of the total, respectively (Lassaletta et al., 2014). Nitrogen surpluses result in losses of reactive N to the environment in the form of NH_3 (Groenestein et al., 2019), nitrous oxide (N_2O) and nitric oxide (NO) emissions (Xiao-tang and Chong, 2017), and nitrate (NO_3^-) leaching (Trolove et al., 2019). Ammonia emitted to the air may cause soil acidification after N deposition (Allen et al., 2011). Nitrous oxide is a greenhouse gas (GHG) with a global warming potential 310 times greater than that of carbon dioxide (CO_2) on a 100-year time horizon (UNFCCC, 2020). NO_3^- leaching may result in eutrophication (Smolders et al., 2010).

Strategies are needed to improve N use efficiency (NUE) from the range of N sources used in the agriculture and reduce losses of N to the environment. However, these strategies need to be based on a sound understanding of the soil processes and factors that control N cycling. In the soil system, N fixation, mineralisation, immobilisation, ammonification, nitrification and denitrification are the major biological processes of the N cycle (Fig. 2.1) (Subbarao et al., 2015). Among these biological processes, nitrification and subsequent denitrification are largely responsible for the transformation of ammonium (NH_4^+) to NO_3^- and NO_3^- consumption, along with GHG, NO, dinitrogen (N_2) emissions and NO_3^- leaching (Ciganda et al., 2018; Loick et al., 2016; Nair et al., 2020). This dissertation addresses one key soil process (nitrification), and the following literature review sets this in a wider context with reference to the other major soil N cycling processes. The literature review focusses on 1) an introduction to the nitrification and denitrification processes; 2) current strategies to inhibit soil nitrification, e.g. synthetic nitrification inhibitors (NIs) and biological NIs; 3) factors controlling the efficacy of synthetic and

biological NIs; 4) advantages and disadvantages of the NIs; 5) knowledge gaps in our current understanding.

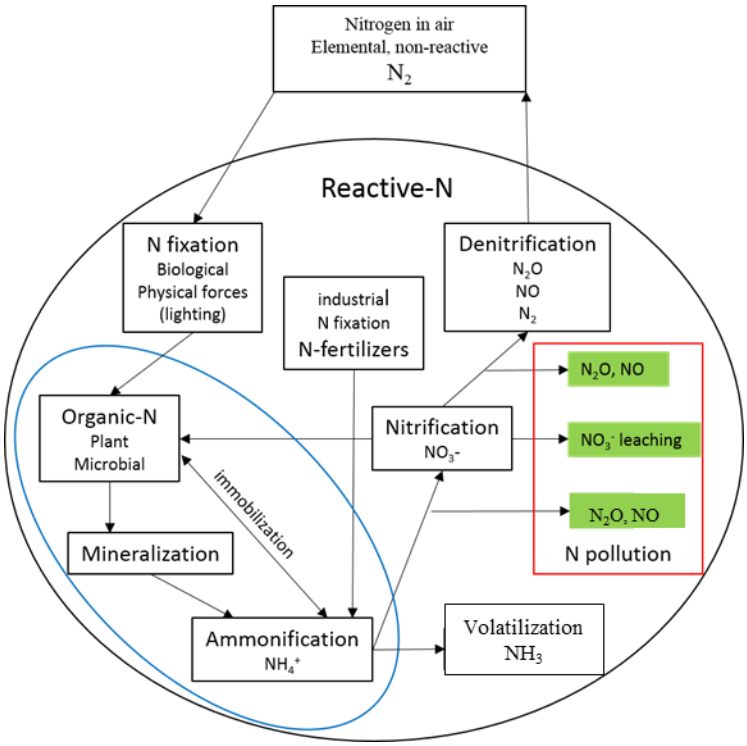

Fig. 2.1 Major nitrogen cycling processes and flows of nitrogen in and from soil (Subbarao et al., 2015).

2.2. Nitrification and denitrification

Nitrification is a two-step microbially mediated process carried out by chemo-autotrophic nitrifying bacteria, first oxidising NH_4^+ to nitrite (NO_2^-) and then oxidising NO_2^- to NO_3^- (Firestone and Davidson, 1989). In *Nitrosomonas europaea*, ammonia monooxygenase (AMO) and hydroxylamine (NH_2OH) oxidoreductase (HAO) catalyse the oxidation of NH_4^+ to NH_2OH, and NH_2OH to NO_2^-, respectively (Eq. 2.1, 2.2) (Sayavedra-Soto et al., 1996). Two groups of soil microorganisms, ammonia oxidising bacteria (AOB) (mainly *Nitrosomonas* spp. and *Nitrosospira* spp.) and ammonia oxidising archaea (AOA), are largely responsible for the biological oxidation of NH_4^+ to NO_3^- (Leininger et al., 2006).

$$NH_3 + O_2 + 2H^+ + 2e^- \xrightarrow{\text{ammonia mono-oxygenase}} NH_2OH + H_2O \qquad \text{Eq. (2.1)}$$

$$NH_2OH + H_2O \xrightarrow{NH_2OH\ \text{oxidoreductase}} NO_2^- + 5H^+ + 4e^- \qquad \text{Eq. (2.2)}$$

$$NO_2^- + H_2O \xrightarrow{\text{nitrite oxidoreductase}} NO_3^- + 2H^+ + 2e^- \quad \text{Eq. (2.3)}$$

2.2.1. Factors affecting nitrification in soil

Soil texture (Sahrawat, 2008), moisture content (Nugroho et al., 2007), aeration (Downing and Nerenberg, 2008), pH (Li et al., 2018; Nugroho et al., 2007) and temperature (Breuer et al., 2002; Zhang et al., 2019) are key controls of the nitrification process. These factors are addressed below.

Soil texture: Pihlatie et al. (2004) found that contribution of nitrification to N_2O production in peat, clay and loamy sand was variable under different soil moisture condition. Soil clay and sand had a significant influence on soil nitrification, percentage of clay was negatively related to the soil nitrification rates in soils maintained continuously moist, however, the relationship was positive in soils that had been dried or rewetted (Strong et al., 1999). Previous study showed that clay and sand had a significant influence on nitrification, but silt did not affect soil nitrification (Sahrawat, 2008).

Moisture content and aeration. Nitrification rate is believed to increase with increasing soil moisture to a certain content and decline when moisture is above it (Wu et al., 2017), with an optimal soil moisture content of 60% water filled pore space (WFPS) (Mosier et al., 1996). For example, increased soil moisture content elevated soil nitrification rates in a broad range of alpine soils, up to a value of 75% water holding capacity (WHC) (Osborne et al., 2016), and gross nitrification was negatively corrected to increased rates of WFPS (ranging from 37% to 62%) due to the simulated rainfall in tropical rain-forest soils (Breuer et al., 2002). Moisture content and aeration are inversely related, with decreasing oxygen content in the soil when moisture content is increased (Sahrawat, 2008). Nitrifying bacteria produce nitrate in well-aerated soils, with maximum rates of nitrification being achieved when oxygen is about 20% of the air (similar to the oxygen concentration in the atmosphere) (Sahrawat, 2008).

pH. Significantly higher nitrification of fertiliser N was observed in soils pH>7.5 (89%) than pH< 6.0 (39%) (Kyveryga et al., 2004). This was also observed in urine-fertilised soils, where soil N_2O emission was significantly higher in the acid treatment (pH=5.0) than the alkaline treatment (pH=6.5) (Robinson et al., 2014). Some studies have shown that AOA growth is favoured in more acidic soil,

whilst AOB growth is favoured by more alkaline soil pH (Li et al., 2018; Robinson et al., 2014). However, other studies have contradicted this, e.g. Che et al. (2015) showed enhanced nitrification in an acidic [Anhui] soil (pH=4.64) in China primarily due to enhancement of AOB rather than AOA.

Temperature. Temperature affects soil nitrification rates by shifting the abundance and composition of AOA and AOB (Ouyang et al., 2017; Zhang et al., 2019) and stimulating enzymatic activity (Taylor et al., 2017). Breuer et al. (2002) showed a positive relationship between gross nitrification and the increase of soil temperature (ranging from 15-23 ℃). However, temperature was not seen to be related with nitrification rates in all soils (Osborne et al., 2016), and this study also found that AOB not AOA were the dominant ammonia oxidisers. Ouyang et al. (2017) found that the proportion of nitrification due to AOA increased with temperature, which was lowest at 5 ℃ and was near to 100% at 50 ℃, with the optimum temperature of 41 ℃ for AOA and 31 ℃ for AOB. Nitrification peaked at 35-40 °C in Chromosol and Dermosol soils (Australian soil classification) (Lai et al., 2019). A recent study showed that elevated temperatures (from 15 to 20 ℃) had a pronounced effect on soil nitrification by altering abundance of AOA and AOB, as well as the composition of AOA but not the AOB (Zhang et al., 2019).

Denitrification refers to the dissimilatory reduction of one or both of the ionic N oxides (NO_3^- and NO_2^-) to the gaseous oxides (NO, N_2O and N_2), which are catalysed by the sequential activity of the enzymes NO_3^- reductase, NO_2^- reductase, NO reductase and N_2O reductase (Knowles, 1982). Firstly, the respiratory NO_3^- reduction and the dissimilatory reduction of NO_3^- to NH_3, is catalysed by a membrane-bound or periplasmic NO_3^- reductase encoded by the *narGHJI* operon or the *napABC* operon, respectively (Philippot and Højberg, 1999). The second step ($NO_2^- \rightarrow NO$) is catalysed by two different types of nitrite reductases (Nir), either a cytochrome *cd1* encoded by *nirS* or a Cu-containing enzyme encoded by *nirK* (Kandeler et al., 2006). The reduction of N_2O to N_2 is the final step and is carried out by the N_2O reductase encoded by the *nosZ* gene in denitrifying microorganisms (Wang et al., 2017). The general requirements for biological denitrification are i) anaerobic conditions or restricted supply of O_2, ii) N oxides as terminal electron acceptors, iii) electron donors such as available organic C compounds, and iv) the bacteria, fungi, other denitrifying eukaryote or archaea possessing metabolic activity (Saggar et al., 2013). Soil factors that affect denitrification include, C availability (Dlamini et

al., 2020), soil mineral N supply (Senbayram et al., 2019), O_2 supply and water content (Gillam et al., 2008), and soil pH (Saleh-Lakha et al., 2009). While the main environment factors that affect denitrification are, soil temperature (Braker et al., 2010; Lai et al., 2019), rainfall (Saggar et al., 2013), soil drying-rewetting and freezing-thawing (Mørkved et al., 2006), and availability of trace metals (Pintathong et al., 2009). Anaerobic and autotrophic ammonium-oxidizing (anammox) bacteria also play an essential part in N removal, which obtain energy from the conversion of NH_4^+ and NO_2^- into N_2 catalysed by the nitrite oxidoreductase, hydrazine hydrolase and hydrazine oxidoreductase (Jetten et al., 2009; Peng et al., 2020; Tong et al., 2020).

Ammonium is readily bound to the negatively charged clay surfaces and functional groups of soil organic matter; in contrast, NO_3^- has a negative charge (and is generated via nitrification) and is highly mobile so is prone to movement through the soil profile to watercourses (Sahrawat, 1989). The losses of N during nitrification and subsequent denitrification reduces the NUE and are sources of both air and water pollutions. However, denitrification cannot take place without the substrate NO_3^- generated via nitrification. Thus, inhibiting nitrification rates is one of effective ways to improve NUE, reduce GHG, NO emissions and NO_3^- leaching.

2.3. Synthetic nitrification inhibitors

2.3.1. Concept and the mode of inhibitory action

Synthetic NIs have been confirmed to reduce or delay the conversion rate of NH_4^+ to NO_3^-, e.g. dicyandiamide (DCD) (Monaghan et al., 2013), 3,4-dimethylpyrazol-phosphate (DMPP) (Gilsanz et al., 2016), 2-Amino-4-chloro-6-methylpyrimidine (nitrapyrin) (Niu et al., 2018), and allylthiourea (AT) (He and Ji, 2020) and the newly identified NI, 3,4 dimethylpyrazol succinic acid (DMPSA) (Pacholski et al., 2016). Most synthetic NIs are known to inhibit nitrification by suppressing only the AMO enzymatic pathway in *Nitrosomonas* (Subbarao et al., 2013; Zakir et al., 2008) (Table 2.1), due to: 1) direct binding and interaction with AMO; 2) interference with the reductant supply to AMO activity; 3) oxidation of substrates to give products that are high reactive and/or inactive to AMO and/or other enzymes (McCarty, 1999; Subbarao et al., 2006b). Among these synthetic NIs, DCD and DMPP have

been the most widely researched in the past few decades (Abalos et al., 2014; Gilsanz et al., 2016; Menéndez et al., 2012).

2.3.2. DCD

Dicyandiamide application decreases soil N_2O emissions from N-fertilised soil (urine, animal slurry and chemical fertilisers), with the variable efficacy of inhibition depending on the application concentration (Zaman and Blennerhassett, 2010), soil properties (Cui et al., 2011; McGeough et al., 2016), temperature (McGeough et al., 2016) and soil moisture content (Di et al., 2014). Previous studies also confirmed that the application of DCD could decrease NO_3^- leaching by 21%-69% (Cameron et al., 2014; Cameron and Di, 2002; Monaghan et al., 2009; Zaman et al., 2009). Researchers have found that the application of DCD can reduce calcium (Ca), potassium (K) and magnesium (Mg) leaching by 50%, 65% and 52% respectively, due to the decreased leaching loss of NO_3^- under urine patches, and follows from their reduced requirement as counter ions in the drainage water (Di and Cameron, 2004). Few studies have explored the effects of DCD on soil CO_2 and methane (CH_4) emissions. Weiske et al. (2001) find that DCD reduced the CO_2 emission from N-fertilised soil by an average of 7% (non-fertilised 10%). However, DCD failed to affect CH_4 emissions (Cardenas et al., 2016; Weiske et al., 2001). Due to the ability of DCD to reduce N losses, herbage production can be increased by 0-30% with DCD application (Cameron et al., 2014; Cameron and Di, 2002; Zaman et al., 2009), and this most likely due to reduced NO_3^- leaching, rather than reduced N_2O losses (which usually represent only small losses, <2% of applied N). Normally, 5 to 15 kg DCD ha^{-1} soil is applied to soil to explore the effects on nitrification inhibition (Di and Cameron, 2006; Gilsanz et al., 2016; Zaman and Blennerhassett, 2010), with the efficacy lasting for a different time depending on the soil properties (water content, soil temperature, soil pH and organic matter) (McGeough et al., 2016; Puttanna et al., 1999) and environment factors (e.g. rainfall) (Marsden et al., 2016b).

2.3.3. DMPP

Applying DMPP in N-fertilized fields has also shown to decrease nitrification rates, resulting in decreasing N_2O emissions (Lam et al., 2018; Scheer et al., 2014; ten Huf and Olfs, 2020), maintenance of relatively higher NH_4^+-N concentration and lower NO_3^--N concentrations in soil (WU et al., 2007; Xu et al., 2019) and leachates (Li et al., 2008), via reduction of NO_3^- leaching (WU et al., 2007). Research suggests that application rates of 0.5 to 1.5 kg ha^{-1} are sufficient to achieve optimal nitrification inhibition in the case of DMPP (Zerulla et al., 2001). 3,4-dimethylpyrazol-phosphate has been confirmed to slow soil nitrification by inhibiting the abundance of AOB but not the AOA (Kleineidam et al., 2011; Shi et al., 2016). In addition, 1% DMPP application has decreased the population of AOB, the activity of nitrate reductase (NaR) and nitrite reductase (NiR) by 24.5%-30.9%, 14.9%-43.5% and 14.7%-31.6%, respectively (Li et al., 2008). In the study of Li et al. (2019), 9 kg ha^{-1} (0.5% of the urea-N application rate) of DMPP was effective in mitigating N_2O emissions by directly inhibiting both ammonia oxidising (AOB) and denitrifying microbes (*nirS* and *nirK*). However, DMPP has no effect on denitrifying enzyme activity (DEA) (Muller et al., 2002). A new nitrification inhibitor, 3,4-dimethylpyrazole succinic (DMPSA), has been found to be alternative to DMPP for reducing N_2O emissions from wheat crops under humid Mediterranean conditions (Huérfano et al., 2016).

Studies have been conducted to compare the effects of DCD and DMPP on N_2O emissions (Gilsanz et al., 2016; Suter et al., 2010), crop productivity (Fangueiro et al., 2009), AOA and AOB (Chen et al., 2014; Di and Cameron, 2011; Gong et al., 2013). A meta-analysis conducted by Abalos et al. (2014) confirmed that DCD application increased crop productivity and NUE compared to the control, but with varying degrees of success for crop productivity, however, the addition of DMPP increased NUE but not crop productivity. Dicyandiamide and DMPP treatments significantly decreased the annual emissions by 35% and 38% in wheat-maize cropping system (Liu et al., 2013). Compared with the urea treatment, the annual crop yield and aboveground biomass increased by 8.5%-9.1% (1.1-1.2 ton ha^{-1} yr^{-1}) and 8.6%-9.7% (2.8-3.2 ton ha^{-1} yr^{-1}) for the DCD and DMPP treatments, respectively (Liu et al., 2013). A dry matter yield of 8698 kg ha^{-1} was obtained when DMPP was applied at the greater rate against only 7444 kg ha^{-1} obtained with the greater rate of DCD (Fangueiro et al., 2009).

2.3.4. Factors influencing the efficacy of NIs

Soil texture. Soil texture influences the effectiveness of synthetic NIs, mainly due to the soil inorganic (sand, clay) or organic (organic matter) constituents (Guardia et al., 2018; McGeough et al., 2016). For example, the relative NO_2^- formation declined and the efficacy of DMPP increased when sand content of soils were higher (Barth et al., 2001). The efficacy of DCD in inhibiting net NO_3^- production was best correlated with soil Cu and % clay, which decreased as soil Cu concentrations and percentage of clay increased (McGeough et al., 2016). The meta-analysis from Li et al. (2018) found that NIs were more effective in reducing soil N_2O emissions in fine-textured soils (77%) compared with coarse (59%) or medium-textured (44%) soils, and in soils with intermediate (10-40 g/kg) soil organic carbon (SOC) (63%) compared to lower (32%) or higher SOC (50%). The efficiency of NIs was also driven by the interaction with soil types (Guardia et al., 2018). In an incubation experiment, grassland soils had higher native total N concentrations than the arable soils, hence the inhibition of net NO_3^- production by DCD was lower and this resulted in an overall inhibition in N_2O emissions of 58% and 81% for grassland and arable soils, respectively (McGeough et al., 2016). 3,4-dimethylpyrazol-phosphate had higher efficacy in neutral and alkaline wheat and vegetable soils, compared with pasture soils (Shi et al., 2016). Researchers have found that the influence of inorganic constituents or organic matter can be explained by their sorption which has the ability of reducing inhibitors' mobility, bioactivity and volatility (Barth et al., 2001; Marsden et al., 2016b). In addition, the nitrification inhibition may be reduced by the presence of fresh organic matter, which acted as source of energy to stimulate microbial activity and thus resulted in faster degradation of inhibitors (Puttanna et al., 1999).

Soil pH. The efficacy of NIs on soil nitrification and ammonia oxidiser population are variable under different soil pH (Liu et al., 2015; Robinson et al., 2014). For example, DCD application did reduce soil peak N_2O emissions in acid, native and alkaline soils, but the effectiveness of DCD in reducing total N_2O emissions was highly effective in the acidic treatment (pH=5.0) than that in the native (pH=6.0) and alkaline (pH≥6.5) treatments (Robinson et al., 2014). Inhibitors (Benzotriazole, *o*-Nitrophenol, *m*-Nitroaniline and DCD) retained higher soil NH_4^+ concentrations when soil pH decreased from 8.3/8.2 to 5.4 (Puttanna et al., 1999). Acetylene (C_2H_2) completely blocked nitrification

in acid (pH=4.6), neutral (pH=7.0) and alkaline soils (pH=8.0) (ranging from 97.1-100%), while DMPP was shown to be more effective in the neutral soil (93.5%), followed by the alkaline (85.1%) and acid soil (70.5%) (Liu et al., 2015). Attention needs to be paid to the effects of soil pH on nitrification, e.g. strong acidity is known to inhibit AOB and/or AOA growth (Li et al., 2018; Sahrawat, 2008).

Temperature. Numerous researchers comparing the nitrification inhibition of NIs at different soil temperatures (ranging from 5 to 35 ℃) have shown that NIs were more effective at lower temperatures that maximised their longevity (Guardia et al., 2018; Lan et al., 2018; McGeough et al., 2016; Menéndez et al., 2012). Soil temperature can affect the mineralisation of NIs, with higher mineralisation rates at increasing temperature (Guardia et al., 2018; Kelliher et al., 2008; McGeough et al., 2016). Kelliher et al. (2014) has provided a relationship between time for concentration of DCD in soil to halve (t½, days) and the mean soil temperature (T, °C): t½ = 54-1.8 T, but under field conditions t½ was about half that under laboratory conditions. The persistence of DCD was strongly inversely related to temperature, with the measured half-life across all soils decreasing with increasing temperature (McGeough et al., 2016). Dicyandiamide and DMPP mineralisation were also confirmed to be strongly influenced by temperature (increasing with temperature), however, their overall effectiveness was highly influenced by the temperature, and not necessarily linked with the kinetics of mineralisation of these NIs (Guardia et al., 2018).

Moisture and aeration. Research has found that the nitrification inhibition efficacy of *o*-nitrophenol and *m*-nitroaniline was maximum at 60% WHC, however, DCD showed an increasing efficacy as soil moisture levels decreased (Puttanna et al., 1999). Chen et al. (2010) showed that DMPP application slowed nitrification appreciably when the soil was at 40% WFPS at 25 ℃, but was less effective at 60% WFPS. Results from the study of Menéndez et al. (2012) indicated that DMPP best performed under extreme environmental conditions (cold and wet conditions or hot and dry conditions). In soil, both moisture and O_2 are critical factors influencing the activity of nitrifiers and therefore, nitrification rates (Barrena et al., 2017). Soil aeration determines the degradation rate of DCD which was greater when the soil was aerobic (Balaine et al., 2015). However, the relationship between moisture and O_2 status is too close to distinguish their individual roles in the efficacy of NIs.

Inhibitors properties. Nitrification inhibitors differ in volatility and water solubility, which has resulted in the diversity of their effectiveness, mobility and persistence. For gaseous NIs (such as carbon disulphide (CS_2) and C_2H_2), the high volatility and rapid dispersion through soil pores makes them more effective, but less persistent, and therefore generally not suitable in the field (Ashworth et al., 1977). Commonly researched NIs (DCD and DMPP) are non-volatile, and the mobility of both NI are similar, but a greater sorption for DCD in comparison to DMPP was found in organic and mineral soils (Marsden et al., 2016b). The efficacy of NIs is strongly influenced by their mineralisation rates which increase with increasing soil temperature (Kelliher et al., 2008; McGeough et al., 2016; Menéndez et al., 2012). Marsden et al. (2016) suggest that the efficacy of NIs is influenced more by differences in microbial uptake and mineralisation rates than by differences in sorption and desorption rates.

Management and other factors. A previous study showed that nitrification inhibition in a loamy soil was more pronounced when NH_4^+ and DMPP were applied as fertiliser granules compared to the application as solution (Brath et al., 2008). The efficacy of NIs on yield and/or NUE tended to be diminished in areas of high rainfall (>1200 mm) (Li et al., 2018). A similar distribution of DCD and DMPP was observed up to a soil depth of 15 cm following a simulated rainfall event (40 mm) in a sandy loam, sandy clay loam and Sapric Histosol (Marsden et al., 2016b). The meta-analysis conducted by Abalos et al. (2014) found that the effects of NIs on soil crop productivity was significantly larger for the highest N fertiliser rate (≥300 kg N ha^{-1}) compared with the lowest N fertiliser (≤150 kg N ha^{-1}), and irrigated systems showed a significantly higher response than rainfed systems to the application of NIs in terms of crop productivity, although these two water management classes did not significantly affect the efficacy of NIs on NUE. The same meta-analysis showed that the effectiveness of NIs to increase the crop productivity and NUE was significantly higher for forage crops than cereals (Abalos et al., 2014). However, Li et al. (2018) showed that NIs had similar impact on N losses mitigation regardless of irrigation or rainfed system. A meta-analysis from Yang et al. (2016) also supported that the efficiency of NIs positively varies with N fertiliser application rates for higher N fertiliser rates input often causing high N losses (Yang et al., 2016). This is also supported by Li et al. (2018), in which the greater reduction in N_2O loss by NIs was observed with the higher baseline of N_2O emission (>20 kg

N_2O-N ha^{-1}). In addition, the application of the DMPP was more effective in reducing N_2O emissions in long-term intensive cultivation soil than in the short-term cultivation soil (Li et al., 2019).

2.3.5. Limitation and unintended consequences of synthetic NIs

In general, synthetic NIs play an important role in improving NUE, reducing GHG emissions, NO_3^- leaching and increasing crop productivity. But the leaching of synthetic NIs from the active zone of nitrification, leads to inconsistent performance under field conditions within laboratory conditions, e.g. DCD (Marsden et al., 2016b; Upadhyay et al., 2011). In addition, the high cost and potential for environmental contamination of synthetic NIs have been additional factors that have limited their commercial use in agriculture system. In 2012, traces of DCD were discovered in infant/baby formula milk products from New Zealand (Lucas, 2013), after which DCD was voluntarily withdrawn from commercial use in New Zealand. There has been speculation about how DCD contaminated the milk supply, and studies have shown that DCD can be acquired and metabolised by graminaceous plants (Marsden et al., 2015). However, plant uptake of DCD was probably not a significant route of NI contamination, which was more likely the result of direct ingestion of DCD on pasture and soil surfaces, which provides a direct route of DCD entry into the food chain. Thus, developing biological NIs may be a sound alternate strategy with greater public acceptance.

2.4. Biological nitrification inhibition

2.4.1. Concept and the mode of nitrification action

Biological nitrification inhibition (BNI) is termed of a plant-mediated rhizosphere process where NIs are produced in plant roots and shoots and released from roots to suppress nitrifier activity in soil (Subbarao et al., 2006a). *Brachiaria humidicola* is a common tropical pasture grass that produce substantial biological NIs from its root (Gopalakrishnan et al., 2007; Subbarao et al., 2009) and shoot tissues (Subbarao et al., 2008). Linoleic acid (LA) and linolenic acid (LN) have been isolated and identified as biological NIs from the shoot tissue of *Brachiaria humidicola*. The BNI compounds in the root tissue and exudate of *Brachiaria humidicola* have been identified as brachialactone (which

contributes 60-90% of the inhibitory activity released from the roots of this tropical grass), methyl p-coumarate and methyl ferulate (Gopalakrishnan et al., 2007; Subbarao et al., 2009). Biological NIs have also been isolated from sorghum root exudates (Subbarao et al., 2013; Tesfamariam et al., 2014; Zakir et al., 2008), rice root exudates (Sun et al., 2016), wheat root exudates and root tissue extracts (O'Sullivan et al., 2016; Subbarao et al., 2007b). They have also been found in seeds of *Pongamia glabra* vent (Sahrawat, 1981; Sahrawat and Mukerjee, 1977), degradation products of cruciferous tissues (Bending and Lincoln, 2000) and *Pinus ponderosa* leaves (White, 1988), and researchers are searching for more compounds (Table 2.1).

The bioassay in which the *Nitrosomonas* culture is incubated with the NIs in the presence and absence of NH_2OH can be used to determine the NIs effects on AMO or HAO pathways (Subbarao et al., 2006a). Most BNI compounds released by the plants inhibit nitrification by suppressing both AMO and HAO enzymatic pathways, whilst most synthetic NIs only inhibit the AMO enzymatic pathways (Table 2.1). The vast majority of the wheat landraces tested caused some level of inhibition, but BNI compounds were not identified and their effects on AMO or HAO pathways were not tested (O'Sullivan et al., 2016). In addition, BNI compound released by rice toot exudates, 1,9-decanediol, have been confirmed to block the AMO pathway and possess an 80% effective dose (ED_{80}) of 90 ng μl^{-1} (Sun et al., 2016). Previous studies show that HAO enzymatic pathways are suppressed by: 1) the inactivity of HAO by hydroxyethyl, phenyl-, and methyl- hydrazine 2) hydrogen peroxide's reaction with the active site of ferric HAO, which destroys the activity and heme P460 of HAO irreversibly; and 3) organo-hydrazines that attack P460 active site (Subbarao, 2006).

Table 2.1 Biological nitrification inhibitors identified from plants and their mode of inhibition action.

Nitrification inhibitors	Isolated from	Inhibit AMO or HAO	References
Biological nitrification inhibitors			
Linoleic acid (LA)	*B. humidicola* shoot tissue	AMO and HAO	(Subbarao et al., 2008)
Methyl linoleate (LA-ME)	*B. humidicola* shoot tissue	AMO and HAO	(Subbarao et al., 2008)
Ethyl linoleate (LA-EE)	*B. humidicola* shoot tissue	AMO and HAO	(Subbarao et al., 2008)
Linolenic acid (LN)	*B. humidicola* shoot tissue	AMO and HAO	(Subbarao et al., 2008)
Methyl Linolenic (LN-ME)	*B. humidicola* shoot tissue	AMO and HAO	(Subbarao et al., 2008)
Brachialactone	*B. humidicola* root exudate	AMO and HAO	(Subbarao et al., 2009)
Methyl p-coumarate	*B. humidicola* root tissue	NA	(Gopalakrishnan et al., 2007)
Methyl ferulate	*B. humidicola* root tissue	NA	(Gopalakrishnan et al., 2007)
Sorgoleone	Sorghum root exudate	AMO and HAO	(Subbarao et al., 2013)
Methyl 3-(4-hydroxyphenyl) propionate (MHPP)	Sorghum root exudate	AMO	(Zakir et al., 2008)
Sakuranetin	Sorghum root exudate	AMO and HAO	(Subbarao et al., 2013)
1,9-Decanediol	Rice root exudates	AMO	(Sun et al., 2016)
NA	Wheat root exudates	HAO	(Subbarao et al., 2007b)
NA	Wheat root tissue extracts	NA	(O'Sullivan et al., 2016)
Karanjin (3-methoxy furano-2,3,7,8-flavone)	Seeds of Pongamia glabra Vent.	NA	(Sahrawat and Mukerjee, 1977)
Isothiocyanate	Degradation of cruciferous tissues	NA	(Bending and Lincoln, 2000)
Limonene	Pinus ponderosa leaf	AMO	(White, 1988)
Synthetic nitrification inhibitors			
Dicyandiamide (DCD)		AMO	(Zakir et al., 2008)
3, 4-dimethylpyrazol-phosphate (DMPP)		AMO	(Benckiser et al., 2013)
2-chloro-6-(trichloromethyl)-pyridine (Nitrapyrin)		AMO	(Subbarao et al., 2013)
Allylthiourea (AT)		AMO	(Subbarao et al., 2013)

NA: not applicable

2.4.2. Methodology for the detection of biological NIs

The oldest method to detect NIs is to grow pure cultures of ammonia oxidising organisms for 5 to 7 days and track their NO_2^- production rates in the presence and absence of NIs, but this approach is labour intensive and time consuming (O'Sullivan et al., 2017). In 1998, a bioluminescence assay using *Nitrosomonas europaea* for rapid and sensitive detection of NIs was developed, that involved introducing an expression vector derived from *Vibrio harveyi* for the *luxAB* genes into *Nitrosomonas*

europaea; the expression of luxAB genes produces bioluminescence which has a close relationship with ammonia-oxidising activity (Iizumi et al., 1998). Subbarao et al. (2006a) adopted and modified the bioluminescence assay (developed in 1998) to detect and quantify BNI compounds released from roots of plants. The amount of BNI activity released by the roots of *Brachiaria humidicola* ranges from 15-25 AT unit g^{-1} root dry wt day^{-1} (one AT unit: 0.22 µM AT containing 18.9 mM of NH_4^+) (Subbarao et al., 2006a). A number of species including tropical and temperature pastures, cereals and legumes were tested for BNI capacity in their root exudate, and the specific BNI (AT unit g^{-1} root dry wt) ranged from 0 to 18.3 AT units (Subbarao et al., 2007a). This bioluminescence assay to detect and quantify nitrification inhibition is fast, but it is difficult to obtain and cultivate the genetically modified reporter bacterium which is not for commercial sale and requires regulated (PC2) laboratory conditions (O'Sullivan et al., 2017).

In 2016, a rapid, simple and accurate colorimetric microplate assay combined with the Griess assay was developed to detect and quantify biological NIs in plants (O'Sullivan et al., 2017). The method uses 'native' nitrifier cultures (pure cultures of AOB) and microplate detection to track the rate of NO_2^- production using a segmented flow analyser in the presence and absence of NIs collected from root exudates, which indicates the metabolic activity of targeting organism directly. The colour change reaction in the colorimetric microplate assay is stable, nevertheless, different exudate collection solutions (NH_4Cl, NH_4Cl with methanol, $CaCl_2$, nutrient solution used for hydroponic growth, and *Nitrosomonas europaea* growth medium minus $NaHCO_3$) result in significantly different BNI capacity of the root exudates (O'Sullivan et al., 2017). Karwat et al. (2019) have verified that nitrate reductase activity (NRA) data in leaves of *Brachiaria humidicola* reflects *in vivo* performance of BNI when complemented with established BNI methodologies under greenhouse and field conditions.

2.4.3. Factors influencing the release of biological NIs

Growth stage of plants. For the plants, stages of growth and shoot N content influence the excretion and activity of biological NIs (Subbarao et al., 2006a). A bioluminescence assay to detect BNI activity released from roots of *Brachiaria humidicola* during 24 h showed that the total BNI

capacity increased from 14 AT units to 36 AT units between the 10 and 50 days after transferring two weeks old plants (days after sowing) to hydroponics. However, the specific BNI activity decreased during this same time period (Subbarao et al., 2006a). Zakir et al. (2008) found that the release of BNI compounds increased with growth stage using a bioluminescence assay using recombinant *Nitrosomonas europaea*.

Added N form. The N forms in the root environment play an important role in the release of BNI activity (Subbarao et al., 2007c; Zhang et al., 2019). The release of BNI compounds from roots is enhanced when plants are grown with NH_4^+ (nearly three times greater) compared with NO_3^- (Subbarao et al., 2007c). This has also been supported by the study of Zhang et al. (2019), in which the release of 1,9-decanediol from the root of rice is enhanced by low to moderate concentrations of NH_4^+ ($\leq$1.0 mM). In addition, the secretion of 1,9-decanediol was triggered in the whole root system, even though only a part of the root system was exposed to NH_4^+ (Zhang et al., 2019). The release of BNI compounds from sorghum is also stimulated by the presence of NH_4^+ and increases with the concentration of NH_4^+ supply (Zakir et al., 2008).

Soil pH. In rhizosphere, the presence of NH_4^+ or low pH of the root environment alone did not have a major effect on the release of biological NIs, but together have a synergistic effect (Subbarao et al., 2007c). Low pH stimulates the release of 1,9-decanediol, but NH_4NO_3 was applied as a N source during the root exudate collection from rice (Zhang et al., 2019). At least three active components have been identified from roots of *Brachiaria humidicola*: Type-I is stable to pH changes ranging from 3.0 to 10, which is the major proportion of BNI compounds in the presence of NH_4^+; Type-II temporarily loses its inhibitory effect at a threshold pH of >4.5 and the inhibitory effect is re-established when the root exudate pH is adjusted to <4.5; the inhibitory effect of Type-III is irreversibly lost if the pH of the root exudate reaches >10.0 (Type-II and Type-III are major BNI compounds in the absence of NH_4^+) (Subbarao et al., 2007c). However, the decrease in rhizosphere pH stimulates the release of hydrophilic-biological NIs, but has no effect on the release of hydrophobic-BNIs released from sorghum roots (Di et al., 2018).

2.5. Knowledge gaps in our current understanding

A bioluminescence assay using recombinant *Nitrosomonas europaea* has been widely used to determine the BNI capacity, inhibitory effects (transformation of NH_4^+ to NO_3^-) and mode of action (inhibition of HAO or AMO pathways) of root exudates collected from *Brachiaria humidicola* genotypes (Subbarao et al., 2006a), rice varieties (Sun et al., 2016), sorghum (Zakir et al., 2008) and wheat (O'Sullivan et al., 2016). Specific compounds that have been identified as biological NIs have been applied at a range of concentration (ranging from 0 to 1000 mg NI kg^{-1} dry soil) to explore their effects on nitrification rates and ammonia oxidisers, e.g. LA and LN (Subbarao et al., 2008), 1,9-decanediol (Lu et al., 2019) and MHPP (Nardi et al., 2013), indicating that only higher doses of specific compounds application (≥ 500 mg NI kg^{-1} dry soil or 350 mg NI-C kg^{-1} dry soil) result in significant nitrification inhibition and reduction of the abundance of AOB and/or AOA. In recent years, some *Brachiaria* species have been grown to explore their effects on soil N_2O emissions, AOB and AOA abundance when grazed with cattle, indicating the potential of forage grasses with high BNI activity to reduce N losses in grazed pastures (Byrnes et al., 2017; Simon et al., 2020).

However, little is known about the effects of biological NIs on soil GHG emissions, and N gases other than N_2O, e.g. NO and N_2. Less is known about the factors that influence the efficacy of biological NIs on nitrification rates, e.g. biological NIs concentrations and their stability. Moreover, even less is known about the mechanism of biological NIs to inhibit nitrification. Hence, the main aims of this thesis are to i) determine the effects of biological NIs on soil nitrification and GHG emissions, ii) explore the factors controlling the efficacy of biological NIs, and iii) clarify the mechanism of BNI through studying the microbial populations and using ^{14}C-labelling of specific BNI compounds.

2.6. References

Abalos, D., Jeffery, S., Sanz-Cobena, A., Guardia, G., Vallejo, A., 2014. Meta-analysis of the effect of urease and nitrification inhibitors on crop productivity and nitrogen use efficiency. Agriculture, Ecosystems and Environment 189, 136–144. doi:10.1016/j.agee.2014.03.036

Allen, A.G., Machado, C.M.D., Cardoso, A.A., 2011. Measurements and modeling of reactive nitrogen deposition in southeast Brazil. Environmental Pollution 159, 1190–1197. doi:10.1016/j.envpol.2011.02.002

Ashworth, J., Briggs, G.G., Evans, A.A., Matula, J., 1977. Inhibition of nitrification by nitrapyrin, carbon disulphide and trithiocarbonate. Journal of the Science of Food and Agriculture 28, 673–683. doi:10.1002/jsfa.2740280804

Balaine, N., Clough, T.J., Kelliher, F.M., van Koten, C., 2015. Soil aeration affects the degradation rate of the nitrification inhibitor dicyandiamide. Soil Research 53, 137. doi:10.1071/SR14162

Barrena, I., Menéndez, S., Correa-Galeote, D., Vega-Mas, I., Bedmar, E.J., González-Murua, C., Estavillo, J.M., 2017. Soil water content modulates the effect of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on nitrifying and denitrifying bacteria. Geoderma 303, 1–8. doi:10.1016/j.geoderma.2017.04.022

Barth, G., Von Tucher, S., Schmidhalter, U., 2001. Influence of soil parameters on the effect of 3,4-dimethylpyrazole-phosphate as a nitrification inhibitor. Biology and Fertility of Soils 34, 98–102. doi:10.1007/s003740100382

Benckiser, G., Christ, E., Herbert, T., Weiske, A., Blome, J., Hardt, M., 2013. The nitrification inhibitor 3,4-dimethylpyrazole-phosphat (DMPP) - quantification and effects on soil metabolism. Plant and Soil 371, 257–266. doi:10.1007/s11104-013-1664-6

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

Braker, G., Schwarz, J., Conrad, R., NW, M., KS, P., DC, W., JHC, C., J, G., LP, N., MA, V., 2010. Influence of temperature on the composition and activity of denitrifying soil communities. FEMS Microbiology Ecology 61, no-no. doi:10.1111/j.1574-6941.2010.00884.x

Brath, G., Von Tucher, S., Schmidhalter, U., 2008. Effectiveness of 3,4-Dimethylpyrazole Phosphate as Nitriflcation Inhibitor in Soil as Influenced by Inhibitor Concentration, Application Form, and Soil Matric Potential1 1 Project supported by the Federal Ministry of Education and Research, Bonn, Germany. Pedosphere 18, 378–385. doi:10.1016/S1002-0160(08)60028-4

Breuer, L., Kiese, R., Butterbach-Bahl, K., 2002. Temperature and Moisture Effects on Nitrification Rates in Tropical Rain-Forest Soils. Soil Science Society of America Journal 66, 834–844. doi:10.2136/sssaj2002.8340

Byrnes, R.C., Nùñez, J., Arenas, L., Rao, I., Trujillo, C., Alvarez, C., Arango, J., Rasche, F., Chirinda, N., 2017. Biological nitrification inhibition by Brachiaria grasses mitigates soil nitrous oxide emissions from bovine urine patches. Soil Biology and Biochemistry 107, 156–163. doi:10.1016/j.soilbio.2016.12.029

Cameron, K., Di, H., Moir, J., 2014. Dicyandiamide (DCD) effect on nitrous oxide emissions, nitrate leaching and pasture yield in Canterbury, New Zealand. New Zealand Journal of Agricultural Research 57, 251–270. doi:10.1080/00288233.2013.797914

Cameron, K.C., Di, H.J., 2002. The use of a nitrification inhibitor, dicyandiamide (DCD), to decrease nitrate leaching and nitrous oxide emissions in a simulated grazed and irrigated grassland. Soil Use and Management 18, 395–403. doi:10.1079/SUM2002151

Cardenas, L.M., Misselbrook, T.M., Hodgson, C., Donovan, N., Gilhespy, S., Smith, K.A., Dhanoa, M.S., Chadwick, D., 2016. Effect of the application of cattle urine with or without the nitrification inhibitor DCD, and dung on greenhouse gas emissions from a UK grassland soil. Agriculture, Ecosystems and Environment 235, 229–241. doi:10.1016/j.agee.2016.10.025

Che, J., Zhao, X.Q., Zhou, X., Jia, Z.J., Shen, R.F., 2015. High pH-enhanced soil nitrification was associated with ammonia-oxidizing bacteria rather than archaea in acidic soils. Applied Soil Ecology 85, 21–29. doi:10.1016/j.apsoil.2014.09.003

Chen, D., Suter, H.C., Islam, A., Edis, R., 2010. Influence of nitrification inhibitors on nitrification and nitrous oxide (N2O) emission from a clay loam soil fertilized with urea. Soil Biology and Biochemistry 42, 660–664. doi:10.1016/j.soilbio.2009.12.014

Chen, Q., Qi, L., Bi, Q., Dai, P., Sun, D., Sun, C., Liu, W., Lu, L., Ni, W., Lin, X., 2014. Comparative effects of 3,4-dimethylpyrazole phosphate (DMPP) and dicyandiamide (DCD) on ammonia-oxidizing bacteria and archaea in a vegetable soil. Applied Microbiology and Biotechnology 99, 477–487. doi:10.1007/s00253-014-6026-7

Ciganda, V.S., López-Aizpún, M., Repullo, M.A., Wu, D., Terra, J.A., Elustondo, D., Clough, T., Cardenas, L.M., 2018. Soil nitrous oxide emissions from grassland: Potential inhibitor effect of hippuric acid. Journal of Plant Nutrition and Soil Science 40–47. doi:10.1002/jpln.201700393

Cui, M., Sun, X., Hu, C., Di, H.J., Tan, Q., Zhao, C., 2011. Effective mitigation of nitrate leaching and nitrous oxide emissions in intensive vegetable production systems using a nitrification inhibitor, dicyandiamide. Journal of Soils and Sediments 11, 722–730. doi:10.1007/s11368-011-0357-0

Di, H.J., Cameron, K.C., 2011. Inhibition of ammonium oxidation by a liquid formulation of 3,4-Dimethylpyrazole phosphate (DMPP) compared with a dicyandiamide (DCD) solution in six new Zealand grazed grassland soils. Journal of Soils and Sediments 11, 1032–1039. doi:10.1007/s11368-011-0372-1

Di, H.J., Cameron, K.C., 2006. Nitrous oxide emissions from two dairy pasture soils as affected by different rates of a fine particle suspension nitrification inhibitor, dicyandiamide. Biology and Fertility of Soils 42, 472–480. doi:10.1007/s00374-005-0038-5

Di, H.J., Cameron, K.C., 2004. Effects of the nitrification inhibitor dicyandiamide on potassium, magnesium and calcium leaching in grazed grassland. Soil Use and Management 20, 2–7. doi:10.1079/SUM2003205

Di, H.J., Cameron, K.C., Podolyan, A., Robinson, A., 2014. Effect of soil moisture status and a nitrification inhibitor, dicyandiamide, on ammonia oxidizer and denitrifier growth and nitrous oxide emissions in a grassland soil. Soil Biology and Biochemistry 73, 59–68. doi:10.1016/j.soilbio.2014.02.011

Di, T., Afzal, M.R., Yoshihashi, T., Deshpande, S., Zhu, Y., Subbarao, G.V., 2018. Further insights into underlying mechanisms for the release of biological nitrification inhibitors from sorghum roots. Plant and Soil 423, 99–110. doi:10.1007/s11104-017-3505-5

Dlamini, J.C., Chadwick, D., Hawkins, J.M.B., Martinez, J., Scholefield, D., Ma, Y., Cárdenas, L.M., 2020. Evaluating the potential of different carbon sources to promote denitrification.

Downing, L.S., Nerenberg, R., 2008. Effect of oxygen gradients on the activity and microbial community structure of a nitrifying, membrane-aerated biofilm. Biotechnology and Bioengineering 101, 1193–1204. doi:10.1002/bit.22018

Fangueiro, D., Fernandes, A., Coutinho, J., Moreira, N., Trindade, H., 2009. Influence of Two Nitrification Inhibitors (DCD and DMPP) on Annual Ryegrass Yield and Soil Mineral N Dynamics after Incorporation with Cattle Slurry. Communications in Soil Science and Plant Analysis 40, 3387–3398. doi:10.1080/00103620903325976

FAO, 2019. World Fertilizer Trends and Outlook to 2022.

Firestone, M.K., Davidson, E.A., 1989. Microbiologial Basis of NO and N2O production and consumption in soil. Exchange of Trace Gases between Terrestrial Ecosystems and the Atmosphere 7–21. doi:10.1017/CBO9781107415324.004

Gillam, K.M., Zebarth, B.J., Burton, D.L., 2008. Nitrous oxide emissions from denitrification and the partitioning of gaseous losses as affected by nitrate and carbon addition and soil aeration 133–143.

Gilsanz, C., Báez, D., Misselbrook, T.H., Dhanoa, M.S., Cárdenas, L.M., 2016. Development of emission factors and efficiency of two nitrification inhibitors, DCD and DMPP. Agriculture, Ecosystems and Environment 216, 1–8. doi:10.1016/j.agee.2015.09.030

Gong, P., Zhang, L.L., Wu, Z.J., Chen, Z.H., Chen, L.J., 2013. Responses of Ammonia-Oxidizing

Bacteria and Archaea in Two Agricultural Soils to Nitrification Inhibitors DCD and DMPP: A Pot Experiment. Pedosphere 23, 729–739. doi:10.1016/S1002-0160(13)60065-X

Gopalakrishnan, S., Subbarao, G. V., Nakahara, K., Yoshihashi, T., Ito, O., Maeda, I., Ono, H., Yoshida, M., 2007. Nitrification inhibitors from the root tissues of Brachiaria humidicola, a tropical grass. Journal of Agricultural and Food Chemistry 55, 1385–1388. doi:10.1021/jf062593o

Groenestein, C.M., Hutchings, N.J., Haenel, H.D., Amon, B., Menzi, H., Mikkelsen, M.H., Misselbrook, T.H., Bruggen, C. Van, Kupper, T., Webb, J., 2019. Comparison of ammonia emissions related to nitrogen use ef fi ciency of livestock production in Europe. Journal of Cleaner Production 211, 1162–1170. doi:10.1016/j.jclepro.2018.11.143

Guardia, G., Marsden, K.A., Vallejo, A., Jones, D.L., Chadwick, D.R., 2018. Determining the influence of environmental and edaphic factors on the fate of the nitrification inhibitors DCD and DMPP in soil. Science of the Total Environment 624, 1202–1212. doi:10.1016/j.scitotenv.2017.12.250

He, X., Ji, G., 2020. Responses of AOA and AOB activity and DNA/cDNA community structure to allylthiourea exposure in the water level fluctuation zone soil. Environmental Science and Pollution Research. doi:10.1007/s11356-020-07952-9

Huérfano, X., Fuertes-Mendizábal, T., Fernández-Diez, K., Estavillo, J.M., González-Murua, C., Menéndez, S., 2016. The new nitrification inhibitor 3,4-dimethylpyrazole succinic (DMPSA) as an alternative to DMPP for reducing N2O emissions from wheat crops under humid Mediterranean conditions. European Journal of Agronomy 80, 78–87. doi:10.1016/j.eja.2016.07.001

Iizumi, T., Mizumoto, M., Nakamura, K., 1998. A Bioluminescence Assay Using Nitrosomonas europaea for Rapid and Sensitive Detection of Nitrification Inhibitors A Bioluminescence Assay Using Nitrosomonas europaea for Rapid and Sensitive Detection of Nitrification Inhibitors. Applied and Environmental Microbiology 64, 3656–3662.

Jetten, M.S.M., Niftrik, L. Van, Strous, M., Kartal, B., Keltjens, J.T., Op Den Camp, H.J.M., 2009. Biochemistry and molecular biology of anammox bacteria biochemistry and molecular biology of anammox bacteria M.S.M. Jetten et al. Critical Reviews in Biochemistry and Molecular Biology 44, 65–84. doi:10.1080/10409230902722783

Kandeler, E., Deiglmayr, K., Tscherko, D., Bru, D., Philippot, L., 2006. Abundance of narG, nirS, nirK, and nosZ genes of denitrifying bacteria during primary successions of a glacier foreland. Applied and Environmental Microbiology 72, 5957–5962. doi:10.1128/AEM.00439-06

Karwat, H., Sparke, M.A., Rasche, F., Arango, J., Nuñez, J., Rao, I., Moreta, D., Cadisch, G., 2019. Nitrate reductase activity in leaves as a plant physiological indicator of in vivo biological nitrification inhibition by Brachiaria humidicola. Plant Physiology and Biochemistry 137, 113–120. doi:10.1016/j.plaphy.2019.02.002

Kelliher, F.M., Clough, T.J., Clark, H., Rys, G., Sedcole, J.R., 2008. The temperature dependence of dicyandiamide (DCD) degradation in soils: A data synthesis. Soil Biology and Biochemistry 40, 1878–1882. doi:10.1016/j.soilbio.2008.03.013

Kelliher, F.M., van Koten, C., Kear, M.J., Sprosen, M.S., Ledgard, S.F., de Klein, C.A.M., Letica, S.A., Luo, J., Rys, G., 2014. Effect of temperature on dicyandiamide (DCD) longevity in pastoral soils under field conditions. Agriculture, Ecosystems and Environment 186, 201–204. doi:10.1016/j.agee.2014.01.026

Kleineidam, K., Košmrlj, K., Kublik, S., Palmer, I., Pfab, H., Ruser, R., Fiedler, S., Schloter, M., 2011. Chemosphere Influence of the nitrification inhibitor 3 , 4-dimethylpyrazole phosphate (DMPP) on ammonia-oxidizing bacteria and archaea in rhizosphere and bulk soil. Chemosphere 84, 182–186. doi:10.1016/j.chemosphere.2011.02.086

Knowles, R., 1982. Denitrification. Microbiological Reviews 46, 43–70.

doi:10.1128/CMR.5.4.356.Updated

Kyveryga, P.M., Blackmer, A.M., Ellsworth, J.W., Isla, R., 2004. Soil pH Effects on Nitrification of Fall-Applied Anhydrous Ammonia. Soil Science Society of America Journal 68, 545–551. doi:10.2136/sssaj2004.5450

Lai, T. V., Farquharson, R., Denton, M.D., 2019. High soil temperatures alter the rates of nitrification, denitrification and associated N2O emissions. Journal of Soils and Sediments 19, 2176–2189. doi:10.1007/s11368-018-02238-7

Lam, S.K., Suter, H., Davies, R., Bai, M., Mosier, A.R., Sun, J., Chen, D., 2018. Direct and indirect greenhouse gas emissions from two intensive vegetable farms applied with a nitrification inhibitor. Soil Biology and Biochemistry 116, 48–51. doi:10.1016/j.soilbio.2017.10.008

Lan, T., Suter, H., Liu, R., Yuan, S., Chen, D., 2018. Effects of nitrification inhibitors on gross N nitrification rate, ammonia oxidizers, and N2O production under different temperatures in two pasture soils. Environmental Science and Pollution Research 25, 28344–28354. doi:10.1007/s11356-018-2873-6

Lassaletta, L., Billen, G., Grizzetti, B., Anglade, J., Garnier, J., 2014. 50 Year Trends in Nitrogen Use Efficiency of World Cropping Systems: the Relationship Between Yield and Nitrogen Input To Cropland. Environmental Research Letters 9, 105011. doi:10.1088/1748-9326/9/10/105011

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C., Schleper, C., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442, 806–809. doi:10.1038/nature04983

Li, H., Liang, X., Chen, Y., Lian, Y., Tian, G., Ni, W., 2008. Effect of nitrification inhibitor DMPP on nitrogen leaching, nitrifying organisms, and enzyme activities in a rice-oilseed rape cropping system. Journal of Environmental Sciences 20, 149–155. doi:10.1016/S1001-0742(08)60023-6

Li, J., Shi, Y., Luo, J., Li, Y., Wang, L., Lindsey, S., 2019. Effects of 3,4-dimethylpyrazole phosphate (DMPP) on the abundance of ammonia oxidizers and denitrifiers in two different intensive vegetable cultivation soils. Journal of Soils and Sediments 19, 1250–1259. doi:10.1007/s11368-018-2155-4

Li, T., Zhang, W., Yin, J., Chadwick, D., Norse, D., Lu, Y., Liu, X., Chen, X., Zhang, F., Powlson, D., Dou, Z., 2018. Enhanced-efficiency fertilizers are not a panacea for resolving the nitrogen problem. Global Change Biology 24, e511–e521. doi:10.1111/gcb.13918

Li, Y., Chapman, S.J., Nicol, G.W., Yao, H., 2018. Nitrification and nitrifiers in acidic soils. Soil Biology and Biochemistry 116, 290–301. doi:10.1016/j.soilbio.2017.10.023

Liu, C., Wang, K., Zheng, X., 2013. Effects of nitrification inhibitors (DCD and DMPP) on nitrous oxide emission, crop yield and nitrogen uptake in a wheat-maize cropping system. Biogeosciences 10, 2427–2437. doi:10.5194/bg-10-2427-2013

Liu, R., Hayden, H., Suter, H., He, J., Chen, D., 2015. The effect of nitrification inhibitors in reducing nitrification and the ammonia oxidizer population in three contrasting soils. Journal of Soils and Sediments 15, 1113–1118. doi:10.1007/s11368-015-1086-6

Loick, N., Dixon, E.R., Abalos, D., Vallejo, A., Matthews, G.P., J.McGeough, K., Well, R., Watson, C., L.Laughlin, R.J., Cardenas, L.M., 2016. Denitrification as a source of nitric oxide emissions from incubated soil cores from a UK grassland soil. Soil Biology and Biochemistry 95, 1–7. doi:10.1016/j.soilbio.2015.12.009

Lu, Y., Zhang, X., Jiang, J., Kronzucker, H.J., Shen, W., Shi, W., 2019. Effects of the biological nitrification inhibitor 1,9-decanediol on nitrification and ammonia oxidizers in three agricultural soils. Soil Biology and Biochemistry 129, 48–59. doi:10.1016/j.soilbio.2018.11.008

Lucas, G.N., 2013. Dicyandiamide contamination of milk powders. Sri Lanka Journalof Child Health 42, 63–64. doi:10.4038/sljch.v42i2.5624

Marsden, K.A., Marín-Martínez, A.J., Vallejo, A., Hill, P.W., Jones, D.L., Chadwick, D.R., 2016. The mobility of nitrification inhibitors under simulated ruminant urine deposition and rainfall: a comparison between DCD and DMPP. Biology and Fertility of Soils 52, 491–503. doi:10.1007/s00374-016-1092-x

Marsden, K.A., Scowen, M., Hill, P.W., Jones, D.L., Chadwick, D.R., 2015. Plant acquisition and metabolism of the synthetic nitrification inhibitor dicyandiamide and naturally-occurring guanidine from agricultural soils. Plant and Soil 395, 201–214. doi:10.1007/s11104-015-2549-7

McCarty, G.W., 1999. Modes of action of nitrification inhibitors. Biology and Fertility of Soils. doi:10.1007/s003740050518

McGeough, K.L., Watson, C.J., Müller, C., Laughlin, R.J., Chadwick, D.R., 2016. Evidence that the efficacy of the nitrification inhibitor dicyandiamide (DCD) is affected by soil properties in UK soils. Soil Biology and Biochemistry 94, 222–232. doi:10.1016/j.soilbio.2015.11.017

Menéndez, S., Barrena, I., Setien, I., González-Murua, C., Estavillo, J.M., 2012. Efficiency of nitrification inhibitor DMPP to reduce nitrous oxide emissions under different temperature and moisture conditions. Soil Biology and Biochemistry 53, 82–89. doi:10.1016/j.soilbio.2012.04.026

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

Monaghan, R.M., Smith, L.C., Ledgard, S.F., 2009. The effectiveness of a granular formulation of dicyandiamide (DCD) in limiting nitrate leaching from a grazed dairy pasture. New Zealand Journal of Agricultural Research 52, 145–159. doi:10.1080/00288230909510499

Mørkved, P.T., Dörsch, P., Henriksen, T.M., Bakken, L.R., 2006. N2O emissions and product ratios of nitrification and denitrification as affected by freezing and thawing. Soil Biology and Biochemistry 38, 3411–3420. doi:10.1016/j.soilbio.2006.05.015

Mosier, A.R., Duxbury, J.M., Freney, J.R., Heinemeyer, O., Minami, K., 1996. Nitrous Oxide Emissions from Agricultural Fields: Assessment, Measurement and Mitigation, in: Progress in Nitrogen Cycling Studies. Springer Netherlands, Dordrecht, pp. 589–602. doi:10.1007/978-94-011-5450-5_97

Muller, C., Stevens, R.J., Laughlin, R.J., Azam, F., Ottow, J.C.G., 2002. The nitrification inhibitor DMPP had no effect on denitrifying enzyme activity. Soil Biology and Biochemistry 34, 1825–1827. doi:10.1016/S0038-0717(02)00165-7

Nair, D., Baral, K.R., Abalos, D., Strobel, B.W., Petersen, S.O., 2020. Nitrate leaching and nitrous oxide emissions from maize after grass-clover on a coarse sandy soil: Mitigation potentials of 3,4-dimethylpyrazole phosphate (DMPP). Journal of Environmental Management 260. doi:10.1016/j.jenvman.2020.110165

Nardi, P., Akutsu, M., Pariasca-Tanaka, J., Wissuwa, M., 2013. Effect of methyl 3-4-hydroxyphenyl propionate, a Sorghum root exudate, on N dynamic, potential nitrification activity and abundance of ammonia-oxidizing bacteria and archaea. Plant and Soil 367, 627–637. doi:10.1007/s11104-012-1494-y

Niu, Y., Luo, J., Liu, D., Müller, C., Zaman, M., Lindsey, S., Ding, W., 2018. Effect of biochar and nitrapyrin on nitrous oxide and nitric oxide emissions from a sandy loam soil cropped to maize. Biology and Fertility of Soils 54, 645–658. doi:10.1007/s00374-018-1289-2

Nugroho, R.A., Röling, W.F.M., Laverman, A.M., Verhoef, H.A., 2007. Low nitrification rates in acid scots pine forest soils are due to pH-related factors. Microbial Ecology 53, 89–97. doi:10.1007/s00248-006-9142-9

O'Sullivan, C.A., Duncan, E.G., Whisson, K., Treble, K., Ward, P.R., Roper, M.M., 2017. A

colourimetric microplate assay for simple, high throughput assessment of synthetic and biological nitrification inhibitors. Plant and Soil 413, 275–287. doi:10.1007/s11104-016-3100-1

O'Sullivan, C.A., Fillery, I.R.P., Roper, M.M., Richards, R.A., 2016. Identification of several wheat landraces with biological nitrification inhibition capacity. Plant and Soil 404, 61–74. doi:10.1007/s11104-016-2822-4

Osborne, B.B., Baron, J.S., Wallenstein, M.D., 2016. Moisture and temperature controls on nitrification differ among ammonia oxidizer communities from three alpine soil habitats. Frontiers of Earth Science 10, 1–12. doi:10.1007/s11707-015-0556-x

Ouyang, Y., Norton, J.M., Stark, J.M., 2017. Ammonium availability and temperature control contributions of ammonia oxidizing bacteria and archaea to nitrification in an agricultural soil. Soil Biology and Biochemistry 113, 161–172. doi:10.1016/j.soilbio.2017.06.010

Pacholski, A., Berger, N., Bustamante, I., Ruser, R., Guardia, G., Mannheim, T., 2016. Effects of the novel nitrification inhibitor DMPSA on yield, mineral N dynamics and N2O emissions 2–5.

Peng, M.W., Guan, Y., Liu, J.H., Chen, L., Wang, H., Xie, Z.Z., Li, H.Y., Chen, Y.P., Liu, P., Yan, P., Guo, J.S., Liu, G., Shen, Y., Fang, F., 2020. Quantitative three-dimensional nondestructive imaging of whole anaerobic ammonium-oxidizing bacteria. Journal of Synchrotron Radiation 27, 753–761. doi:10.1107/S1600577520002349

Philippot, L., Højberg, O., 1999. Dissimilatory nitrate reductases in bacteria. Biochimica et Biophysica Acta - Gene Structure and Expression 1446, 1–23. doi:10.1016/S0167-4781(99)00072-X

Pihlatie, M., Syväsalo, E., Simojoki, A., Esala, M., Regina, K., 2004. Contribution of nitrification and denitrification to N2O production in peat, clay and loamy sand soils under different soil moisture conditions. Nutrient Cycling in Agroecosystems 70, 135–141. doi:10.1023/B:FRES.0000048475.81211.3c

Pintathong, P., Richardson, D.J., Spiro, S., Choorit, W., 2009. Influence of metal ions and organic carbons on denitrification activity of the halotolerant bacterium, Paracoccus pantotrophus P16 a strain from shrimp pond. Electronic Journal of Biotechnology 12, 1–11. doi:10.2225/vol12-issue2-fulltext-12

Puttanna, K., Gowda, N.M.N., Rao, E.V.S.P., 1999. Effect of concentration , temperature , moisture , liming and organic matter on the efficacy of the nitrification inhibitors benzotriazole , o - nitrophenol , m -nitroaniline and dicyandiamide 196464, 251–257.

Robinson, A., Di, H.J., Cameron, K.C., Podolyan, A., He, J., 2014. The effect of soil pH and dicyandiamide (DCD) on N2O emissions and ammonia oxidiser abundance in a stimulated grazed pasture soil. Journal of Soils and Sediments 14, 1434–1444. doi:10.1007/s11368-014-0888-2

Saggar, S., Jha, N., Deslippe, J., Bolan, N.S., Luo, J., Giltrap, D.L., Kim, D.G., Zaman, M., Tillman, R.W., 2013. Denitrification and N2O: N2 production in temperate grasslands: Processes, measurements, modelling and mitigating negative impacts. Science of the Total Environment 465, 173–195. doi:10.1016/j.scitotenv.2012.11.050

Sahrawat, K.L., 2008. Factors affecting nitrification in soils. Communications in Soil Science and Plant Analysis 39, 1436–1446. doi:10.1080/00103620802004235

Sahrawat, K.L., 1989. Effects of Nitrification Inhibitors on Nitrogen Transformations, Other Than Nitrification, in Soils. Advances in Agronomy 42, 279–309. doi:10.1016/S0065-2113(08)60527-1

Sahrawat, K.L., 1981. Comparison of Karanjin with other nitrification inhibitors for retardation of nitrification of urea N in soil. Plant and Soil 59, 495–498. doi:10.1007/BF02184555

Sahrawat, K.L., Mukerjee, S.K., 1977. Nitrification inhibitors. Plant and Soil 36, 27–36.

Saleh-Lakha, S., Shannon, K.E., Henderson, S.L., Goyer, C., Trevors, J.T., Zebarth, B.J., Burton, D.L., 2009. Effect of pH and temperature on denitrification gene expression and activity in Pseudomonas mandelii. Applied and Environmental Microbiology 75, 3903–11. doi:10.1128/AEM.00080-09

Sayavedra-Soto, L.A., Hommes, N.G., Russell, S.A., Arp, D.J., 1996. Induction of ammonia monooxygenase and hydroxylamine oxidoreductase mRNAs by ammonium in Nitrosomonas europaea. Molecular Microbiology 20, 541–548. doi:10.1046/j.1365-2958.1996.5391062.x

Scheer, C., Rowlings, D.W., Firrel, M., Deuter, P., Morris, S., Grace, P.R., 2014. Impact of nitrification inhibitor (DMPP) on soil nitrous oxide emissions from an intensive broccoli production system in sub-tropical Australia. Soil Biology and Biochemistry 77, 243–251. doi:10.1016/j.soilbio.2014.07.006

Senbayram, M., Budai, A., Bol, R., Chadwick, D., Marton, L., Gündogan, R., Wu, D., 2019. Soil NO 3− level and O 2 availability are key factors in controlling N 2 O reduction to N 2 following long-term liming of an acidic sandy soil. Soil Biology and Biochemistry. doi:10.1016/j.soilbio.2019.02.009

Shi, X., Hu, H., He, J., Chen, D., Suter, H.C., 2016. Effects of 3,4-dimethylpyrazole phosphate (DMPP) on nitrification and the abundance and community composition of soil ammonia oxidizers in three land uses. Biology and Fertility of Soils 52, 927–939. doi:10.1007/s00374-016-1131-7

Simon, P.L., Dieckow, J., Zanatta, J.A., Ramalho, B., Ribeiro, R.H., van der Weerden, T., de Klein, C.A.M., 2020. Does Brachiaria humidicola and dicyandiamide reduce nitrous oxide and ammonia emissions from cattle urine patches in the subtropics? Science of the Total Environment 720, 137692. doi:10.1016/j.scitotenv.2020.137692

Smolders, A.J.P., Lucassen, E.C.H.E.T., Bobbink, R., Roelofs, J.G.M., Lamers, L.P.M., 2010. How nitrate leaching from agricultural lands provokes phosphate eutrophication in groundwater fed wetlands: the sulphur bridge. Biogeochemistry 98, 1–7. doi:10.1007/s10533-009-9387-8

Strong, D.T., Sale, P.W.G., Helyar, K.R., 1999. The influence of the soil matrix on nitrogen mineralisation and nitrification. IV. Texture. Australian Journal of Soil Research 37, 329–344. doi:10.1071/S98043

Subbarao, G.V., Yoshihashi, T., Worthington, M., Nakahara, K., Ando, Y., Sahrawat, K.L., Rao, I.M., Lata, J.C., Kishii, M., Braun, H.J., 2015. Suppression of soil nitrification by plants. Plant Science. doi:10.1016/j.plantsci.2015.01.012

Subbarao, G. V., Ishikawa, T., Ito, O., Nakahara, K., Wang, H.Y., Berry, W.L., 2006a. A bioluminescence assay to detect nitrification inhibitors released from plant roots: A case study with Brachiaria humidicola. Plant and Soil 288, 101–112. doi:10.1007/s11104-006-9094-3

Subbarao, G. V., Ito, O., Sahrawat, K.L., Berry, W.L., Nakahara, K., Ishikawa, T., Watanabe, T., Suenaga, K., Rondon, M., Rao, I.M., 2006b. Scope and Strategies for Regulation of Nitrification in Agricultural Systems—Challenges and Opportunities. Critical Reviews in Plant Sciences 25, 303–335. doi:10.1080/07352680600794232

Subbarao, G. V., Nakahara, K., Hurtado, M.P., Ono, H., Moreta, D.E., Salcedo, A.F., Yoshihashi, A.T., Ishikawa, T., Ishitani, M., Ohnishi-Kameyama, M., Yoshida, M., Rondon, M., Rao, I.M., Lascano, C.E., Berry, W.L., Ito, O., 2009. Evidence for biological nitrification inhibition in Brachiaria pastures. Proceedings of the National Academy of Sciences 106, 17302–17307. doi:10.1073/pnas.0903694106

Subbarao, G. V., Rondon, M., Ito, O., Ishikawa, T., Rao, I.M., Nakahara, K., Lascano, C., Berry, W.L., 2007a. Biological nitrification inhibition (BNI) - Is it a widespread phenomenon? Plant and Soil 294, 5–18. doi:10.1007/s11104-006-9159-3

Subbarao, G. V., Tomohiro, B., Masahiro, K., Osamu, I., Samejima, H., Wang, H.Y., Pearse, S.J., Gopalakrishnan, S., Nakahara, K., Zakir Hossain, A.K.M., Tsujimoto, H., Berry, W.L., 2007b.

Can biological nitrification inhibition (BNI) genes from perennial Leymus racemosus (Triticeae) combat nitrification in wheat farming? Plant and Soil 299, 55–64. doi:10.1007/s11104-007-9360-z

Subbarao, G. V., Wang, H.Y., Ito, O., Nakahara, K., Berry, W.L., 2007c. NH4+ triggers the synthesis and release of biological nitrification inhibition compounds in Brachiaria humidicola roots. Plant and Soil 290, 245–257. doi:10.1007/s11104-006-9156-6

Subbarao, G. V, Nakahara, K., Ishikawa, T., Ono, H., Yoshida, M., Yoshihashi, T., Zhu, Y., Zakir, H.A.K.M., Deshpande, S.P., Hash, C.T., Sahrawat, K.L., 2013. Biological nitrification inhibition (BNI) activity in sorghum and its characterization. Plant Soil 366, 243–259. doi:10.1007/s11104-012-1419-9

Subbarao, G. V, Nakahara, K., Ishikawa, T., Yoshihashi, T., Ito, O., Ono, H., Ohnishi-Kameyama, M., Yoshida, M., Kawano, N., Berry, W.L., 2008. Free fatty acids from the pasture grass Brachiaria humidicola and one of their methyl esters as inhibitors of nitrification. Plant and Soil 313, 89–99. doi:10.1007/s11104-008-9682-5

Sun, L., Lu, Y., Yu, F., Kronzucker, H.J., Shi, W., 2016. Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency. New Phytologist 212, 646–656. doi:10.1111/nph.14057

Suter, H., Chen, D., Li, H., Edis, R., Walker, C., Victoria, N., 2010. Comparison of the ability of the nitrification inhibitors DCD and DMPP to reduce nitrification and N 2 O emissions from nitrogen fertilisers 84–87.

Taylor, A.E., Giguere, A.T., Zoebelein, C.M., Myrold, D.D., Bottomley, P.J., 2017. Modeling of soil nitrification responses to temperature reveals thermodynamic differences between ammonia-oxidizing activity of archaea and bacteria. ISME Journal 11, 896–908. doi:10.1038/ismej.2016.179

ten Huf, M., Olfs, H.W., 2020. Effect of the nitrification inhibitor DMPP on nitrous oxide emissions and the stabilization of ammonium following the injection of dairy slurry and digestate in a soil-column experiment. Journal of Plant Nutrition and Soil Science 129–135. doi:10.1002/jpln.201900025

Tesfamariam, T., Yoshinaga, H., Deshpande, S.P., Srinivasa Rao, P., Sahrawat, K.L., Ando, Y., Nakahara, K., Hash, C.T., Subbarao, G. V., 2014. Biological nitrification inhibition in sorghum: The role of sorgoleone production. Plant and Soil 379, 325–335. doi:10.1007/s11104-014-2075-z

Tong, T., Li, B., Xie, S., 2020. Anaerobic ammonium-oxidizing bacteria in river water treatment wetland. Folia Microbiologica 65, 315–322. doi:10.1007/s12223-019-00726-y

Trolove, S., Thomas, S., van der Klei, G., Beare, M., Cichota, R., Meenken, E., 2019. Nitrate leaching losses during pasture renewal – Effects of treading, urine, forages and tillage. Science of the Total Environment 651, 1819–1829. doi:10.1016/j.scitotenv.2018.09.333

United Nations Framework Convention on Climate Change (UNFCCC), 2020. Global Warming Potentials (IPCC Second Assessment Report).

Upadhyay, R.K., Patra, D.D., Tewari, S.K., 2011. Natural nitrification inhibitors for higher nitrogen use efficiency, crop yield, and for curtailing global warming. Journal of Tropical Agriculture 49, 19–24.

Wang, Q., Hu, H.W., Shen, J.P., Du, S., Zhang, L.M., He, J.Z., Han, L.L., 2017. Effects of the nitrification inhibitor dicyandiamide (DCD) on N2Oemissions and the abundance of nitrifiers and denitrifiers in two contrasting agricultural soils. Journal of Soils and Sediments 17, 1635–1643. doi:10.1007/s11368-016-1633-9

Weiske, A., Benckiser, G., Herbert, T., Ottow, J.C.G., 2001. Influence of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) in comparison to dicyandiamide (DCD) on nitrous

oxide emissions, carbon dioxide fluxes and methane oxidation during 3 years of repeated application in field experiments. Biology and Fertility of Soils 34, 109–117. doi:10.1007/s003740100386

White, C.S., 1988. Nitrification Inhibition by Monoterpenoids: Theoretical Mode of Action Based on Molecular Structures. Ecology 69, 1631–1633. doi:10.2307/1941663

Wu, D., Cárdenas, L.M., Calvet, S., Brüggemann, N., Loick, N., Liu, S., Bol, R., 2017. The effect of nitrification inhibitor on N2O, NO and N2emissions under different soil moisture levels in a permanent grassland soil. Soil Biology and Biochemistry 113, 153–160. doi:10.1016/j.soilbio.2017.06.007

WU, S. fu, WU, L. huan, SHI, Q. wei, WANG, Z. qiang, CHEN, X. you, LI, Y. shan, 2007. Effects of a new nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on nitrate and potassium leaching in two soils. Journal of Environmental Sciences 19, 841–847. doi:10.1016/S1001-0742(07)60140-5

Xiao-tang, J.U., Chong, Z., 2017. Nitrogen cycling and environmental impacts in upland agricultural soils in North China : A review. Journal of Integrative Agriculture 16, 2848–2862. doi:10.1016/S2095-3119(17)61743-X

Xu, J., Zhu, T., Xue, W., Ni, D., Sun, Y., Yang, J., Xu, L., Chen, X., Li, H., Liu, M., 2019. Influences of nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) and application method on nitrogen dynamics at the centimeter-scale. European Journal of Soil Biology 90, 44–50. doi:10.1016/j.ejsobi.2018.12.004

Yang, M., Fang, Y., Sun, D., Shi, Y., 2016. Efficiency of two nitrification inhibitors (dicyandiamide and 3, 4-dimethypyrazole phosphate) on soil nitrogen transformations and plant productivity: A meta-analysis. Scientific Reports 6, 1–10. doi:10.1038/srep22075

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

Zaman, M., Blennerhassett, J.D., 2010. Effects of the different rates of urease and nitrification inhibitors on gaseous emissions of ammonia and nitrous oxide, nitrate leaching and pasture production from urine patches in an intensive grazed pasture system. Agriculture, Ecosystems and Environment 136, 236–246. doi:10.1016/j.agee.2009.07.010

Zaman, M., Saggar, S., Blennerhassett, J.D., Singh, J., 2009. Effect of urease and nitrification inhibitors on N transformation, gaseous emissions of ammonia and nitrous oxide, pasture yield and N uptake in grazed pasture system. Soil Biology and Biochemistry 41, 1270–1280. doi:10.1016/j.soilbio.2009.03.011

Zerulla, W., Barth, T., Dressel, J., Erhardt, K., Horchler von Locquenghien, K., Pasda, G., R??dle, M., Wissemeier, A., 2001. 3,4-Dimethylpyrazole phosphate (DMPP) - A new nitrification inhibitor for agriculture and horticulture. An introduction. Biology and Fertility of Soils 34, 79–84. doi:10.1007/s003740100380

Zhang, Q., Li, Y., He, Y., Brookes, P.C., Xu, J., 2019. Elevated temperature increased nitrification activity by stimulating AOB growth and activity in an acidic paddy soil. Plant and Soil 445, 71–83. doi:10.1007/s11104-019-04052-7

Zhang, X., Lu, Y., Yang, T., Kronzucker, H.J., Shi, W., 2019. Factors influencing the release of the biological nitrification inhibitor 1,9-decanediol from rice (Oryza sativa L.) roots. Plant and Soil 436, 253–265. doi:10.1007/s11104-019-03933-1

Chapter 3: Biological nitrification inhibitors linoleic acid and 1,9-decanediol are ineffective at inhibiting nitrification and ammonia oxidisers in a highly nitrifying soil

Yan Ma[1,*], Davey L. Jones[1,2], Laura Cardenas[3], Qing Chen[4], David R. Chadwick[1,5]

[1] *School of Natural Science, Bangor University, Bangor, Gwynedd, LL57 2UW, UK*

[2] *SoilsWest, UWA School of Agriculture and Environment, The University of Western Australia, Perth, WA 6009, Australia*

[3] *Sustainable Agriculture Sciences Department, North Wyke, Okehampton, Devon, EX20 2SB, UK*

[4] *College of Resources and Environmental Sciences, China Agricultural University, Beijing 100193, P.R.China*

[5] *Interdisciplinary Research Centre for Agriculture Green Development in Yangtze River Basin, Southwest University, Chongqing, China*

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Abstract

Biological nitrification inhibitors (NIs) are considered to be an environmentally friendly and cost-effective strategy to inhibit soil nitrification and reduce nitrogen (N) losses, and increasing numbers of biological NIs have been isolated and identified from crops and grasses. In this study, a highly nitrifying soil (sandy clay loam textured Eutric Cambisol) was used to explore the effectiveness of newly identified biological NIs (linoleic acid, LA and 1,9-decanediol) versus a proven synthetic NI (dicyandiamide, DCD) on soil nitrification and ammonia oxidisers. Results show that DCD application retained added NH_4^+ in the soil, with inhibited conversion to NO_3^-, and significantly reduced cumulative nitrous oxide (N_2O) emissions (52.3-65.1%). Dicyandiamide also inhibited ammonia oxidising bacteria (AOB) gene abundance rather than ammonia oxidising archaea (AOA). Linoleic acid applied at a concentration of 12.7 or 127 mg kg^{-1} dry soil (equivalent to 10 or 100 mg kg^{-1} wet soil) had no effect on both soil nitrification and ammonia oxidiser gene abundance. The 1,9-decanediol application significantly reduced soil NO_3^- concentration, but did not affect soil NH_4^+ concentration, N_2O emissions or ammonia oxidiser gene abundance. We conclude that LA or 1, 9-decanediol applied at a concentration of 12.7 or 127 mg kg^{-1} dry soil was ineffective at inhibiting soil nitrification and ammonia oxidiser in a highly nitrifying soil.

Key words: biological nitrification inhibitor, linoleic acid, 1,9-decanediol, dicyandiamide, nitrous oxide, ammonia oxidiser

3.1. Introduction

During the last five decades, the global nitrogen (N) fertiliser supply has increased dramatically, but the global average N use efficiency (NUE) (percentage of applied N recovered in the crop) is relatively low, with ca. 53% of the fertiliser being lost either in gaseous (N emissions) or aqueous forms (nitrate (NO_3^-) leaching and dissolved organic N) (Gardiner et al., 2016; Lassaletta et al., 2014). These N losses from the plant-soil system not only result in low resource use efficiency, but also contribute to

important environmental problems (e.g. eutrophication, global warming, ozone depletion, air pollution) (Galloway et al., 2008; Schlesinger, 2009).

Nitrification is a two-step microbially mediated process, where ammonium (NH_4^+) is first oxidised to nitrite (NO_2^-) and subsequently to NO_3^- by nitrifying bacteria (Firestone and Davidson, 1989). Nitrification is a key soil process that controls the supply of NO_3^- that is then available for plant uptake, or for subsequent use as a substrate for denitrification and N_2O loss or can be lost from the rhizosphere via leaching (Lam et al., 2017). To increase NUE and minimize N losses, the targeting of chemicals such as synthetic nitrification inhibitors (NIs) have been explored and widely used to reduce nitrification in agriculture system (Jumadi et al., 2019; Zerulla et al., 2001).

Dicyandiamide (DCD), one of the most widely used synthetic NIs, has been developed to slow nitrification and reduce N losses in agriculture system (Monaghan et al., 2013; Robinson et al., 2014; Sharma and Prasad, 1995). During the nitrification process, NH_4^+ is first oxidised to hydroxylamine (NH_2OH) by ammonia monooxygenase (AMO) (a copper-containing enzyme), and then NH_2OH is oxidised to NO_2^- by hydroxylamine oxidoreductase (HAO) (Subbarao et al., 2007a). Research has shown that DCD only suppresses the AMO pathway in *Nitrosomonas* (Zakir et al., 2008), and DCD is effective in inhibiting ammonia oxidising bacteria (AOB) and archaea (AOA) which are important for N_2O emissions (Robinson et al., 2014). However, the application of DCD suffers from a series of challenges, including the variable responses to soil types and moisture/temperature regimes (Marsden et al., 2016b; McGeough et al., 2016; Menéndez et al., 2012), high cost for spatially-targeting NI application in the field (Luo et al., 2015; Minet et al., 2018; Welten et al., 2014), lack of chemical stability (Guardia et al., 2018; Marsden et al., 2016b) and potential food safety risk (Lucas, 2013).

The natural ability of a plant to inhibit soil nitrification by releasing inhibitors from roots is termed of biological nitrification inhibition (BNI) (Subbarao et al., 2006a). *Brachiaria humidicola*, a species of tropical pasture grass, has the ability to produce nitrification inhibitory compounds in its shoot and roots tissues, and release biological NIs from its roots (Gopalakrishnan et al., 2007; Subbarao et al., 2009, 2008). Linoleic acid (LA) and linolenic acid (LN) have been identified as predominant BNI compounds from the shoot tissue of *Brachiaria humidicola* (Subbarao et al., 2008). NH_4^+-N-fertilised soil treated with LA and LN at a concentration of 50 to 1000 mg kg^{-1} soil has shown the maintenance

of soil inorganic N in the form of NH_4^+ and decreased the NO_3^- accumulation, with a NO_3^- formation inhibition rate of 16.6-87.5% and 16.6-90.9%, respectively (Subbarao et al., 2008). Research has shown that the effectiveness of LA and LN is via the blocking of both the AMO and HAO enzymatic pathways in *Nitrosomonas* (Subbarao et al., 2008). However, little is known about the effectiveness of LA and LN on greenhouse gas (GHG) emissions and ammonia oxidisers.

In addition to *Brachiaria humidicola*, more biological NIs have been isolated and identified in other agricultural crops. Rice is one of the most important food crops for humans and is grown worldwide. Sun et al. (2016) have explored the BNI potential of 19 rice varieties on the key nitrifying bacterium *Nitrosomonas europaea*. A new biological NI, 1,9-decanediol, has been identified and has shown to block the AMO pathway of ammonia oxidation (Sun et al., 2016). Synthetic 1,9-decanediol was applied at a range of concentration (100, 500 and 1000 mg kg^{-1} dry soil) to explore its effect on nitrification and ammonia oxidisers (Lu et al., 2019). High doses of 1,9-decanediol application (500 and 1000 mg kg^{-1} dry soil) have been shown to suppress nitrification in agricultural soils by impeding AOA and AOB, with highest inhibition rates shown in the acidic red soil (43.0% and 58.1%), followed by the alkaline fluvo-aquic soil (25.6% and 37.0%) and then the neutral paddy soil (20.1% and 35.7%) (Lu et al., 2019). In addition, 1,9-decanediol can significantly reduce N_2O emissions especially in the acidic red soil (Lu et al., 2019). Hence there is potential for this BNI to be synthesised and used in other soils, climates and cropping systems throughout the world.

Previous studies using LA and 1, 9-decanediol have focussed on the transformation of NH_4^+ to NO_3^-, but little is known about the effectiveness of these biological NIs on GHG emissions (N_2O and CO_2) and especially the ammonia oxidisers. In addition, less in known about the effects of biological NIs on soil nitrification in a highly nitrifying soil after N fertiliser application. Therefore, this study aimed to compare the effect of biological (LA and 1,9-decanediol) and synthetic (DCD) NIs on soil NH_4^+ and NO_3^- concentrations, GHG (N_2O and CO_2) emissions, and ammonia oxidisers in a highly nitrifying soil after NH_4^+ fertiliser application. We hypothesised that 1) soils will retain higher NH_4^+ and lower NO_3^- concentrations in the DCD treatments than the LA and 1,9-decanediol; and 2) the application of LA, 1,9-decanediol and DCD will decrease soil N_2O emissions due to the inhibition of AOA and/or AOB.

3.2. Materials and methods

3.2.1. Soil sampling and properties

A sandy clay loam textured Eutric Cambisol (moisture content=19.4%; organic matter=6.7%; pH (H_2O)=5.9; EC=125.2 µS cm^{-1}; total carbon (C)=26.5 g kg^{-1} dry soil; total N=2.5 g kg^{-1} dry soil; C/N ratio of 10.6; NH_4^+-N=1.7 mg kg^{-1} dry soil; NO_3^--N=30.4 mg kg^{-1} dry soil) was collected from a fertilised grassland in North Wales (53°24'N, 4°02'W). The pasture had not been grazed for > 3 months. This soil was selected as it is known to have a high nitrification rate (Jones et al., 2004) and it had not been previously exposed to LA, 1,9-decanediol and DCD. Intact blocks (20×20 cm, 0-10 cm depth) were collected at 3 locations and retained as three replicates. After sampling, the soil from each replicate was sieved through a 2 mm sieve and mixed, before storing at 4 °C for 4 days in loosely sealed bags before starting the incubation experiment.

3.2.2. Experimental design and management

To compare the effects of LA and 1,9-decanediol with proven NI, DCD, on soil nitrification (NH_4^+ and NO_3^- concentrations), N_2O and CO_2 emissions, and ammonia oxidisers, synthetic compounds were applied to a highly nitrifying soil at the same concentrations within a 48-d incubation. The soil was added to containers (400 g field most soil to each container; volume, 850 mL; length × width × height, 137×104×120 mm). Nitrification inhibitors were applied to the soil at the concentration rate of 0, 12.7 and 127 mg kg^{-1} dry soil (equivalent to 0 10 and 100 mg kg^{-1} wet soil) with N fertiliser applied in the form of NH_4Cl at the concentration of 127 mg N kg^{-1} dry soil (equivalent to 100 mg kg^{-1} wet soil). Treatments were applied as follow: 1) LA 12.7, 2) 1,9-decanediol 12.7, 3) DCD 12.7 (where LA, 1,9-decanediol or DCD were applied at 12.7 mg kg^{-1} dry soil); 4) LA 127, 5) 1,9-decanediol 127, 6) DCD 127 (where LA, 1,9-decanediol or DCD were applied at 127 mg kg^{-1} dry soil); 7) control (no NIs, N fertiliser application only).

To aid uniform application and dispersion of these small doses of inhibitor compounds to the soil, the compounds were mixed with sand first, as follow. First, LA and 1,9-decanediol was dissolved in a

small amount of ethanol (50 µl ethanol g^{-1} sand), and mixed with sterilised (105 °C, 16 h) fine quartz sand (0.065 g sand g^{-1} soil) to ensure the ethanol coated it as evenly as possible. Then, the NI-labelled sand was evaporated to dryness under a stream of air, making sure the sand was mixed regularly before mixing it into the soil. In the DCD treatments, DCD was dissolved in the same amount of distilled water and mixed with sterilised fine quartz sand as described above. As for the control, no NIs were added to the soil, but same amount of sterilised fine quartz sand was mixed evenly with the soil.

During the incubation period, the soil water status was maintained at 60% water filled pore space (WFPS) to optimise conditions for nitrification (Mosier et al., 1996). At the start of the experiment, 127 mg N kg^{-1} dry soil as NH_4Cl was dissolved in distilled water (to maintain 60% WFPS), and then applied to the surface of soil. Throughout the monitoring period, containers were covered with parafilm, to allow gas exchange but to retain moisture. Every three days, pots were weighed, and deionised water was added if it was necessary to maintain its humidity. Soils were incubated in a temperature-controlled room (in the dark) at 10 °C, the mean annual air temperature for the soil collection site (Hill et al., 2015).

3.2.3. Soil sampling and analysis

There were two sets of containers: one set of containers was used for soil sampling, and another set of containers was used for GHG sampling. Soil samples were collected twice during the first two weeks after treatments application. Afterwards, soil sampling continued at a frequency of once per week. At each sampling time, fresh soil (5 g) was extracted with 25 ml of 0.5 M K_2SO_4 in an orbital shaker (200 rev min^{-1}, 1 h). The extracts were centrifuged (4000 rpm, 10 min), filtered through a Whatman No.1 filter paper, and then stored at -20 °C to await for analysis for NH_4^+-N (Mulvaney, 1996) and NO_3^--N (Miranda et al., 2001) concentrations. Inhibition of NO_3^- formation was estimated as Eq. (3.1) (Subbarao et al., 2007b).

$$\text{Inhibition of } NO_3^- \text{ formation} = \left(1 - \frac{NO_3^- \text{–N concentration in treatment}}{NO_3^- \text{–N concentration in control}}\right) \times 100\% \quad (3.1)$$

Subsamples of soil were taken at d 4, d 8, d 21, d 34 and d 48 to determine the AOA and AOB gene abundance, and stored at -80 °C prior to DNA extraction. Soil DNA was extracted from 0.25 g of defrosted soil by using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to the protocol

of manufacturer. The purity and concentration of extracted soil DNA were determined by the Nanodrop spectrophotometer ND-1000 (Labtech, UK). Polymerase chain reaction (PCR) followed by melting curve analysis to confirm PCR product specificity were carried out on real-time quantitative PCR (QPCR) using the QuantStudio™ 6 flex real-time PCR system (Thermo Fisher Scientific, UK). The 10 µL reaction mixture composed of 5 µL TB Green Premix Ex Taq (TaKaRa, Tokyo, Japan), 0.15 µL of each primer, 0.2 µL ROX Reference dye, 1 µL template DNA and 3.5 µL of sterilised deionised water. The primers sets and thermal conditions were the same as list in Wang et al. (2017). Standard curves were generated by using a tenfold serial dilution of plasmids harbouring target genes.

3.2.4. GHG sampling and analysis

In the first week, the GHG samples were collected every two days, and then at a frequency of twice per week for the following two weeks. Afterwards, gas samples were collected once per week until the end of incubation period. Air-tight lids fitted with septum were used to close the incubation containers. At 0 and 60 min after the lids were closed, gas samples from the headspace were collected using the syringes (20 ml) fitted with hypodermic needles, and transferred to pre-evacuated 20 ml headspace glass vials fitted with rubber butyl septa crimp caps. Greenhouse gas concentrations were determined by gas chromatography (GC) (Clarus 580 GC; PerkinElmer Corp., Waltham, MA), which was equipped with an electron capture detector (ECD) for N_2O detection and a flame ionization detector (FID) for CO_2 detection. Standards of N_2O and CO_2 were collected and analysed at the same time as the GHG samples. Daily gaseous fluxes were estimated as the slope of the linear regression between concentrations at the two times taking into account the temperature and the ratio between chamber volume and soil surface area (MacKenzie et al., 1998). The cumulative gaseous fluxes were calculated as Li et al. (2016).

The gas concentration in the headspace over one hour was assumed to be linear, as linearity was confirmed before the incubation (see Appendix 1). Four headspace gas samples were taken within one hour (0, 20, 40 and 60 min after the lids were closed) from 4 replicate vessels filled with same amount of soil and NH_4Cl at the same %WFPS and temperature, and analysed as described above. The N_2O

(y=0.0025x+0.33, R^2=0.995) and CO_2 (y=10.6 x+533.7, R^2=0.997) concentrations (y is ppm) in headspace were confirmed to be linear over time (x is min).

3.2.5. Statistical analysis

The repeated measurement analysis of variance (RMANOVA) was applied to determine the effect of the different NIs (LA, 1,9-decanediol or DCD) concentrations on soil NH_4^+, NO_3^- concentrations, and daily gaseous fluxes (N_2O and CO_2) throughout the monitoring period. A one-way ANOVA was used to test the effect of LA, 1,9-decanediol and DCD application on cumulative N_2O and CO_2 emissions after 48 d incubation, and AOA and AOB gene copies on each sampling day. All statistical analyses were performed in SPSS Statistics 25.0 (IBM Inc., Armonk, NY).

3.3. Results

3.3.1. Soil ammonium concentrations

The soil NH_4^+-N concentrations varied significantly with time during the monitoring period in all LA, 1,9-decanediol and DCD treatments (P_{time}<0.001, Table 3.1). The soil NH_4^+-N concentrations increased (peaked at d 8) after N application and then decreased during the incubation in the control, LA and DCD treatments (Fig. 3.1a, c). In the 1,9-decanediol treatments, the NH_4^+-N concentrations decreased after N application, but then increased by d 4, and then decreased again until the end of the experiment (Fig. 3.1b). The application of LA and 1,9-decanediol did not affect the NH_4^+-N concentrations significantly (P>0.05). During the incubation period, the NH_4^+-N concentrations decreased from 128.3 mg N kg^{-1} dry soil to 11.0 mg N kg^{-1} dry soil (ranging from 9.4 to 12.4 mg N kg^{-1} dry soil) in the control, LA and 1,9-decanediol treatments. In the DCD treatments, soil NH_4^+-N concentrations remained significantly higher in the DCD 12.7 (P<0.001) and DCD 127 (P<0.001) treatments, and increased as the concentrations of DCD increased, reaching 76.1 and 97.2 mg N kg^{-1} dry soil respectively at the end of the incubation experiment.

3.3.2. Soil nitrate concentrations

In the control, LA (Fig. 3.1d) and 1,9-decanediol (Fig. 3.1e) treatments, the NO_3^--N concentration increased gradually from d 1 to d 34, decreased during the following 7 days, and then remained constant until the end of the experiment. During the monitoring period, LA did not affect the NO_3^--N concentration significantly (Table 3.1, $P>0.05$), with the NO_3^- concentration increasing from 23.11 to 65.3 mg kg^{-1} dry soil (ranging from 63.8 to 66.8 mg kg^{-1} dry soil). The application of 1,9-decanediol ($P<0.01$) and DCD ($P<0.001$) decreased the NO_3^--N concentration significantly, compared to the control. The NO_3^--N concentration increased with the increasing concentration of 1,9-decanediol, with the average inhibition of NO_3^- formation of 13.0% and 6.7% in the 1,9-decanediol 12.7 and 1,9-decanediol 127 treatments (Fig. 3.1h), respectively. Dicyandiamide application showed much higher inhibition of NO_3^- formation, with the average rate of 46.8% and 68% in the DCD 12.7 and DCD 127 treatments, respectively (Fig. 3.1i).

Table 3.1 Repeated measurement analysis of variance on soil NH_4^+ and NO_3^- concentrations, N_2O and CO_2 emissions in the LA, LN and DCD treatments

Source	NI		Time		NI × Time	
	df	*F*	*df*	*F*	*df*	*F*
				LA		
NH_4^+	2	0.2	7	312.0***	14	4.5***
NO_3^-	2	4.7	7	462.2***	14	3.6**
N_2O emission	2	0.4	10	20.8***	20	5.7***
CO_2 emission	2	6.5*	10	22.1***	20	1.8*
				1,9-decanediol		
NH_4^+	2	0.3	7	130.7***	14	1.7
NO_3^-	2	21.7**	7	263.5***	14	4.0***
N_2O emission	2	0.9	10	6.5***	20	1.5
CO_2 emission	2	2.8	10	17.8***	20	2.6**
				DCD		
NH_4^+	2	53.5***	7	49.2***	14	1.9
NO_3^-	2	6782.4***	7	180.8***	14	78.7***
N_2O emission	2	82.3***	10	26.0***	20	5.0***
CO_2 emission	2	0.4	10	7.6***	20	1.7

*$P<0.05$, **$P<0.01$, *** $P<0.001$.

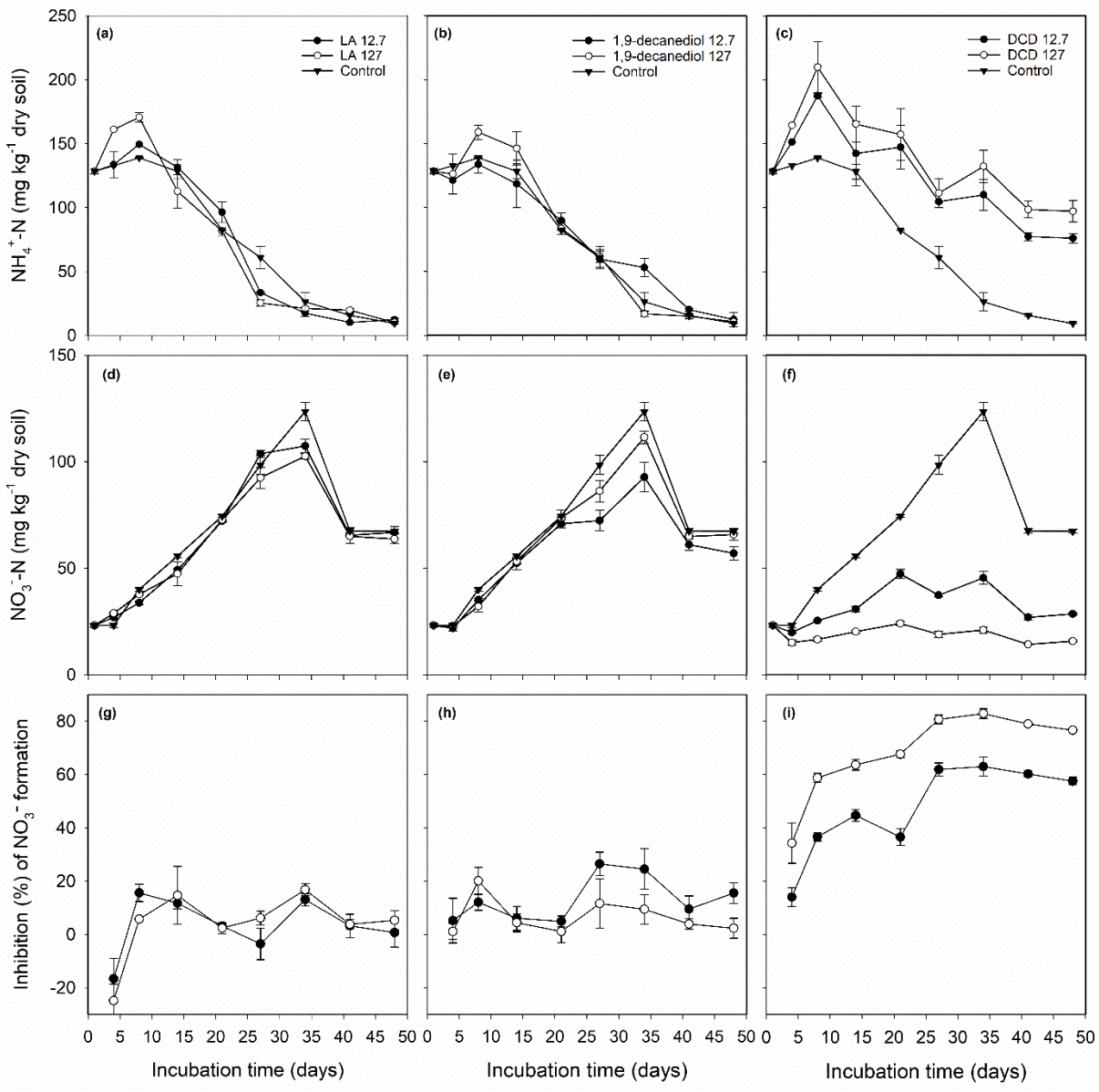

Fig. 3.1 Effect of different concentrations of LA (panels a, d, g), 1,9-decanediol (panels b, e, h) and DCD (panels c, f, i) on soil NH_4^+-N, NO_3^--N and inhibition of NO_3^- formation during a 48-d incubation at 10 °C. Error bars represent standard error of the mean (n=3).

3.3.3. Nitrous oxide emissions

Throughout the incubation period, the daily N_2O emissions varied significantly in all treatments (control, LA, 1,9-decanediol and DCD treatments) (Fig. 3.2, Table 3.1, P_{time}<0.001). In the 1,9-decanediol and DCD treatments, the daily N_2O emission increased dramatically after the N application, and decreased in the following 2 days, then remained stable until the end of the experiment. During the

monitoring period, the application of LA and 1,9-decanediol did not affect the daily N_2O emissions significantly (P>0.05). However, DCD significantly (P<0.001) decreased the daily N_2O emissions. The cumulative N_2O emissions followed the order: LA 12.7 (386.2 µg N kg^{-1} dry $soil^{-1}$) > 1,9-decanediol 12.7/127 (296.8/264.7 µg N kg^{-1} dry $soil^{-1}$) > DCD 12.7/127 (156.3/114.5 µg N kg^{-1} dry $soil^{-1}$) (Fig. 3.3a). However, there was no significant difference between the control and LA treatments, or between the control and 1,9-decanediol treatments.

3.3.4. Carbon dioxide emissions

In the LA, 1,9-decanediol and DCD treatments, the daily CO_2 emissions varied significantly with the incubation time (P_{time}<0.001, Table 3.1). The daily CO_2 emissions increased rapidly to the first peak after the NIs and N application, and reached another peak between d 8 and d 16, then remained stable at a lower emissions rate until the end of the experiment in the LA 127, 1,9-decanediol and DCD treatments (Fig. 3.4). While in the control and LA 12.7 treatments, the daily CO_2 emissions decreased dramatically after the NIs and N application. From d 8 to the end of the incubation period, the daily CO_2 emissions showed a similar pattern to the LA 127, 1,9-decanediol and DCD treatments. The cumulative CO_2 emissions in the LA 127 and 1,9-decanediol 12.7 treatments was significantly higher than that in the control and DCD treatments, reaching 301.7 mg C kg^{-1} dry soil and 299.9 mg C kg^{-1} dry soil in the LA 127 and 1,9-decanediol 12.7 treatments, respectively (Fig. 3.3b).

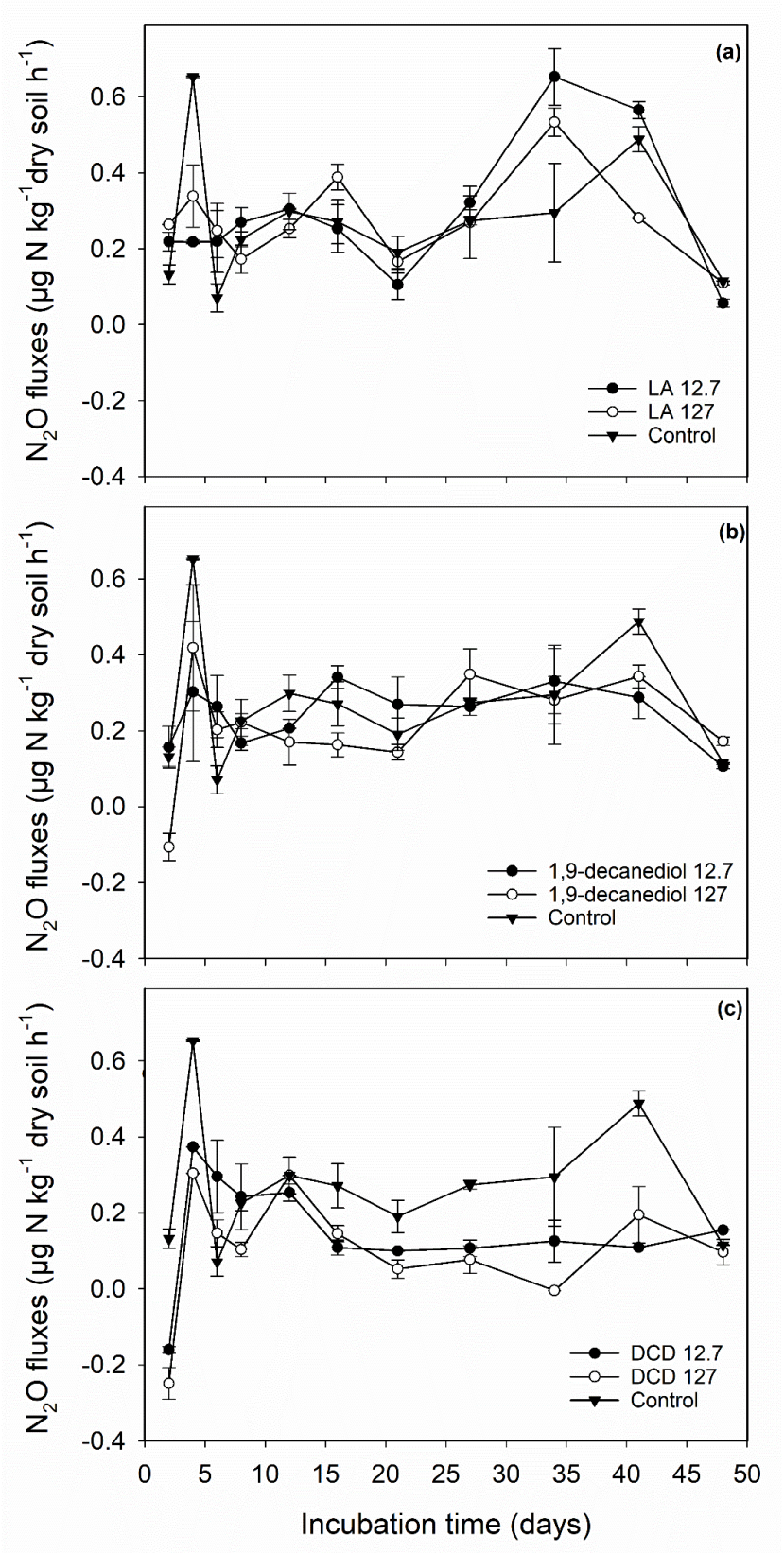

Fig. 3.2 Effect of different concentrations of LA (panel a), 1,9-decanediol (panel b) and DCD (panel c) on soil N_2O emissions during a 48-d incubation at 10 °C. Error bars represent standard error of the mean (n=3).

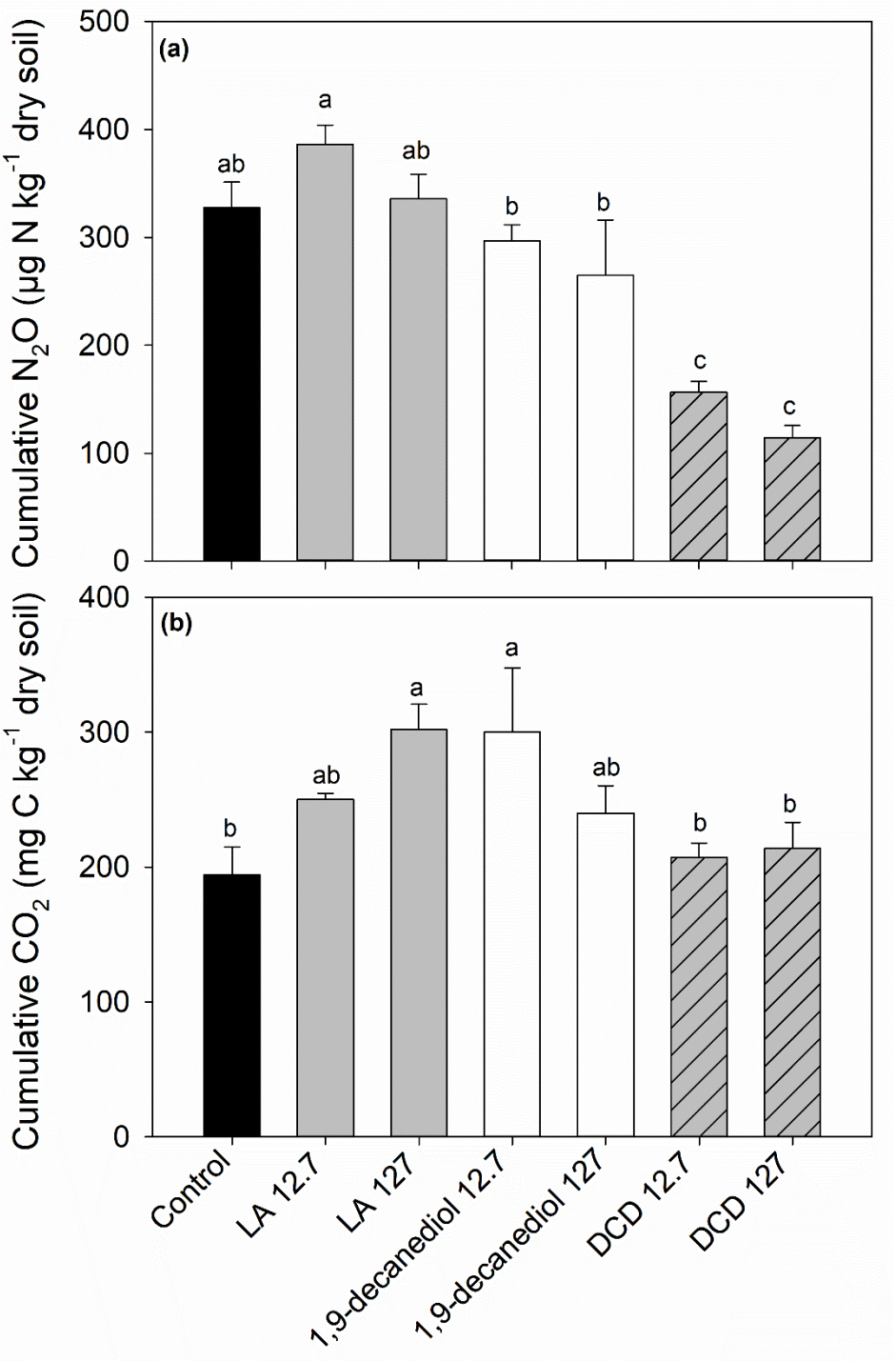

Fig. 3.3 Effect of LA, 1,9-decanediol and DCD on soil cumulative N_2O (panel a) and CO_2 (panel b) emissions during a 48-d incubation at 10 °C. Error bars represent standard error of the mean (n=3). Different letters indicate significant differences between treatments at $P<0.05$ by LSD (n=3).

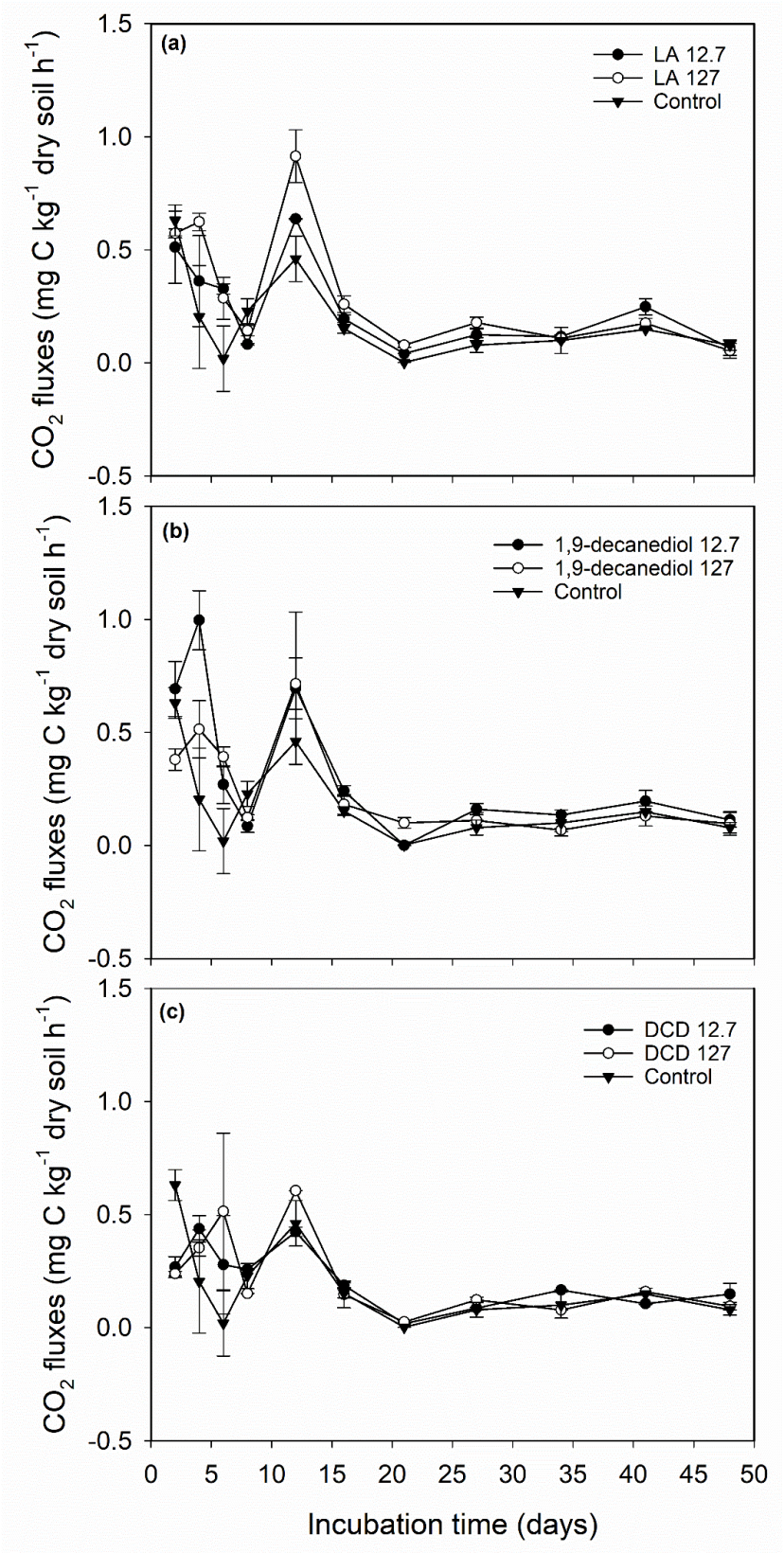

Fig. 3.4 Effect of different concentrations of LA (panel a), 1,9-decanediol (panel b) and DCD (panel c) on soil CO_2 emissions during a 48-d incubation at 10 °C. Error bars represent standard error of the mean (n = 3).

3.3.5. AOA and AOB gene abundance

During the incubation, there were no significant differences in gene abundance between the control and the NI (LA, 1,9-decanediol and DCD) treatments, except for d 21, when the application of LA, DCD and higher concentration of 1,9-decanediol (127 mg kg^{-1} dry soil) increased the AOA gene abundance (Fig. 3.5a). Unlike the DCD treatment, the AOB gene copies increased gradually in the LA, 1,9-decanediol treatments during the 48-d incubation (Fig. 3.5b). No significant differences were observed in the control, LA 12.7 and 1,9-decanediol 12.7 treatments in the AOB gene copies during the incubation. The application of LA (d 4, 34, P<0.05-0.01) and 1,9-decanediol (d 21, 34, P<0.05-0.01) at the higher concentration (127 mg kg^{-1} dry soil) increased the AOB population growth significantly. After the 48-d incubation, AOB population growth was significantly reduced in the DCD treatments compared with the control, and all the LA and 1,9-decanediol treatments , being 2.29×10^6 copies g^{-1} dry soil in the DCD 12.7 (51.3%, P<0.001) and 2.77×10^6 copies g^{-1} dry soil DCD 127 (48.0%, P<0.001) treatments. The AOB gene copy numbers were greater than that of AOA during the monitoring period.

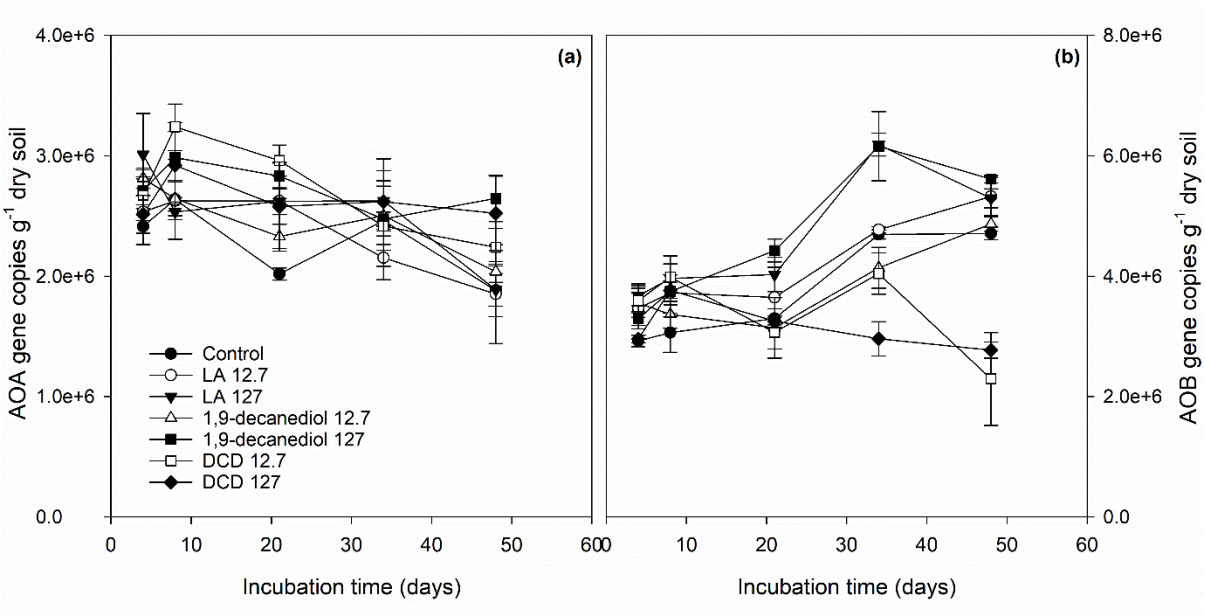

Fig. 3.5 Effect of LA, 1,9-decanediol and DCD on gene copies of AOA (panel a), AOB (panel b) during a 48-d incubation at 10 °C. Error bars represent standard error of the mean (n=3).

3.4. Discussion

Dicyandiamide was shown to be much more effective than LA and 1,9-decanediol at inhibiting the transformation of NH_4^+ to NO_3^- and suppressing the N_2O emissions in this study. The application of DCD retained high soil NH_4^+ and resulted in low NO_3^- concentrations (Fig. 3.1c, f), and significantly inhibited the N_2O emissions (Fig. 3.3). The mitigating effect of DCD on soil nitrification and N_2O emissions was consistent with previous studies (Gilsanz et al., 2016; McGeough et al., 2016; Robinson et al., 2014). Dicyandiamide may be an N source when applied to the soil (66.7% N in DCD), however, the relatively lower mineralisation (5.5% and 2.7% mineralisation rate when DCD applied at 12.7 and 127 mg kg^{-1} dry soil, see chapter 4, Fig. 4.4) indicated that only 0.5 and 2.3 mg N kg^{-1} dry soil may result from the biodegradation of DCD in the DCD 12.7 and DCD 127 treatments, respectively. This provided the evidence that the main N source was the NH_4^+ fertiliser application and not the DCD mineralisation, and that the retained higher soil NH_4^+ concentration was due to inhibition of nitrification. Other studies indicate that the effectiveness of DCD is strongly related to the temperature, and that half-lives of DCD across many soils decrease with increasing temperature (Di and Cameron, 2004; Kelliher et al., 2008; McGeough et al., 2016). The relatively lower incubation temperature (10 °C) may explain the stability of DCD in this study.

The majority of N_2O is produced by microbial nitrification and denitrification as part of soil N cycle (Wrage et al., 2001). The relatively high NH_4^+ and lower NO_3^- concentration of the soil, combined with inhibited AOB *amoA* gene abundance indicates that the lower N_2O emissions in the DCD treatments were likely due to the inhibited nitrification process. Dicyandiamide has been shown to inhibit the AOB by binding to the active sites of the copper-containing AMO metalloenzyme required by AOB (Amberger, 1989), but recently also to inhibit the AOA in acidic soils (Lehtovirta-Morley et al., 2013; Zhang et al., 2012). In this study, DCD significantly decreased the AOB gene abundance, but there was no effect on the AOA gene abundance at the end of the incubation, which was consistent with studies that have shown DCD inhibits nitrification through influencing AOB rather than AOA (Chen et al., 2014; O'Callaghan et al., 2010).

Nevertheless, both LA and 1,9-decanediol had no effect on the soil NH_4^+ concentration, and only 1,9-decanediol decreased soil NO_3^- concentration in this study (Fig. 3.1, Table 3.1). In addition, neither the LA or 1,9-decanediol application affected the daily and cumulative N_2O emissions compared with the control (Fig. 3.2, 3, Table 3.1). The effectiveness of NIs depends on the several key factors, including concentration, ease of microbial degradation and soil properties (Lu et al., 2019; McGeough et al., 2016). Previous studies have indicated that LA applied at ≥200 mg kg^{-1} dry soil retained added N in the NH_4^+ form, and that LA applied at 50 and 100 mg kg^{-1} dry soil had no effect on the NH_4^+ concentration and decreased the NO_3^- concentration slightly compared with the control which is consistent with our results (Subbarao et al., 2008). High application rates of 1.9-decanediol (≥500 mg kg^{-1} dry soil) have resulted in strong inhibition of nitrification, although efficacy varied significantly between soil types (Lu et al., 2019). The concentration of LA and 1,9-decanediol applied in this study (≤127 mg kg^{-1} dry soil) was much lower than these effective doses reported.

In addition, the application of LA and 1,9-decanediol at a rate of ≤127 mg kg^{-1} dry soil did not affect the AOA and AOB gene abundance, which may explain the lack of nitrification inhibition. We hypothesised that the rapid mineralisation rate of LA (46.9-53.2% within 38 d) and 1,9-decanediol compared with DCD (2.7-5.5% within 38 d) (see Chapter 4, Fig. 4.4) may be an explanation for the reduced efficacy of LA and 1,9-decanediol. Although ^{14}C-labelled 1,9-decanediol was not possible, and hence we were not able to determine the mineralisation rate of 1,9-decanediol, the higher CO_2 emissions in the 1,9-decanediol 12.7 treatments compared with the control and DCD treatments provided evidence of the higher mineralisation rate of 1,9-decanediol, due to the positive linear relationship between the ^{14}C-CO_2 and CO_2 (see Chapter 4, Fig. 4.4).

3.5. Conclusions

In this highly nitrifying soil, DCD was effective at inhibiting nitrification (soil retained high NH_4^+ concentration and low NO_3^- concentration) and reduced cumulative N_2O emission by inhibiting AOB gene abundance rather than AOA. However, neither LA or 1,9-decanediol applied at ≤127 mg kg^{-1} dry soil was effective in regulating nitrification, and may potentially increase CO_2 emissions.

3.6. Acknowledgments

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3.7. References

Amberger, A., 1989. Research on dicyandiamide as a nitrification inhibitor and future outlook. Communications in Soil Science and Plant Analysis 20, 1933–1955. doi:10.1080/00103628909368195

Chen, Q., Qi, L., Bi, Q., Dai, P., Sun, D., Sun, C., Liu, W., Lu, L., Ni, W., Lin, X., 2014. Comparative effects of 3,4-dimethylpyrazole phosphate (DMPP) and dicyandiamide (DCD) on ammonia-oxidizing bacteria and archaea in a vegetable soil. Applied Microbiology and Biotechnology 99, 477–487. doi:10.1007/s00253-014-6026-7

Di, H.J., Cameron, K.C., 2004. Effects of temperature and application rate of a nitrification inhibitor, dicyandiamide (DCD), on nitrification rate and microbial biomass in a grazed pasture soil. Australian Journal of Soil Research 42, 927. doi:10.1071/SR04050

Firestone, M.K., Davidson, E.A., 1989. Microbiologial Basis of NO and N2O production and consumption in soil. Exchange of Trace Gases between Terrestrial Ecosystems and the Atmosphere 7–21. doi:10.1017/CBO9781107415324.004

Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the Nitrogen Cycle. Science 320, 889–892. doi:10.1126/science.1136674

Gardiner, C.A., Clough, T.J., Cameron, K.C., Di, H.J., Edwards, G.R., de Klein, C.A.M., 2016. Potential for forage diet manipulation in New Zealand pasture ecosystems to mitigate ruminant urine derived N2O emissions: a review. New Zealand Journal of Agricultural Research. doi:10.1080/00288233.2016.1190386

Gilsanz, C., Báez, D., Misselbrook, T.H., Dhanoa, M.S., Cárdenas, L.M., 2016. Development of emission factors and efficiency of two nitrification inhibitors, DCD and DMPP. Agriculture, Ecosystems and Environment 216, 1–8. doi:10.1016/j.agee.2015.09.030

Gopalakrishnan, S., Subbarao, G. V., Nakahara, K., Yoshihashi, T., Ito, O., Maeda, I., Ono, H., Yoshida, M., 2007. Nitrification inhibitors from the root tissues of Brachiaria humidicola, a tropical grass. Journal of Agricultural and Food Chemistry 55, 1385–1388. doi:10.1021/jf062593o

Guardia, G., Marsden, K.A., Vallejo, A., Jones, D.L., Chadwick, D.R., 2018. Determining the influence of environmental and edaphic factors on the fate of the nitrification inhibitors DCD and DMPP in soil. Science of the Total Environment 624, 1202–1212. doi:10.1016/j.scitotenv.2017.12.250

Hill, P.W., Garnett, M.H., Farrar, J., Iqbal, Z., Khalid, M., Soleman, N., Jones, D.L., 2015. Living roots magnify the response of soil organic carbon decomposition to temperature in temperate grassland. Global Change Biology 21, 1368–1375. doi:10.1111/gcb.12784

Jones, D.L., Shannon, D., Murphy, D. V, Farrar, J., 2004. Role of dissolved organic nitrogen (DON) in soil N cycling in grassland soils. Soil Biology and Biochemistry 36, 749–756. doi:10.1016/j.soilbio.2004.01.003

Jumadi, O., Hala, Y., Iriany, R.N., Makkulawu, A.T., Baba, J., 2019. Combined effects of nitrification inhibitor and zeolite on greenhouse gas fluxes and corn growth.

Kelliher, F.M., Clough, T.J., Clark, H., Rys, G., Sedcole, J.R., 2008. The temperature dependence of dicyandiamide (DCD) degradation in soils: A data synthesis. Soil Biology and Biochemistry 40, 1878–1882. doi:10.1016/j.soilbio.2008.03.013

Lam, S.K., Suter, H., Mosier, A.R., Chen, D., 2017. Using nitrification inhibitors to mitigate agricultural N2O emission: a double-edged sword? Global Change Biology 23, 485–489. doi:10.1111/gcb.13338

Lassaletta, L., Billen, G., Grizzetti, B., Anglade, J., Garnier, J., 2014. 50 Year Trends in Nitrogen Use Efficiency of World Cropping Systems: the Relationship Between Yield and Nitrogen Input To Cropland. Environmental Research Letters 9, 105011. doi:10.1088/1748-9326/9/10/105011

Lehtovirta-Morley, L.E., Verhamme, D.T., Nicol, G.W., Prosser, J.I., 2013. Effect of nitrification inhibitors on the growth and activity of Nitrosotalea devanaterra in culture and soil. Soil Biology and Biochemistry 62, 129–133. doi:10.1016/j.soilbio.2013.01.020

Li, X., Sørensen, P., Olesen, J.E., Petersen, S.O., 2016. Evidence for denitrification as main source of N 2 O emission from residue-amended soil. Soil Biology and Biochemistry 92, 153–160. doi:10.1016/j.soilbio.2015.10.008

Lu, Y., Zhang, X., Jiang, J., Kronzucker, H.J., Shen, W., Shi, W., 2019. Effects of the biological nitrification inhibitor 1,9-decanediol on nitrification and ammonia oxidizers in three agricultural soils. Soil Biology and Biochemistry 129, 48–59. doi:10.1016/j.soilbio.2018.11.008

Lucas, G.N., 2013. Dicyandiamide contamination of milk powders. Sri Lanka Journalof Child Health 42, 63–64. doi:10.4038/sljch.v42i2.5624

Luo, J., Ledgard, S., Wise, B., Welten, B., Lindsey, S., Judge, A., Sprosen, M., 2015. Effect of dicyandiamide (DCD) delivery method, application rate, and season on pasture urine patch nitrous oxide emissions. Biology and Fertility of Soils 51, 453–464. doi:10.1007/s00374-015-0993-4

MacKenzie, A.F., Fan, M.X., Cadrin, F., 1998. Nitrous oxide emission in three years as affected by tillage, corn- soybean-alfalfa rotations, and nitrogen fertilization. Journal of Environmental Quality 27, 698–703. doi:10.2134/jeq1998.00472425002700030029x

Marsden, K.A., Marín-Martínez, A.J., Vallejo, A., Hill, P.W., Jones, D.L., Chadwick, D.R., 2016. The mobility of nitrification inhibitors under simulated ruminant urine deposition and rainfall: a comparison between DCD and DMPP. Biology and Fertility of Soils 52, 491–503. doi:10.1007/s00374-016-1092-x

McGeough, K.L., Watson, C.J., Müller, C., Laughlin, R.J., Chadwick, D.R., 2016. Evidence that the efficacy of the nitrification inhibitor dicyandiamide (DCD) is affected by soil properties in UK soils. Soil Biology and Biochemistry 94, 222–232. doi:10.1016/j.soilbio.2015.11.017

Menéndez, S., Barrena, I., Setien, I., González-Murua, C., Estavillo, J.M., 2012. Efficiency of nitrification inhibitor DMPP to reduce nitrous oxide emissions under different temperature and moisture conditions. Soil Biology and Biochemistry 53, 82–89. doi:10.1016/j.soilbio.2012.04.026

Minet, E.P., Ledgard, S.F., Grant, J., Murphy, J.B., Krol, D.J., Lanigan, G.J., Luo, J., Richards, K.G., 2018. Feeding dicyandiamide (DCD) to cattle: An effective method to reduce N2O emissions from urine patches in a heavy-textured soil under temperate climatic conditions. Science of the Total Environment 615, 1319–1331. doi:10.1016/j.scitotenv.2017.09.313

Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A Rapid, Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite. Nitric Oxide 5, 62–71. doi:10.1006/NIOX.2000.0319

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

38. doi:10.1016/j.agee.2013.04.019

Mosier, A.R., Duxbury, J.M., Freney, J.R., Heinemeyer, O., Minami, K., 1996. Nitrous Oxide Emissions from Agricultural Fields: Assessment, Measurement and Mitigation, in: Progress in Nitrogen Cycling Studies. Springer Netherlands, Dordrecht, pp. 589–602. doi:10.1007/978-94-011-5450-5_97

Mulvaney, R.L., 1996. Nitrogen—Inorganic Forms, in: Methods of Soil Analysis Part 3—Chemical Methods. Soil Science Society of America, American Society of Agronomy, pp. 1123–1184. doi:10.2136/sssabookser5.3.c38

O'Callaghan, M., Gerard, E.M., Carter, P.E., Lardner, R., Sarathchandra, U., Burch, G., Ghani, A., Bell, N., 2010. Effect of the nitrification inhibitor dicyandiamide (DCD) on microbial communities in a pasture soil amended with bovine urine. Soil Biology and Biochemistry 42, 1425–1436. doi:10.1016/j.soilbio.2010.05.003

Robinson, A., Di, H.J., Cameron, K.C., Podolyan, A., He, J., 2014. The effect of soil pH and dicyandiamide (DCD) on N2O emissions and ammonia oxidiser abundance in a stimulated grazed pasture soil. Journal of Soils and Sediments 14, 1434–1444. doi:10.1007/s11368-014-0888-2

Schlesinger, W.H., 2009. On the fate of anthropogenic nitrogen. Proceedings of the National Academy of Sciences 106, 203–208. doi:10.1073/pnas.0810193105

Sharma, S.N., Prasad, R., 1995. Use of nitrification inhibitors (neem and DCD) to increase N efficiency in maize-wheat cropping system. Fertilizer Research 44, 169–175. doi:10.1007/BF00750923

Subbarao, G. V., Ishikawa, T., Ito, O., Nakahara, K., Wang, H.Y., Berry, W.L., 2006. A bioluminescence assay to detect nitrification inhibitors released from plant roots: A case study with Brachiaria humidicola. Plant and Soil. doi:10.1007/s11104-006-9094-3

Subbarao, G. V., Nakahara, K., Hurtado, M.P., Ono, H., Moreta, D.E., Salcedo, A.F., Yoshihashi, A.T., Ishikawa, T., Ishitani, M., Ohnishi-Kameyama, M., Yoshida, M., Rondon, M., Rao, I.M., Lascano, C.E., Berry, W.L., Ito, O., 2009. Evidence for biological nitrification inhibition in Brachiaria pastures. Proceedings of the National Academy of Sciences 106, 17302–17307. doi:10.1073/pnas.0903694106

Subbarao, G. V., Rondon, M., Ito, O., Ishikawa, T., Rao, I.M., Nakahara, K., Lascano, C., Berry, W.L., 2007a. Biological nitrification inhibition (BNI) - Is it a widespread phenomenon? Plant and Soil. doi:10.1007/s11104-006-9159-3

Subbarao, G. V., Tomohiro, B., Masahiro, K., Osamu, I., Samejima, H., Wang, H.Y., Pearse, S.J., Gopalakrishnan, S., Nakahara, K., Zakir Hossain, A.K.M., Tsujimoto, H., Berry, W.L., 2007b. Can biological nitrification inhibition (BNI) genes from perennial Leymus racemosus (Triticeae) combat nitrification in wheat farming? Plant and Soil. doi:10.1007/s11104-007-9360-z

Subbarao, G. V, Nakahara, K., Ishikawa, T., 2008. Free fatty acids from the pasture grass Brachiaria humidicola and one of their methyl esters as inhibitors of nitrification 89–99. doi:10.1007/s11104-008-9682-5

Sun, L., Lu, Y., Yu, F., Kronzucker, H.J., Shi, W., 2016. Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency. New Phytologist 212, 646–656. doi:10.1111/nph.14057

Wang, Q., Hu, H.W., Shen, J.P., Du, S., Zhang, L.M., He, J.Z., Han, L.L., 2017. Effects of the nitrification inhibitor dicyandiamide (DCD) on N2Oemissions and the abundance of nitrifiers and denitrifiers in two contrasting agricultural soils. Journal of Soils and Sediments 17, 1635–1643. doi:10.1007/s11368-016-1633-9

Welten, B.G., Ledgard, S.F., Luo, J., 2014. Administration of dicyandiamide to dairy cows via drinking water reduces nitrogen losses from grazed pastures, in: Journal of Agricultural Science. Cambridge University Press, pp. S150–S158. doi:10.1017/S0021859613000634

Wrage, N., Velthof, G.., van Beusichem, M.., Oenema, O., 2001. Role of nitrifier denitrification in the

production of nitrous oxide. Soil Biology and Biochemistry 33, 1723–1732. doi:10.1016/S0038-0717(01)00096-7

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

Zerulla, W., Barth, T., Dressel, J., Erhardt, K., Horchler von Locquenghien, K., Pasda, G., R??dle, M., Wissemeier, A., 2001. 3,4-Dimethylpyrazole phosphate (DMPP) - A new nitrification inhibitor for agriculture and horticulture. An introduction. Biology and Fertility of Soils 34, 79–84. doi:10.1007/s003740100380

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

Chapter 4: Relative efficacy and stability of biological and synthetic nitrification inhibitors in a highly nitrifying soil: evidence of indirect nitrification inhibition by linoleic acid and linolenic acid

Yan Ma[1,*], Davey L. Jones[1,2], Jinyang Wang[1], Laura M. Cardenas[3], David R. Chadwick[1,4]

[1] *School of Natural Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW, UK*

[2] *SoilsWest, UWA School of Agriculture and Environment, The University of Western Australia, Perth, WA 6009, Australia*

[3] *Sustainable Agriculture Sciences Department, North Wyke, Okehampton, Devon, EX20 2SB, UK*

[4] *Interdisciplinary Research Centre for Agriculture Green Development in Yangtze River Basin, Southwest University, Chongqing, China*

[*]**Correspondence:** Yan Ma. Email: mtfy@cau.edu.cn.

Running Title: Apparent biological nitrification inhibition

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Abstract

Biological nitrification inhibition (BNI) is a plant-mediated rhizosphere process where natural nitrification inhibitors (NIs) can be produced and released by roots to suppress nitrifier activity in soil. Although nitrification is one of the critical soil processes in the nitrogen (N) cycle, unrestricted and rapid nitrification in agricultural systems can result in major losses of N from the plant-soil system (i.e. by nitrate (NO_3^-) leaching and gaseous N emissions). In this study, we explored the potential efficacy of biological (linoleic acid, LA and linolenic acid, LN) versus a proven efficient synthetic NI (dicyandiamide, DCD) on N dynamics, N_2O and CO_2 emissions in a highly nitrifying soil. ^{14}C-labelled LA, LN and DCD mineralisation was determined in a parallel experiment to explore the fate of inhibitors after application. We found that LA and LN had no effect on soil NH_4^+ concentrations, but significantly decreased NO_3^- concentrations. Soil that received DCD had lower NO_3^- and higher NH_4^+ concentrations than the control (soil without NIs). Linoleic acid and LN increased the cumulative N_2O and CO_2 emissions when they were applied at high concentrations (635 or 1270 mg kg^{-1} dry soil). Linoleic acid and LN had a much greater mineralisation rates than that of DCD, 47-56%, 37-61% and 2.7-5.5%, respectively after 38-d incubation. We conclude that in contrast to the direct inhibition of nitrification caused by DCD, that addition of high concentrations of LA and LN cause apparent nitrification inhibition by promoting microbial immobilisation of soil NH_4^+ and/or NO_3^-. In contrast to DCD, high concentrations of LA and LN are sources of highly bioavailable carbon (C) in the soil and they may stimulate N_2O loss via denitrification. Future studies on NIs need to clearly differentiate between the direct and potential indirect effects which result from addition of these compounds to soil.

Highlights

- We explored the efficacy and stability of nitrification inhibitors in a highly nitrifying soil.
- This study supports efforts to mitigate N losses and improve nitrogen use efficiency of inputs.
- Addition of LA, LN and DCD can decrease NO_3^- concentration, but their mode of action is different.
- The apparent 'inhibitory' effect of LA and LN on soil NO_3^- concentration could be indirect.

Keywords: nitrification inhibitor; mineralisation; immobilisation; nitrous oxide; carbon dioxide; ^{14}C labelling

4.1. Introduction

In the past decades, the global supply of nitrogen (N) fertilisers has increased dramatically, and is estimated to reach 171 million tonnes in 2020 (FAO, 2017). Chemical fertilisers represent the main global input of N to agriculture soils (61% of the total), with additional N supplied via livestock manures (16%), symbiotic and associative N fixation (18%), and atmospheric N deposition (5%) (Lassaletta et al., 2014). Although the use of synthetic N fertilisers is central to maintaining food security, their use is also strongly associated with many of the world's most serious environmental problems (e.g. marine eutrophication, global warming, ozone depletion and air pollution) (Erisman et al., 2013). These issues are directly associated with the inefficient use of fertiliser N and large losses of N from agricultural systems either in gaseous (e.g. ammonia (NH_3), nitrous oxide (N_2O) and dinitrogen (N_2)) or aqueous forms (dissolved organic N, nitrate (NO_3^-)) (Gardiner et al., 2016). The global average N use efficiency (NUE) (the percentage of applied fertiliser N recovered from the crop) is very low (*ca.* 47%) with little improvement seen in the last 30 years (Lassaletta et al., 2014). There is therefore an urgent need to devise practical and cost-effective solutions to promote greater capture of fertiliser N by crop plants and to minimise N loss pathways (e.g. leaching, surface run-off, denitrification and volatilisation). Of the numerous proposed strategies, one that is particularly promising is the targeting of chemicals to control critical N transformations in the soil N cycle where large N losses are known to occur (e.g. urea $\rightarrow NH_4^+$ and $NH_4^+ \rightarrow NO_3^-$).

Nitrification is a key soil process, responsible for the conversion of ammonia (NH_4^+) to NO_3^- (Firestone and Davidson, 1989). It is a two-step microbially mediated process carried out by chemo-autotrophic nitrifying bacteria, first oxidising NH_4^+ to nitrite (NO_2^-) and then oxidising NO_2^- to NO_3^- (Firestone and Davidson, 1989). Two groups of soil microorganisms, ammonia oxidising bacteria (AOB) (mainly *Nitrosomonas* spp. and *Nitrosospira* spp.) and ammonia oxidising archaea (AOA), are largely responsible for the biological oxidation of NH_4^+ to NO_3^- (Beeckman et al., 2018; Leininger et al., 2006;

Taylor et al., 2010). Nitrification, nitrifier-denitrification and denitrification are primarily biological mediated processes in soil which are responsible for N_2O generation (Gardiner et al., 2016; Hofstra and Bouwman, 2005; Smith et al., 1997; Tubiello et al., 2013). However, denitrification cannot take place without the substrate NO_3^-. Thus, controlling nitrification represents a good potential way to simultaneously improve NUE, reduce greenhouse gas emissions (GHG) and attenuate NO_3^- leaching.

Synthetic nitrification inhibitors (NIs), such as dicyandiamide (DCD), 3,4-dimethylpyrazol-phosphate (DMPP) and 2-chloro-6-(trichloromethyl)-pyridine (Nitrapyrin) have been developed for use in agriculture to help slow nitrification and reduce soil N losses (Li et al., 2008; Menéndez et al., 2012; Weiske et al., 2001; WU et al., 2007). The synthetic NIs specifically suppress the ammonia monooxygenases (AMO) pathway within nitrification (Subbarao et al., 2008). In addition to improving NUE (Monaghan et al., 2013; WU et al., 2007), the application of NIs may also improve the economic and environmental footprint of food production, and in some cases improve agronomic yield (Li et al., 2018b). In the case of DCD, the application of low doses to N-sources applied or deposited to grassland soils (10 to 50 mg kg^{-1} soil) has been shown to reduce N_2O emissions by 26-82% and CO_2 emissions by 7% (Chadwick et al., 2018; Di and Cameron, 2016; Weiske et al., 2001). Despite their proven benefits, however, synthetic NIs suffer from a number of challenges that may limit their adoption. These include: i) lack of chemical stability and variable responses in different soil types and moisture/temperature regimes (Marsden et al., 2016b; McGeough et al., 2016; Menéndez et al., 2012), ii) lack of cost-effective and practical delivery strategies to spatially-targeted NI application in the field (e.g. urine patches) (Ledgard et al., 2008; Luo et al., 2015; Minet et al., 2018, 2016; Welten et al., 2014), and iii) recent evidence that synthetic NIs (e.g. DCD) can contaminate grazed grass (Kim et al., 2012) and be taken up by plants (Marsden et al., 2015), finding their way into the human food chain (Lucas, 2013), resulting in negative public perception.

Biological nitrification inhibition (BNI) is a plant-mediated rhizosphere process where NIs are produced and released from roots that can suppress nitrifier activity in soil (Subbarao et al., 2006a). Harnessing this potential to promote greater NUE is highly desirable and has several benefits over synthetic NIs including: low cost, delivery through the entire root zone, continuous production, greater public acceptability and lower carbon (C) footprint. Most biological NIs released by plants inhibit

nitrification by suppressing both AMO and hydroxylamine oxidoreductase (HAO) enzymatic pathways in *Nitrosomonas* (Table 2.1). *Brachiaria humidicola* is a common tropical pasture grass that contains substantial amounts of biological NIs within its root and shoot tissues (Gopalakrishnan et al., 2007; Subbarao et al., 2009, 2008). Of these biological NIs, brachialactone has been found to contribute 60-90% of the inhibitory activity released from the root (Subbarao et al., 2009). In addition, two other biological NIs (i.e. linoleic acid, LA and linolenic acid, LN) have been identified from the shoot tissue of *Brachiaria humidicola* (Subbarao et al., 2008). When applied to soil as pure compounds, LA and LN have been shown to promote NH_4^+ retention and reduce NO_3^- levels (Subbarao et al., 2008). Most researches have focussed on the effects of biological NIs on soil receiving NH_4^+-based fertiliser (Subbarao et al., 2007a, 2013, 2008; Sun et al., 2016) or urine (Byrnes et al., 2017). However, little is known about the effects of BNIs on 'residual' soil NH_4^+-N, especially that produced in strongly nitrifying soils.

The aims of our study were therefore to: 1) compare the relative effect of LA and LN with DCD on 'residual' NH_4^+ and NO_3^- concentrations in a highly nitrifying soil; 2) evaluate the effect of LA, LN and DCD on N_2O and carbon dioxide (CO_2) emissions from soil; and 3) explore the stability (mineralisation rate) of LA, LN and DCD in soil. In addition, we use our results to explore if reported nitrification inhibition by biological NIs could actually be the result of an indirect effect due to microbial immobilisation of N, stimulated by the addition of available C in LA and LN.

4.2. Materials and methods

4.2.1. Soil properties

A sandy clay loam textured Eutric Cambisol collected from a sheep-grazed fertilised grassland in North Wales was used for this study (53°24'N, 4°02'W) (Table 4.1). This soil was chosen as it is known to possess very high nitrification rates (Jones et al., 2004). The soil had not been previously exposed to LA, LN or DCD, and had not been grazed for > 3 months prior to collection. Four independent replicate soil samples (0-10 cm depth) were collected, and sieved to pass 2 mm, then stored at 4 °C in loosely

sealed bags for 5 days before the incubation experiment was prepared. Each replicate soil sample collected was used as an experimental replicate (n=4).

Soil moisture content was determined after oven drying (105 °C, 24 h), and soil organic matter content determined by loss-on-ignition in a muffle furnace (450 °C, 16 h) (Ball, 1964). Soil pH and electrical conductivity (EC) were measured on fresh soil using standard electrodes (1:2.5 (w/v) soil-to-distilled water). Total soil C and N concentrations were determined on oven-dried soil using a CHN2000 analyser (Leco Corp., St. Joseph, MI). Extractable NH_4^+ and NO_3^- concentrations were measured colorimetrically on 1:5 (w/v) fresh soil-to-1 M KCl extracts, using the methods of Mulvaney (1996) and Miranda et al. (2001), respectively.

Table 4.1 Properties of soils (0-10 cm) used in the incubation experiments.

Soil property	Eutric Cambisol
Moisture content (%)	25.14 ± 0.06
Organic matter (%)	5.26 ± 0.29
pH	5.47 ± 0.01
Electrical conductivity (µS cm^{-1})	103.4 ± 0.49
Total carbon (g kg^{-1} dry soil)	22.13 ± 1.19
Total nitrogen (g kg^{-1} dry soil)	2.33 ± 0.13
NH_4^+-N (mg kg^{-1} dry soil)	4.17 ± 0.05
NO_3^--N (mg kg^{-1} dry soil)	21.29 ± 1.20

Values represent means ± standard error (n=4).

4.2.2. Effect of LA, LN and DCD on soil nitrification

To characterize the effect of LA, LN and DCD on soil nitrification, a soil incubation experiment was conducted. Pure compounds of LA, LN and DCD (Sigma-Aldrich, Gillingham, UK) were added to 450 g of the sandy loam soil in containers (volume: 850 ml; Length × Width × Height: 137 × 104 × 120 mm) at a range of concentrations. LA and LN were applied at 12.7, 127, 635 and 1270 mg kg^{-1} dry soil (equivalent to 10, 100, 500 and 1000 mg kg^{-1} wet soil), In this study we included biological NI treatments at higher application rates than used in Chapter 3. These higher rates of inclusion were similar to some of those used in previous studies that have shown inhibitory effects of specific biological NIs on nitrification rates, measured as a reduction in soil NO_3^- formation compared to the control treatment (Subbarao et al., 2008). Dicyandiamide was added at the concentration of 0 (control), 12.7,

63.5 and 127 mg kg^{-1} dry soil (equivalent to 0, 10, 50 and 100 mg kg^{-1} wet soil) (Subbarao et al., 2006b), 127 mg kg^{-1} dry soil DCD was added to compare its effects with LA and LN under the same concentration. To ensure uniform mixing of the small quantities of NIs in the soil, the NIs were first mixed with sterile fine-grained quartz sand. Firstly, LA and LN were dissolved in a small amount of ethanol, which was then mixed with fine quartz sand (50 µl ethanol g^{-1} sand) and evaporated to dryness under a stream of air. The NI-labelled sand was then mixed into the soil (0.065 g sand g^{-1} wet soil). For the DCD treatments, DCD was dissolved in distilled water and mixed with the same quartz sand and add to soil as described above.

The experiment consisted of two sets of containers. One set of containers was used for regular soil sampling, and another set of containers was used for GHG sampling. Containers (850 ml) containing the NI-labelled soil (450 g soil $container^{-1}$) were covered with Parafilm® (Bemis Inc, Neenah, WI) to allow gas exchange but to retain moisture. Every three days, the containers were weighed and deionised water was added if it was necessary to maintain soil moisture. The containers were incubated in the dark in a temperature-controlled room at 10 °C, the mean annual air temperature in NW Wales (Hill et al., 2015). The soil water status during the experiment was maintained at 60% water filled pore space (WFPS) to optimise conditions for nitrification (Mosier et al., 1996). The incubation experiment lasted 38 d. During that time, soil samples and GHG samples were collected every two or three days during the first two weeks after NI application. Afterwards, sampling continued at a frequency of once or twice per week. Soils in the containers were not disturbed when soil samples were collected.

At each sampling time, soil (5 g) was extracted with 25 ml of 1 M KCl in an orbital shaker at 200 rev min^{-1} (1 h, 20 °C), the extracts were centrifuged (10 min, 3800 *g*), filtered through a Whatman No.1 filter paper, and stored at -20 °C to await analysis for NH_4^+ and NO_3^- as described above. For GHG sampling, air-tight lids fitted with septum were attached to the incubation vessels, and syringes (20 ml) fitted with hypodermic needles were used to collect two gas samples from the headspace (0 and 60 min after the lids were closed). The increase in gas concentration in the headspace was assumed to be linear over 1 h, based on headspace gas analysis of replicated vessels filled with the same quantity of soil at same %WFPS and temperature (see Appendix 2 for details; N_2O, R^2=0.936; CO_2, R^2=0.993). Gas samples were transferred to pre-evacuated 20 ml headspace glass vials fitted with rubber butyl septa

crimp caps. Gas samples were analysed by gas chromatography (GC) (Clarus 580 GC; PerkinElmer Corp., Waltham, MA) equipped with a capillary column and an electron capture detector (ECD) for N_2O detection and a flame ionization detector (FID) for CO_2. Standards of N_2O and CO_2 were placed in vials, stored and analysed at the same time as the samples.

4.2.3. Mineralisation of ^{14}C-labelled LA, LN and DCD within soil

In a parallel experiment, a ^{14}C-labelling approach (Marsden et al., 2016b) was used in the incubation experiment to assess the stability of LA, LN and DCD in soil, i.e. their mineralisation rate. ^{14}C-labelled LA, LN and DCD (American Radiolabelled Chemical Inc., St Louis, MO) were added to 5 g of soil (collected in section 4.2.1) contained in sealed polypropylene tubes (50 ml) using the same method described above (section 4.2.2), and at the same range of concentrations (LA and LN applied at 12.7, 127, 635 and 1270 mg kg^{-1} dry soil; DCD at 12.7, 63.5 and 127 mg kg^{-1} dry soil). Soils were incubated at 10 °C in the dark for 38 d.

At the beginning of the incubation, the ^{14}C activity of substrates solution (^{14}C-labelled LA, LN and DCD) added to the soil was determined by liquid scintillation counting after mixing with HiSafe 3 scintillant (4 ml) (PerkinElmer Corp.). After adding of the ^{14}C-labelled NI to the soil, a vial containing 1 M NaOH (1 ml) was placed above the soil surface to absorb any $^{14}CO_2$ evolved (capture efficiency>95%; Boddy et al., 2007) and the tubes sealed. The $^{14}CO_2$ traps were changed two or three times in the first two weeks after which they were changed weekly. The ^{14}C activity of the NaOH solution was then determined by liquid scintillation counting after mixing with 4 ml HiSafe 3 scintillant. After 38 d, the soil (5 g) was extracted by shaking with either 25 ml ethanol or distilled water (1 h, 200 rev min^{-1}), the extracts were centrifuged (10 min, 3850 *g*) and the ^{14}C of the supernatant was determined by liquid scintillation counting as described above.

4.2.4. Data calculations

The effect of LA, LN and DCD on soil nitrification was characterised after the 38-d incubation study. Treatment effect on soil NO_3^- concentration was estimated as Eq. (4.1) (Subbarao et al., 2007b).

$$\text{Treatment effect on } NO_3^- \text{ concentration} = \left(1 - \frac{NO_3^- \text{–N concentration in treatment}}{NO_3^- \text{–N concentration in control}}\right) \times 100\% \quad (4.1)$$

Fluxes of N_2O and CO_2 were estimated from the slope of the linear regression between headspace concentrations at the two time points as Eq. (4.2) and (4.3) (MacKenzie et al., 1998).

$$F_{N-N_2O} = \frac{28}{22.4} \times \frac{dc}{dt} \times \frac{V \times 60}{W} \quad (4.2)$$

$$F_{C-CO_2} = \frac{12}{22.4} \times \frac{dc}{dt} \times \frac{V \times 60}{W} \quad (4.3)$$

Where F_{N-N_2O} is the flux of N-N_2O in μg kg^{-1} dry soil h^{-1}, F_{C-CO_2} is the flux of C-CO_2 in μg kg^{-1} dry soil h^{-1}, 28 is the molar mass of N in N_2O, 12 is the molar mass of C in CO_2, 22.4 is the molar volume of an idea gas at standard temperature and pressure, $\frac{dc}{dt}$ is the initial rate of change in concentration with time in ppb min^{-1}, V is the volume of the headspace in m^3, W is the dry weight of soil added in the container in kg, 60 converts minutes to hours.

Cumulative N_2O and CO_2 emissions, were calculated from estimated mean daily fluxes as Eq. (4.4) (Li et al., 2016).

$$F_{k+1} = \frac{1}{2}\sum_1^k [\Delta_i \times (f_i + f_{i+1})] \quad (4.4)$$

Where F_{k+1} is the cumulative flux from the d 1 to d (k + 1) in μg N kg^{-1} dry soil or μg C kg^{-1} dry soil, Δ_i is the time interval between the d i and d (i +1) in h, f_i is the mean flux on the d i in in μg kg^{-1} dry soil h^{-1}.

Mineralisation rate of ^{14}C-labelled LA, LN and DCD was determined as Eq. (4.5) (Marsden et al., 2015).

$$\text{Mineralisation rate (\%)} = \frac{^{14}\text{C activity of NaOH solution}}{^{14}\text{C activity of substrate}} \times 100\% \quad (4.5)$$

To explore if reported BNI may be indirect, e.g. as a result of immobilisation of N resulting from the addition of highly available C in the biological NIs, we calculated potential soil microbial N immobilisation indirectly. We used the % C mineralised (from the $^{14}CO_2$ measurements) of the NIs (Fig. 4.4) to estimate the total C available to the soil microbial biomass, using the individual C contents (i.e. based on their molecular structures: LA: $C_{18}H_{32}O_2$, LN: $C_{18}H_{30}O_2$, DCD: $C_2H_4N_4$). The microbial N demand needed to assimilate the C-rich substrates was calculated, in mg N kg^{-1} dry soil (predicted value), using the standard C:N ratio of the soil microbial biomass of 8:1 (G. Chen et al., 2003). Whilst

we recognise there may be some variation in the C:N of the microbial biomass, we based the choice of this ratio (value) on the average from Xu et al. (2013), a global analysis of >3000 data points from the World's major biomass. The observed amount of N immobilisation was calculated indirectly from the extractable soil mineral N measurements as Eq. (4.6) shows, in mg N kg^{-1} dry soil (observed value). These calculations were made on all concentrations for the LA and LN treatments at d 6, d 11, d 14 and d 35.

$$\text{N immobilised} = (NH_4^+ - N + NO_3^- - N \text{ in control}) - (NH_4^+ - N + NO_3^- - N \text{ in treatment}) \quad (4.6)$$

4.2.5. Statistical analysis

A repeated measurement analysis of variance (RMANOVA) was used to test concentrations of NI (LA, LN or DCD) on NH_4^+, NO_3^-, CO_2 flux and treatment effect on soil NO_3^- concentration during the incubation period. A one-way ANOVA was applied to determine the effect of LA, LN or DCD concentrations on cumulative N_2O, CO_2 and mineralisation rate. In addition, a linear regression analysis was undertaken to relate the predicted microbial N immobilisation (predicted value, section 4.2.4) and observed N immobilisation (observed value, section 4.2.4) as a result of added available C in the LA and LN treatments. All statistical analyses were performed in SPSS Statistics 25.0 (IBM Inc., Armonk, NY).

4.3. Results

4.3.1. Soil ammonium concentrations

During the monitoring period, NH_4^+ concentration varied significantly (P_{time}<0.001, Table 4.2) with incubation time and showed a similar trend in the LA, LN and DCD treatments (Fig. 4.1a, b, c). The soil NH_4^+ concentration increased during the first 8 days, then decreased over the following 27 days, with a small additional increase at d 27 in the LA, LN and DCD treatments. During the incubation period, there were no significant effects of LA (P=0.804) or LN (P=0.431) on soil NH_4^+ concentration. The NH_4^+ concentrations in the DCD 10, DCD 50 and DCD 100 treatments remained significantly higher than that in the control (without NI), reaching 4.7 mg N kg^{-1} dry soil, 12.4 mg N kg^{-1} dry soil,

and 15.8 mg N kg^{-1} dry soil after incubation (in the control, 0.8 mg N kg^{-1} dry soil). Throughout the monitoring period, DCD significantly affected soil NH_4^+ concentrations (P<0.001), with soil NH_4^+ concentrations increased as the concentration of DCD increased at almost all sampling days (with the exception of d 6 and d 11).

4.3.2. Soil nitrate concentrations

Soil NO_3^- concentrations increased slowly during the experimental period, and varied significantly (P_{time}<0.001, Table 4.2) with the incubation time in the LA, LN and DCD treatments (Fig. 4.1d, e, f). Compared with the control, the addition of LA (P<0.001), LN (P<0.001) and DCD (P<0.01) significantly decreased soil NO_3^- concentrations. There was almost no effect of the LA 10 treatment on soil NO_3^- concentration (averaging a reduction of 0.6%, Fig. 4.1g). During the monitoring period, the LA 100, LA 500 and LA 1000 treatments resulted in average reductions in soil NO_3^- concentrations of 16.5%, 63.2% and 93.5%, respectively. The concentration of LN required to reduce soil NO_3^- concentration was substantially higher than that for LA (Fig. 4.1h), with the LN 100, LN 500 and LN 1000 treatments resulting in average reductions in soil NO_3^- concentrations of 11.5%, 36.8% and 50.8%. For DCD, the effect on soil NO_3^- concentration significantly increased as DCD concentration increased (P<0.05-0.01, Fig. 4.1i), with soil NO_3^- concentration reductions of 15.0%, 31.1% and 39.6% for the DCD 10, DCD 50 and DCD 100 treatments, respectively.

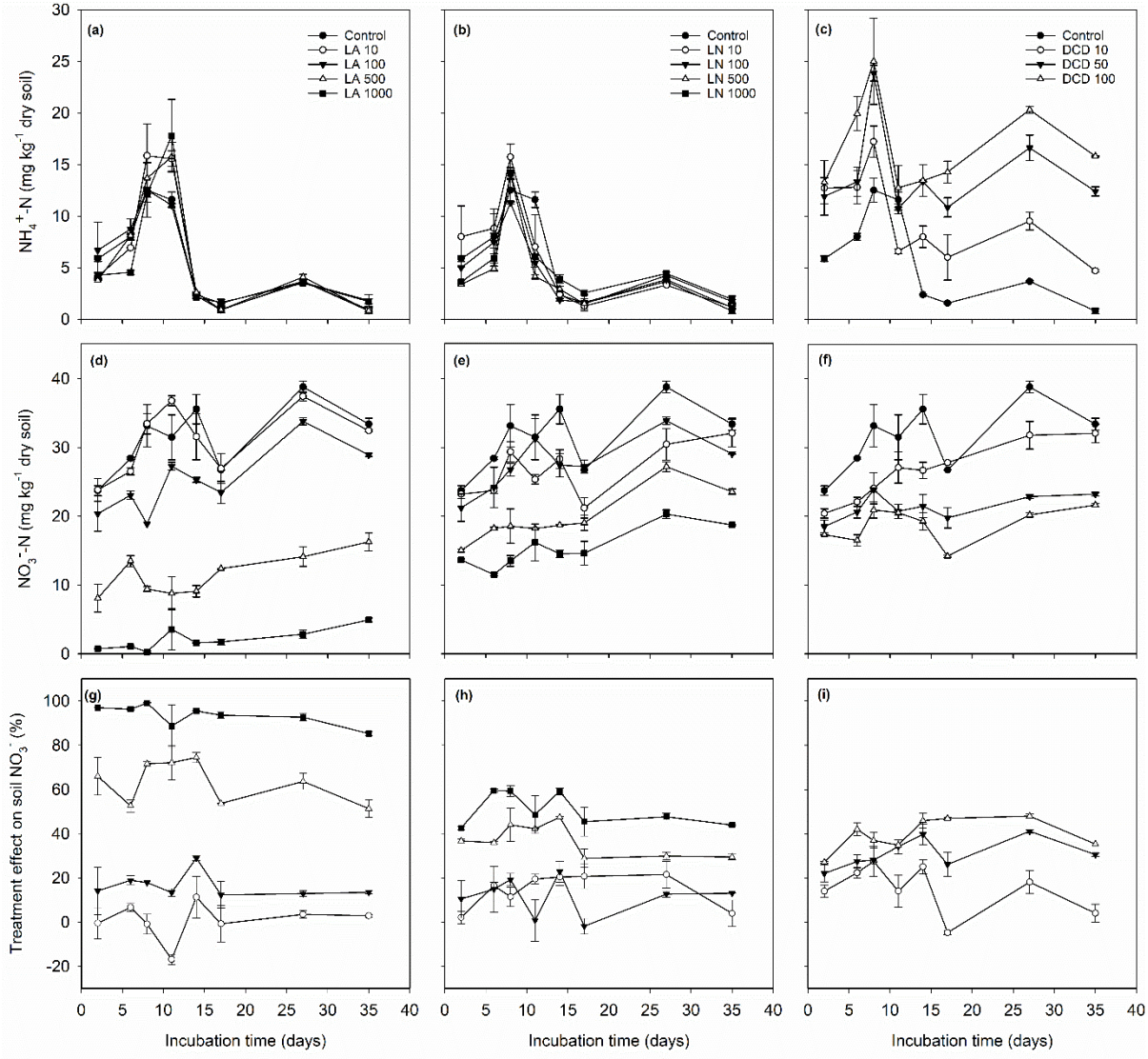

Fig. 4.1 Effect of different concentrations of linoleic acid (LA, panels a, d, g), linolenic acid (LN, panels b, e, h) and DCD (panels c, f, i) on soil NH_4^+, NO_3^- concentrations and treatment effect on soil NO_3^- concentration during a 38-d incubation at 10 °C. Error bars represent standard error of the mean (n=4).

4.3.3. N_2O emissions

Generally, cumulative N_2O emissions in the LA and LN treatments increased as the concentrations increased (Fig. 4.2). In the LA 500 and LA 1000 treatments, the cumulative N_2O emissions were significantly higher than that in the control, LA 10 and LA 100 treatments (P<0.01-0.001), and no significant differences (P>0.05) were observed between the control, LA 10 and LA 100 treatments.

Similar effects were also observed in the LN treatments. After the 38-d incubation, the cumulative N_2O emissions in the LA 500 treatment and LA 1000 treatment were 201 μg N kg^{-1} dry soil and 271 μg N kg^{-1} dry soil, respectively, whilst the cumulative N_2O emissions in the LN 500 and LN 1000 treatments were 138 μg N kg^{-1} dry soil and 156 μg N kg^{-1} dry soil. During the monitoring period, there was no significant effect (P>0.05) of the concentration of DCD on soil cumulative N_2O emission (Fig. 4.2). After 38-d incubation, the cumulative N_2O emissions were 58.1 μg N kg^{-1} dry soil, 87.9 μg N kg^{-1} dry soil, 95.0 μg N kg^{-1} dry soil and 64.7 μg N kg^{-1} dry soil in the control, DCD 10, DCD 50 and DCD 100 treatments, respectively.

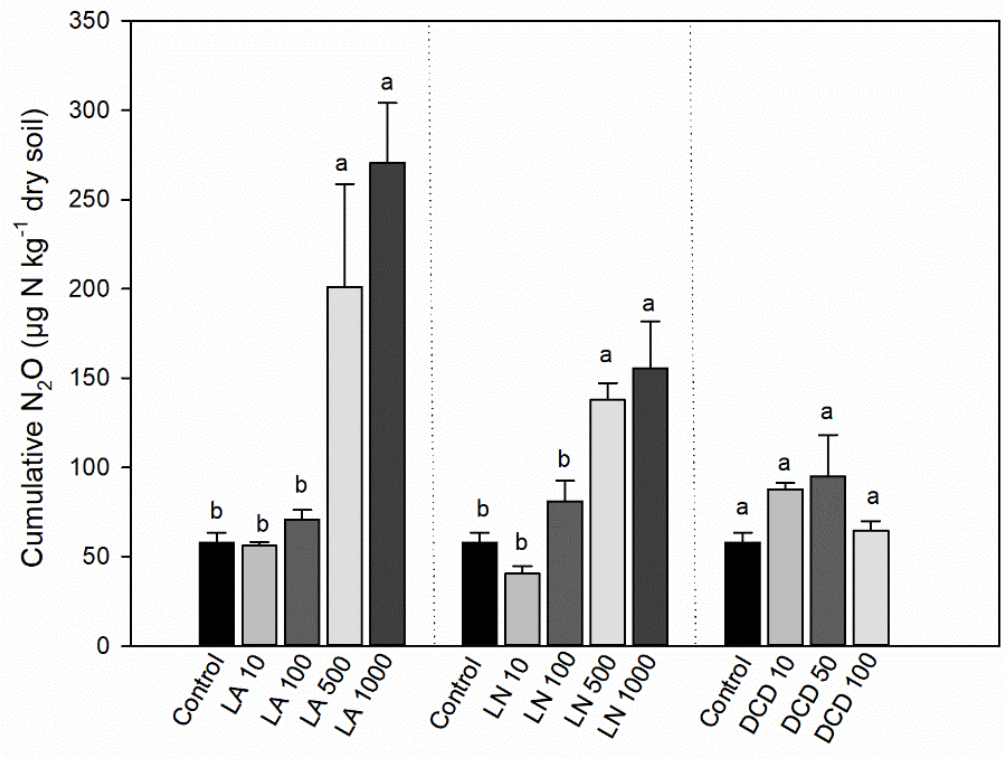

Fig. 4.2 Effect of different concentrations of linoleic acid (LA), linolenic acid (LN) and DCD on cumulative N_2O emissions during a 38-d incubation at 10 °C. Error bars represent standard error of the mean (n=4). Different letters indicate significant differences between treatments at P<0.05 by LSD test.

4.3.4. CO_2 emissions

As shown in Fig. 4.3a, b and c, the daily CO_2 emissions varied significantly (P_{time}<0.001, Table 4.2) with incubation time. In the LA, LN and DCD treatments, daily CO_2 emissions increased rapidly

from d 1 to d 4, and then decreased gradually. At d 4, the peak CO_2 emissions in the LA 500 and LA 1000 treatments were 1.1 mg C kg^{-1} dry soil h^{-1} and 1.6 mg C kg^{-1} dry soil h^{-1}, and were 1.4 mg C kg^{-1} dry soil h^{-1} and 2.1 mg C kg^{-1} dry soil h^{-1} in the LN 500 and LN 1000 treatments, respectively. But in the control, the CO_2 emissions declined rapidly from d 1 to d 6, and then decreased gradually during the remainder of the 38-d incubation period. During the incubation period, daily CO_2 emissions were significantly affected by the application of LA, LN and DCD (P<0.01-0.001).

In the LA 10 treatment, the cumulative CO_2 emissions was significantly (P<0.01) lower, with a reduction rate of 27.7% compared to the control. No significant (P>0.05) effects of LN addition at lower concentrations (Control, LN 10 and LN 100) on cumulative CO_2 emissions were observed. LA and LN applied at 635 and 1270 mg kg^{-1} dry soil significantly (P<0.001) increased the cumulative CO_2 emissions, with an increase of 86.5% and 176% in the LA treatments, and 68.5% and 189% in the LN treatments, respectively. There were no significant differences between the control and DCD 10 treatment (P=0.185), and between the control and DCD 100 treatment (P=0.283). In the DCD 50 treatment, the cumulative CO_2 emission was significantly lower (P<0.01) with a reduction of 26.8%.

Table 4.2 Repeated measurement analysis of variance on soil NH_4^+ and NO_3^- concentrations, treatment effect on soil NO_3^- concentration and CO_2 fluxes in the LA, LN and DCD treatments.

Source	NI		Time		NI × Time	
	df	*F*	*df*	*F*	*df*	*F*
	LA					
NH_4^+	4	0.4	7	113.9***	28	1.8*
NO_3^-	4	423.1***	7	25.5***	28	4.3***
Treatment effect on NO_3^-	3	2772.1***	7	3.8**	21	1.7
CO_2 flux	4	166.3***	8	50.8***	32	10.5***
	LN					
NH_4^+	4	1.1	7	115.1***	28	3.2**
NO_3^-	4	52.0***	7	36.6***	28	2.6**
Treatment effect on NO_3^-	3	67.1**	7	6.7***	21	2.2*
CO_2 flux	4	148.4***	8	62.2***	32	11.9***
	DCD					
NH_4^+	3	87.3***	7	33.7***	21	4.2***
NO_3^-	3	49.0**	7	26.5***	21	4.4***
Treatment effect on NO_3^-	2	82.0**	7	9.1***	14	4.7**
CO_2 flux	3	9.2**	8	23.6***	24	4.5***

$^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001.

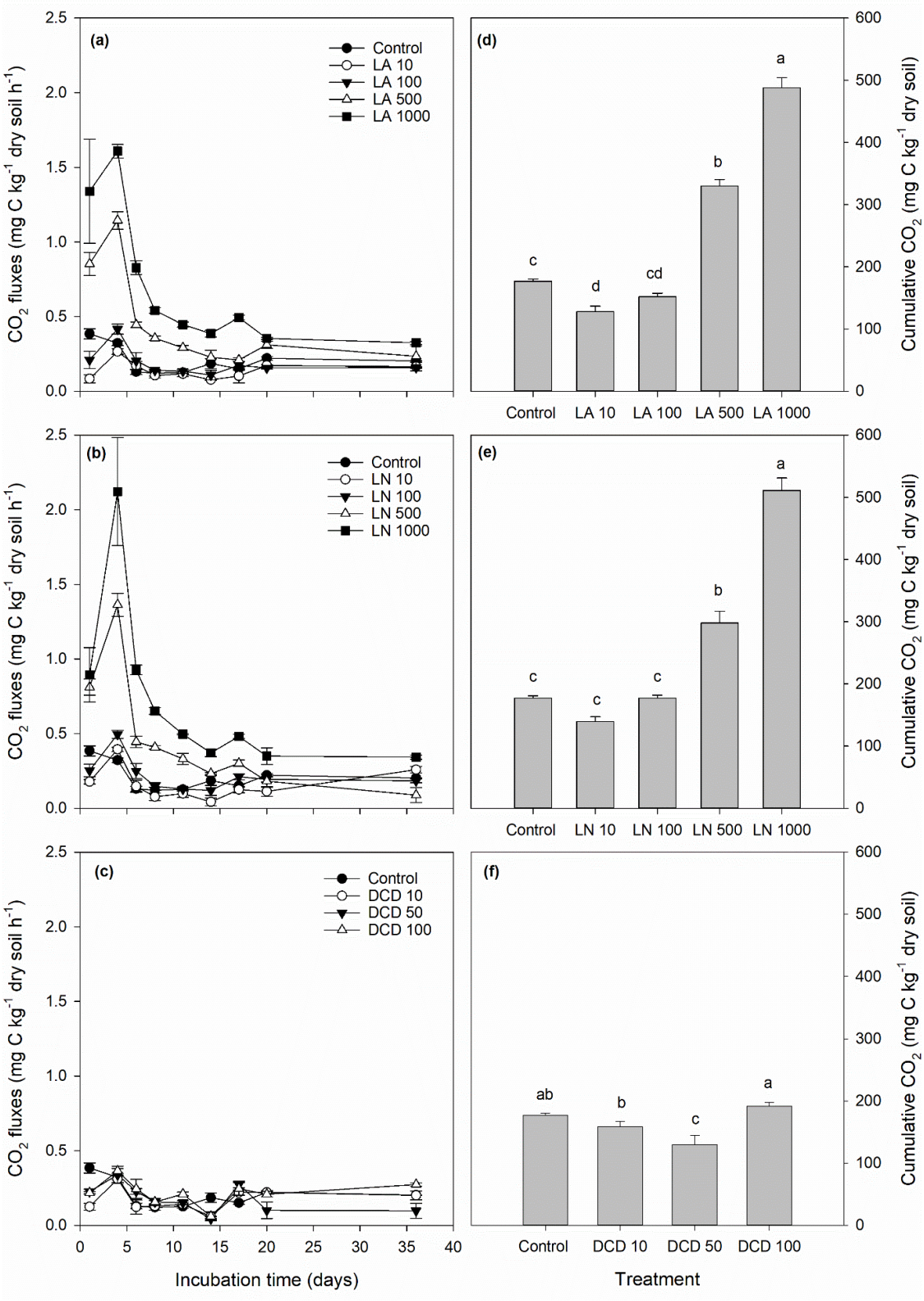

Fig. 4.3 Effect of different concentrations of linoleic acid (LA, panels a, d), linolenic acid (LN, panels b, e) and DCD (panels c, f) on CO_2 fluxes and cumulative CO_2 emissions during a 38-d incubation at 10 °C. Error bars represent standard error of the mean (n=4). Different letters indicate significant differences between treatments at $P<0.05$ by LSD test.

4.3.5. Microbial mineralisation of ^{14}C-labelled LA, LN and DCD

During the incubation period, the overall patterns of LA (Fig. 4.4a) and LN (Fig. 4.4b) mineralisation were similar. The mineralisation of LA and LN were initially rapid (d 1 to d 6) and became progressively slower over the 38-d incubation period. After the 38-d incubation period, the total mineralisation rate averaged 52.6% ranging from 46.9% to 55.7% in the LA treatments, and averaged 50.7% ranging from 36.6% to 60.7% in the LN treatments. In comparison with LA and LN, the mineralisation rate of DCD was much lower (Fig. 4.4c), with a total mineralisation rate of 5.5%, 2.9% and 2.7% in the DCD 10, DCD 50 and DCD 100 treatments after the 38-d incubation.

At the end of the 38-d incubation, the amount of ^{14}C-labelled LA, LN and DCD remaining in the soil were quantified by extracting in water or ethanol (Table 4.3). In the water-based extraction, only 2.1-2.6% of ^{14}C-labelled LA, and 2.7-2.8% of the ^{14}C-labelled LN remained, compared with 20.6-25.3% of the ^{14}C-labelled DCD. In the LA and LN treatments, the quantities detected from the ethanol extraction were greater than that of water extractions, *viz.* 3.9-5.2% ^{14}C-labelled LA, and 4.2-5.5% ^{14}C-labelled LN, with only 3.3-6.8% of the ^{14}C-labelled DCD being detected in the ethanol extractions. In the LA, LN and DCD treatments, 37.2-45.4%, 30.9-55.9% and 64.5-73.2% of the ^{14}C-labelled substrates were not recovered in the water and ethanol extraction, indicating immobilisation of the remaining ^{14}C by the soil biomass. As there is no satisfactory technique (e.g. chloroform-fumigation extraction) for assessing the quantity of isotope contained in the microbial biomass (Glanville et al., 2016), this could not be verified.

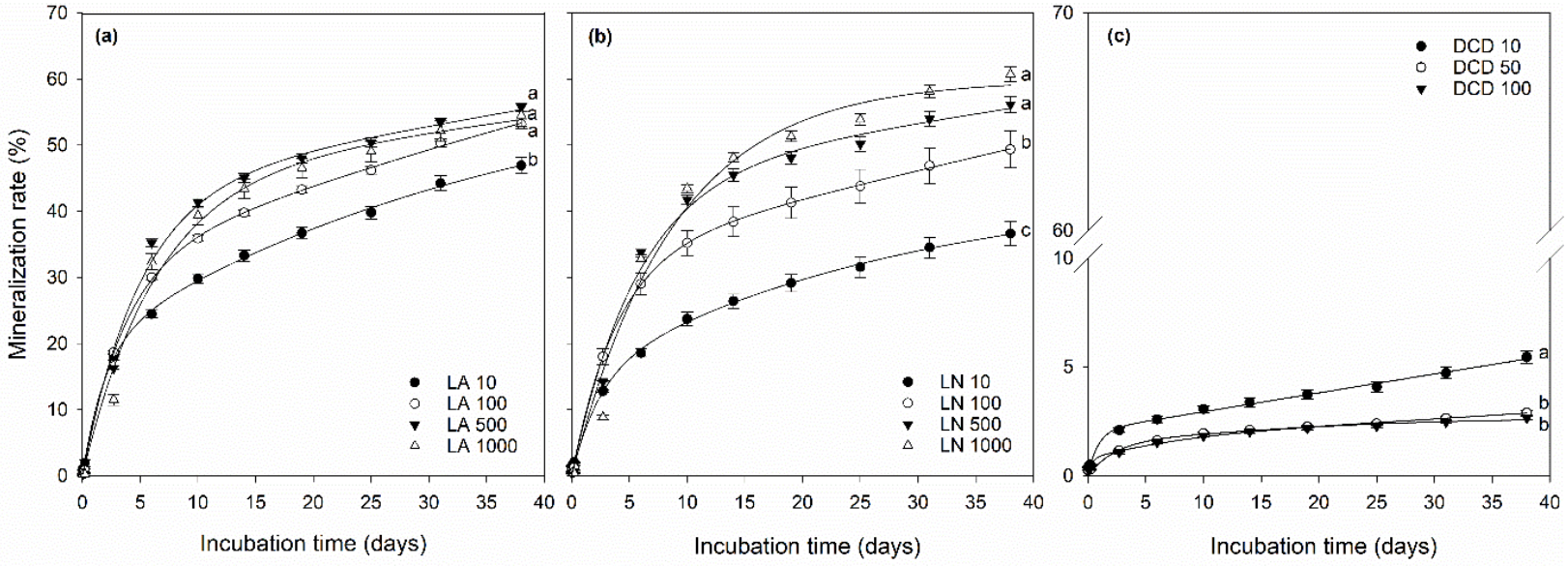

Fig. 4.4 Effect of nitrification inhibitors concentrations on mineralization rate of ^{14}C-labelled linoleic acid (LA, panel a), linolenic acid (LN, panel b) and DCD (panel c) in a sandy clay loam soil during a 38-d incubation at 10 °C. Error bars represent standard error of the mean (n=4). Different letters indicate significant differences between treatments at $P<0.05$ by LSD test.

Table 4.3 ^{14}C-labelled LA, LN and DCD extracted from soil at the end of the 38-d incubation period. Different letters indicate significant differences between treatments for each extractant at $P<0.05$ by LSD. Values represent means ± standard error (n=4).

NI	^{14}C-compound in water (%)	^{14}C-compound in ethanol (%)
LA		
LA 10	2.6±0.4 c	5.1±0.8 ab
LA 100	2.1±0.3 c	4.4±1.2 bc
LA 500	2.6±0.7 c	3.9±1.0 bc
LA 1000	3.1±0.2 c	5.2±0.6 ab
LN		
LN 10	2.8±0.2 c	4.7±0.5 abc
LN 100	2.8±0.3 c	5.5±0.4 ab
LN 500	2.7±0.1 c	4.2±0.5 bc
LN 1000	3.2±0.4 c	5.2±0.3 ab
DCD		
DCD 10	23.2±2.9 ab	6.8±0.4 a
DCD 50	20.6±2.5 b	3.3±0.6 bc
DCD 100	25.2±2.4 a	5.0±0.2 abc

4.3.6. Apparent CO_2 emissions changes in the total amount of $^{14}CO_2$

During the monitoring period, cumulative CO_2 emissions above that of the control treatment (cumulative CO_2 emissions in the LA/LN treatments minus that in the control, y in mg C kg^{-1} dry soil) were significantly related with the amount of $^{14}CO_2$ (x in mg C kg^{-1} dry soil) ($P<0.001$), as measured using the ^{14}C-labelled LA and LN. The relationship for LA was $y=0.62x-27.85$ ($R^2=0.982$) and for LN was $y=0.58x-14.44$ ($R^2=0.982$). The apparent linear relationship suggests that the additional CO_2 emissions in the LA/LN 500 and LA/LN 1000 treatments were mainly associated with the mineralisation of added LA and LN.

4.3.7. Soil microbial N immobilisation

There was a strong linear relationship between the predicted value (potential soil microbial N immobilisation as a result of the added available C in the LA and LN) and observed value (the observed amount of N immobilization) for the LA (Fig. 4.5a, $P<0.001$) and LN treatments (Fig. 4.5b, $P<0.001$). This linear relationship between predicted and observed immobilisation value indicates that at high

concentrations of addition, LA and LN result in microbial N immobilisation of NH_4^+ and/or NO_3^-. This effect was not observed for DCD addition in this study.

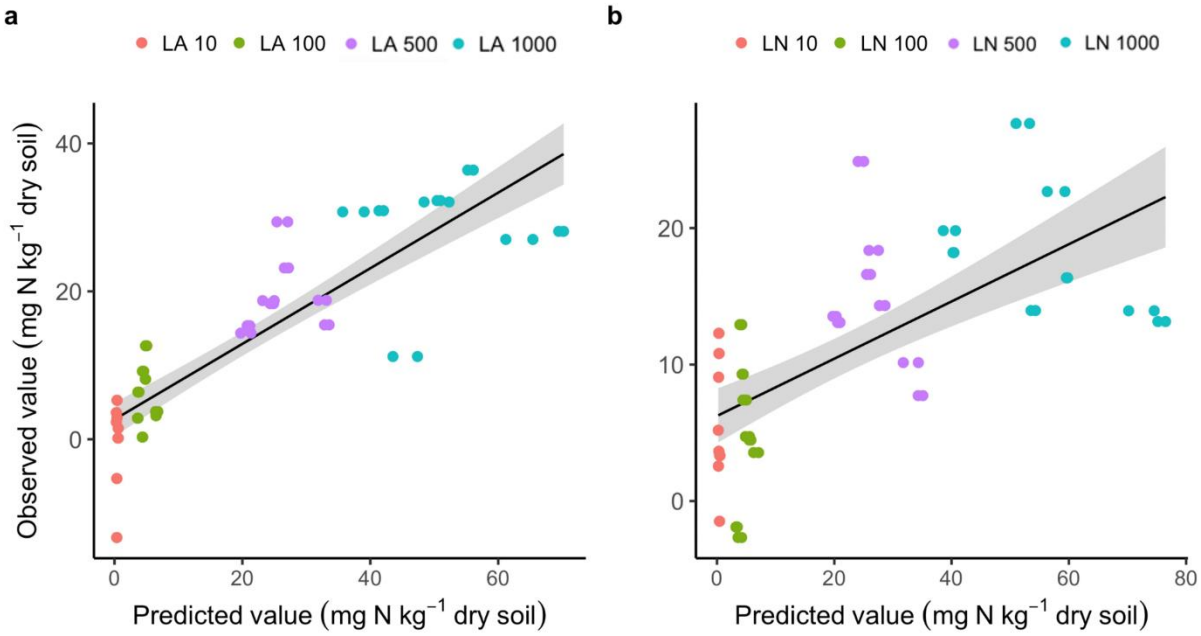

Fig. 4.5 Relationship between predicted and observed N immobilization in the linoleic acid (LA, panel a) and linolenic acid (LN, panel b) treatments. LA: y=0.51x+2.67, R^2=0.74; LN: y=0.21x+6.24, R^2=0.42.

4.4. Discussion

Nitrification inhibitors are capable of delaying the oxidisation of NH_4^+ to NO_3^- effectively to mitigate the negative impact of NO_3^- on the environment (Guo et al., 2013; Subbarao et al., 2008). Previous studies, where an additional source of NH_4^+ has been applied, have indicated that LA and LN show direct nitrification inhibition due to blocking the AMO and HAO enzymatic pathways which play a critical role in the oxidation of NH_4^+ to NO_2^- in *Nitrosomonas* (Subbarao et al., 2008). In this study, with no added NH_4^+ source, and where soil NH_4^+ and NO_3^- concentrations were <6 mg kg^{-1} and <24 mg kg^{-1}, respectively, we observed that the addition of high concentrations of LA and LN decreased soil NO_3^- concentration significantly, but did not have an appreciable effect on the residual NH_4^+ concentration in soil (Fig. 4.1). In contrast, the addition of DCD resulted in high soil NH_4^+ and low

NO_3^- concentrations, corroborating the direct effect of this NI on NO_3^- formation as seen other studies (Chaves et al., 2006; McGeough et al., 2016).

The NO_3^- concentration decreased significantly as expected, but the NH_4^+ concentration did not increase correspondingly in this study. A decline in NH_4^+ supply rather than toxicity of specific compounds to nitrifiers have at times explained low nitrification rates (Schimel et al., 1996), and heterotrophic NO_3^- immobilisation could occur when NH_4^+ concentrations are low (Rice and Tiedje, 1989). Thus, we hypothesise that the apparent nitrification inhibition (i.e. reduction in soil NO_3^- concentration) observed when high concentrations of LA and LN are added to a highly nitrifying soil (with no NH_4^+ amendment) could be the result of microbial immobilisation of N (i.e. an indirect effect), in contrast to the direct inhibition proven for NIs such as DCD (Guo et al., 2013; Subbarao et al., 2008).

The linear relationship between the predicted microbial N immobilisation (predicted value) using the ^{14}C-labelling method and observed N immobilisation (observed value) (Fig. 4.5) provided evidence for the immobilisation effect of LA and LN. Numerous studies have shown that the addition of labile C-rich substrates to soil can increase net N immobilisation, and is an indicator of immediate microbial response to the C substrate (G. Chen et al., 2003; Magill and Aber, 2000; Vinten et al., 2002). The addition of organic C stimulates the growth of soil microorganisms until they become limited by N availability (Garten and Wullschleger, 2000; Martin and Johnson, 1995). The compounds used in this study contained 77% C (for LA and LN) supporting this theory. Compared with DCD, the relatively rapid and high mineralisation of LA and LN indicates that the addition of LA and LN represents an available C source to the soil microorganisms (Fig. 4.4), and the linear relationship between the $^{14}CO_2$ and CO_2-C indicates that the mineralisation of LA and LN was the main source of the CO_2 emissions.

To our knowledge, this is the first study to explore the degradation rates of LA and LN in soil directly, e.g. using ^{14}C-labelled compounds, so the factors that influence the mineralisation of these specific biological NIs have not been quantified previously. The mineralisation rates of LA and LN observed in this study provide a reference for future research studies. The relative low mineralisation rates of DCD are consistent with other studies (e.g. Marsden et al., 2015; Singh et al., 2008). DCD degrades to CO_2 and NH_4^+ via guanylic urea, guanidine and urea (Kelliher et al., 2008; Marsden et al., 2016b). The half-life of DCD is strongly affected by soil temperature (Kelliher et al., 2014, 2008;

McGeough et al., 2016; Singh et al., 2008). Researchers have quantified the relationship between temperature (T) and the time (t) taken for DCD concentration in soil to decline to half its application value ($t_{½}$) as $t_{½}$ (T) = 168 $e^{-0.084T}$ (Kelliher et al., 2008). In this study, the soil was incubated at relative low temperature (10 °C) which may explain the low mineralisation rate of DCD.

In previous studies, researchers have focussed on the effect of LA and LN on soil N transformations (Lu et al., 2019; Subbarao et al., 2008). This is the first study to determine the effect of LA and LN on N_2O emissions. Our results demonstrated that cumulative N_2O emissions were significantly greater in the higher concentration biological NI treatments. Both nitrification and denitrification process are responsible for the N_2O emissions (Gardiner et al., 2016; Hofstra and Bouwman, 2005; Smith et al., 1997). These high N_2O emissions coupled with the lower soil NO_3^- concentrations in the 635 and 1270 mg BNI kg^{-1} dry soil treatments suggest that denitrification, stimulated by the large amount of available C added in the LA and LN, may be another soil process responsible for the apparent nitrification inhibition observed. In this study, DCD did not have a significant effect on the N_2O emissions, which is inconsistent with the fact that DCD can reduce direct soil N_2O emissions by 26% - 91% (Cameron et al., 2014; Cameron and Di, 2002; Kelliher et al., 2008; Smith et al., 2008; Weiske et al., 2001; Zaman et al., 2009). This could be because total N_2O emissions were relatively low and DCD can act as a microbial N source (66.7% N in DCD application) when N inputs are low, as in this study.

High rates of LA and LN application to soil significantly increased soil microbial immobilisation and decreased NO_3^- concentration. However, low NO_3^- concentrations may also be the result of increased N_2O emissions, presumably via denitrification, following the supply of sufficient available C in the two highest additions of the biological NIs. Since there was such as difference in the apparent BNI effect (microbial immobilisation and/or denitrification) between the 127 and 635 mg kg^{-1} BNI treatments, we suggest that further research is needed to explore the appropriate application rates of LA and LN needed to inhibit soil nitrification/increase N immobilisation and decrease GHG emissions at the same time.

4.5. Conclusions

Our results confirmed that the addition of LA, LN and DCD can decrease soil NO_3^- concentration, but their mode of action is different. Our results suggest that the apparent effect of LA and LN on soil NO_3^- concentration could be indirect under low-N conditions (no addition of fertiliser NH_4^+) due to the addition of sufficient labile C in the biological NIs stimulating either i) microbial immobilisation of soil NH_4^+ and/or NO_3^- (under high C/N ratios), and/or ii) denitrification losses, such as N_2O. We also demonstrated that LA and LN were much more rapidly mineralised than DCD in soil. Overall, we suggest that researchers exploring the effectiveness of biological NIs, consider whether any observed effects on NO_3^- concentration are the result of direct nitrification inhibition, or a potential indirect effect via their influence in other related processes, as this has implications for developing effective mitigation strategies for N_2O emission and NO_3^- leaching, and is something that has been overlooked.

4.6. Acknowledgments

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4.7. Declaration of interests

We declare that the authors have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

4.8. Data availability statement

The data presented in this study are available from the corresponding author upon reasonable request.

4.9. References

Ball, D.F., 1964. Loss-on-ignition as an estimate of organic matter and organic carbon in non-calcareous soils. Journal of Soil Science 15, 84–92. doi:10.1111/j.1365-2389.1964.tb00247.x

Beeckman, F., Motte, H., Beeckman, T., 2018. Nitrification in agricultural soils: impact, actors and mitigation. Current Opinion in Biotechnology 50, 166–173. doi:10.1016/j.copbio.2018.01.014

Boddy, E., Hill, P.W., Farrar, J., Jones, D.L., 2007. Fast turnover of low molecular weight components of the dissolved organic carbon pool of temperate grassland field soils 39, 827–835. doi:10.1016/j.soilbio.2006.09.030

Byrnes, R.C., Nùñez, J., Arenas, L., Rao, I., Trujillo, C., Alvarez, C., Arango, J., Rasche, F., Chirinda, N., 2017. Biological nitrification inhibition by Brachiaria grasses mitigates soil nitrous oxide emissions from bovine urine patches. Soil Biology and Biochemistry 107, 156–163. doi:10.1016/j.soilbio.2016.12.029

Cameron, K., Di, H., Moir, J., 2014. Dicyandiamide (DCD) effect on nitrous oxide emissions, nitrate leaching and pasture yield in Canterbury, New Zealand. New Zealand Journal of Agricultural Research 57, 251–270. doi:10.1080/00288233.2013.797914

Cameron, K.C., Di, H.J., 2002. The use of a nitrification inhibitor, dicyandiamide (DCD), to decrease nitrate leaching and nitrous oxide emissions in a simulated grazed and irrigated grassland. Soil Use and Management 18, 395–403. doi:10.1079/SUM2002151

Chadwick, D.R., Cardenas, L.M., Dhanoa, M.S., Donovan, N., Misselbrook, T., Williams, J.R., Thorman, R.E., McGeough, K.L., Watson, C.J., Bell, M., Anthony, S.G., Rees, R.M., 2018. The contribution of cattle urine and dung to nitrous oxide emissions: Quantification of country specific emission factors and implications for national inventories. Science of the Total Environment 635, 607–617. doi:10.1016/j.scitotenv.2018.04.152

Chaves, B., Opoku, A., De Neve, S., Boeckx, P., Van Cleemput, O., Hofman, G., 2006. Influence of DCD and DMPP on soil N dynamics after incorporation of vegetable crop residues. Biology and Fertility of Soils 43, 62–68. doi:10.1007/s00374-005-0061-6

Chen, G., Zhu, H., Zhang, Y., 2003. Soil microbial activities and carbon and nitrogen fixation. Research in Microbiology 154, 393–398. doi:10.1016/S0923-2508(03)00082-2

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

Erisman, J.W., Galloway, J.N., Seitzinger, S., Bleeker, A., Dise, N.B., Petrescu, A.M.R., Leach, A.M., de Vries, W., 2013. Consequences of human modification of the global nitrogen cycle. Philosophical Transactions of the Royal Society B: Biological Sciences 368, 20130116–20130116. doi:10.1098/rstb.2013.0116

FAO, 2017. World fertilizer trends and outlook to 2020 - Summary Report 66. doi:10.1002/job

Firestone, M.K., Davidson, E.A., 1989. Microbiologial Basis of NO and N2O production and consumption in soil. Exchange of Trace Gases between Terrestrial Ecosystems and the Atmosphere 7–21. doi:10.1017/CBO9781107415324.004

Gardiner, C.A., Clough, T.J., Cameron, K.C., Di, H.J., Edwards, G.R., de Klein, C.A.M., 2016. Potential for forage diet manipulation in New Zealand pasture ecosystems to mitigate ruminant urine derived N2O emissions: a review. New Zealand Journal of Agricultural Research. doi:10.1080/00288233.2016.1190386

Garten, C.T., Wullschleger, S.D., 2000. Soil Carbon Dynamics beneath Switchgrass as Indicated by Stable Isotope Analysis. Journal of Environment Quality 29, 645. doi:10.2134/jeq2000.00472425002900020036x

Glanville, H.C., Hill, P.W., Schnepf, A., Oburger, E., Jones, D.L., 2016. Combined use of empirical data and mathematical modelling to better estimate the microbial turnover of isotopically labelled carbon substrates in soil. Soil Biology and Biochemistry 94, 154–168. doi:10.1016/j.soilbio.2015.11.016

Gopalakrishnan, S., Subbarao, G. V., Nakahara, K., Yoshihashi, T., Ito, O., Maeda, I., Ono, H., Yoshida, M., 2007. Nitrification inhibitors from the root tissues of Brachiaria humidicola, a tropical grass. Journal of Agricultural and Food Chemistry 55, 1385–1388. doi:10.1021/jf062593o

Guo, Y.J., Di, H.J., Cameron, K.C., Li, B., Podolyan, A., Moir, J.L., Monaghan, R.M., Smith, L.C., O'Callaghan, M., Bowatte, S., Waugh, D., He, J.Z., 2013. Effect of 7-year application of a nitrification inhibitor, dicyandiamide (DCD), on soil microbial biomass, protease and deaminase activities, and the abundance of bacteria and archaea in pasture soils. Journal of Soils and Sediments 13, 753–759. doi:10.1007/s11368-012-0646-2

Hill, P.W., Garnett, M.H., Farrar, J., Iqbal, Z., Khalid, M., Soleman, N., Jones, D.L., 2015. Living roots magnify the response of soil organic carbon decomposition to temperature in temperate grassland. Global Change Biology 21, 1368–1375. doi:10.1111/gcb.12784

Hofstra, N., Bouwman, A.F., 2005. Denitrification in Agricultural Soils: Summarizing Published Data and Estimating Global Annual Rates. Nutrient Cycling in Agroecosystems 72, 267–278. doi:10.1007/s10705-005-3109-y

Jones, D.L., Shannon, D., Murphy, D. V, Farrar, J., 2004. Role of dissolved organic nitrogen (DON) in soil N cycling in grassland soils. Soil Biology and Biochemistry 36, 749–756. doi:10.1016/j.soilbio.2004.01.003

Kelliher, F.M., Clough, T.J., Clark, H., Rys, G., Sedcole, J.R., 2008. The temperature dependence of dicyandiamide (DCD) degradation in soils: A data synthesis. Soil Biology and Biochemistry 40, 1878–1882. doi:10.1016/j.soilbio.2008.03.013

Kelliher, F.M., van Koten, C., Kear, M.J., Sprosen, M.S., Ledgard, S.F., de Klein, C.A.M., Letica, S.A., Luo, J., Rys, G., 2014. Effect of temperature on dicyandiamide (DCD) longevity in pastoral soils under field conditions. Agriculture, Ecosystems and Environment 186, 201–204. doi:10.1016/j.agee.2014.01.026

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

Lassaletta, L., Billen, G., Grizzetti, B., Anglade, J., Garnier, J., 2014. 50 Year Trends in Nitrogen Use Efficiency of World Cropping Systems: the Relationship Between Yield and Nitrogen Input To Cropland. Environmental Research Letters 9, 105011. doi:10.1088/1748-9326/9/10/105011

Ledgard, S.F., Menneer, J.C., Dexter, M.M., Kear, M.J., Lindsey, S., Peters, J.S., Pacheco, D., 2008. A novel concept to reduce nitrogen losses from grazed pastures by administering soil nitrogen process inhibitors to ruminant animals: A study with sheep. Agriculture, Ecosystems & Environment 125, 148–158. doi:10.1016/J.AGEE.2007.12.006

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C., Schleper, C., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442, 806–809. doi:10.1038/nature04983

Li, H., Liang, X., Chen, Y., Lian, Y., Tian, G., Ni, W., 2008. Effect of nitrification inhibitor DMPP on nitrogen leaching, nitrifying organisms, and enzyme activities in a rice-oilseed rape cropping system. Journal of Environmental Sciences 20, 149–155. doi:10.1016/S1001-0742(08)60023-6

Li, T., Zhang, W., Yin, J., Chadwick, D., Norse, D., Lu, Y., Liu, X., Chen, X., Zhang, F., Powlson, D., Dou, Z., 2018. Enhanced-efficiency fertilizers are not a panacea for resolving the nitrogen problem. Global Change Biology. doi:10.1111/gcb.13918

Li, X., Sørensen, P., Olesen, J.E., Petersen, S.O., 2016. Evidence for denitrification as main source of N 2 O emission from residue-amended soil. Soil Biology and Biochemistry 92, 153–160. doi:10.1016/j.soilbio.2015.10.008

Lu, Y., Zhang, X., Jiang, J., Kronzucker, H.J., Shen, W., Shi, W., 2019. Effects of the biological nitrification inhibitor 1,9-decanediol on nitrification and ammonia oxidizers in three agricultural soils. Soil Biology and Biochemistry 129, 48–59. doi:10.1016/j.soilbio.2018.11.008

Lucas, G.N., 2013. Dicyandiamide contamination of milk powders. Sri Lanka Journal of Child Health 42, 63–64. doi:10.4038/sljch.v42i2.5624

Luo, J., Ledgard, S., Wise, B., Welten, B., Lindsey, S., Judge, A., Sprosen, M., 2015. Effect of dicyandiamide (DCD) delivery method, application rate, and season on pasture urine patch nitrous oxide emissions. Biology and Fertility of Soils 51, 453–464. doi:10.1007/s00374-015-0993-4

MacKenzie, A.F., Fan, M.X., Cadrin, F., 1998. Nitrous oxide emission in three years as affected by tillage, corn- soybean-alfalfa rotations, and nitrogen fertilization. Journal of Environmental Quality 27, 698–703. doi:10.2134/jeq1998.00472425002700030029x

Magill, A.H., Aber, J.D., 2000. Variation in soil net mineralization rates with dissolved organic carbon additions. Soil Biology and Biochemistry 32, 597–601. doi:10.1016/S0038-0717(99)00186-8

Marsden, K.A., Marín-Martínez, A.J., Vallejo, A., Hill, P.W., Jones, D.L., Chadwick, D.R., 2016. The mobility of nitrification inhibitors under simulated ruminant urine deposition and rainfall: a comparison between DCD and DMPP. Biology and Fertility of Soils 52, 491–503. doi:10.1007/s00374-016-1092-x

Marsden, K.A., Scowen, M., Hill, P.W., Jones, D.L., Chadwick, D.R., 2015. Plant acquisition and metabolism of the synthetic nitrification inhibitor dicyandiamide and naturally-occurring guanidine from agricultural soils. Plant and Soil 395, 201–214. doi:10.1007/s11104-015-2549-7

Martin, C.W., Johnson, W.C., 1995. Variation in Radiocarbon Ages of Soil Organic Matter Fractions from Late Quaternary Buried Soils. Quaternary Research 43, 232–237. doi:10.1006/qres.1995.1023

McGeough, K.L., Watson, C.J., Müller, C., Laughlin, R.J., Chadwick, D.R., 2016. Evidence that the efficacy of the nitrification inhibitor dicyandiamide (DCD) is affected by soil properties in UK soils. Soil Biology and Biochemistry 94, 222–232. doi:10.1016/j.soilbio.2015.11.017

Menéndez, S., Barrena, I., Setien, I., González-Murua, C., Estavillo, J.M., 2012. Efficiency of nitrification inhibitor DMPP to reduce nitrous oxide emissions under different temperature and moisture conditions. Soil Biology and Biochemistry 53, 82–89. doi:10.1016/j.soilbio.2012.04.026

Minet, E.P., Ledgard, S.F., Grant, J., Murphy, J.B., Krol, D.J., Lanigan, G.J., Luo, J., Richards, K.G., 2018. Feeding dicyandiamide (DCD) to cattle: An effective method to reduce N2O emissions from urine patches in a heavy-textured soil under temperate climatic conditions. Science of the Total Environment 615, 1319–1331. doi:10.1016/j.scitotenv.2017.09.313

Minet, E.P., Ledgard, S.F., Lanigan, G.J., Murphy, J.B., Grant, J., Hennessy, D., Lewis, E., Forrestal, P., Richards, K.G., 2016. Mixing dicyandiamide (DCD) with supplementary feeds for cattle: An effective method to deliver a nitrification inhibitor in urine patches. Agriculture, Ecosystems and Environment 231, 114–121. doi:10.1016/j.agee.2016.06.033

Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A Rapid, Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite. Nitric Oxide 5, 62–71.

doi:10.1006/NIOX.2000.0319

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

Mosier, A.R., Duxbury, J.M., Freney, J.R., Heinemeyer, O., Minami, K., 1996. Nitrous Oxide Emissions from Agricultural Fields: Assessment, Measurement and Mitigation, in: Progress in Nitrogen Cycling Studies. Springer Netherlands, Dordrecht, pp. 589–602. doi:10.1007/978-94-011-5450-5_97

Mulvaney, R.L., 1996. Nitrogen—Inorganic Forms, in: Methods of Soil Analysis Part 3—Chemical Methods. Soil Science Society of America, American Society of Agronomy, pp. 1123–1184. doi:10.2136/sssabookser5.3.c38

Rice, C.W., Tiedje, J.M., 1989. Regulation of nitrate assimilation by ammonium in soils and in isolated soil microorganisms. Soil Biology and Biochemistry 21, 597–602. doi:10.1016/0038-0717(89)90135-1

Schimel, J.P., Cleve, K. Van, Cates, R.G., Clausen, T.P., Reichardt, P.B., 1996. Effects of balsam poplar (Populus balsamifera) tannins and low molecular weight phenolics on microbial activity in taiga floodplain soil: implications for changes in N cycling during succession. Canadian Journal of Botany 74, 84–90. doi:10.1139/b96-012

Singh, J., Saggar, S., Giltrap, D.L., Bolan, N.S., 2008. Decomposition of dicyandiamide (DCD) in three contrasting soils and its effect on nitrous oxide emission, soil respiratory activity, and microbial biomass—an incubation study. Australian Journal of Soil Research 46, 517. doi:10.1071/SR07204

Smith, K.A., McTaggart, I.P., Tsuruta, H., 1997. Emissions of N_2O and NO associated with nitrogen fertilization in intensive agriculture, and the potential for mitigation. Soil Use and Management 13, 296–304. doi:10.1111/j.1475-2743.1997.tb00601.x

Smith, L.C., de Klein, C.A.M., Monaghan, R.M., Catto, W.D., 2008. The effectiveness of dicyandiamide in reducing nitrous oxide emissions from a cattle-grazed, winter forage crop in Southland, New Zealand. Australian Journal of Experimental Agriculture 48, 160. doi:10.1071/EA07262

Subbarao, G. V., Ishikawa, T., Ito, O., Nakahara, K., Wang, H.Y., Berry, W.L., 2006a. A bioluminescence assay to detect nitrification inhibitors released from plant roots: A case study with Brachiaria humidicola. Plant and Soil. doi:10.1007/s11104-006-9094-3

Subbarao, G. V., Ito, O., Sahrawat, K.L., Berry, W.L., Nakahara, K., Ishikawa, T., Watanabe, T., Suenaga, K., Rondon, M., Rao, I.M., 2006b. Scope and Strategies for Regulation of Nitrification in Agricultural Systems—Challenges and Opportunities. Critical Reviews in Plant Sciences 25, 303–335. doi:10.1080/07352680600794232

Subbarao, G. V., Nakahara, K., Hurtado, M.P., Ono, H., Moreta, D.E., Salcedo, A.F., Yoshihashi, A.T., Ishikawa, T., Ishitani, M., Ohnishi-Kameyama, M., Yoshida, M., Rondon, M., Rao, I.M., Lascano, C.E., Berry, W.L., Ito, O., 2009. Evidence for biological nitrification inhibition in Brachiaria pastures. Proceedings of the National Academy of Sciences 106, 17302–17307. doi:10.1073/pnas.0903694106

Subbarao, G. V., Rondon, M., Ito, O., Ishikawa, T., Rao, I.M., Nakahara, K., Lascano, C., Berry, W.L., 2007a. Biological nitrification inhibition (BNI) - Is it a widespread phenomenon? Plant and Soil. doi:10.1007/s11104-006-9159-3

Subbarao, G. V., Tomohiro, B., Masahiro, K., Osamu, I., Samejima, H., Wang, H.Y., Pearse, S.J., Gopalakrishnan, S., Nakahara, K., Zakir Hossain, A.K.M., Tsujimoto, H., Berry, W.L., 2007b. Can biological nitrification inhibition (BNI) genes from perennial Leymus racemosus (Triticeae) combat nitrification in wheat farming? Plant and Soil. doi:10.1007/s11104-007-9360-z

Subbarao, G. V, Nakahara, K., Ishikawa, T., 2008. Free fatty acids from the pasture grass Brachiaria humidicola and one of their methyl esters as inhibitors of nitrification 89–99. doi:10.1007/s11104-008-9682-5

Subbarao, G. V, Nakahara, K., Ishikawa, T., Ono, H., Yoshida, M., Yoshihashi, T., Zhu, Y., Zakir, H.A.K.M., Deshpande, S.P., Hash, C.T., Sahrawat, K.L., 2013. Biological nitrification inhibition (BNI) activity in sorghum and its characterization. Plant Soil 366, 243–259. doi:10.1007/s11104-012-1419-9

Sun, L., Lu, Y., Yu, F., Kronzucker, H.J., Shi, W., 2016. Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency. New Phytologist 212, 646–656. doi:10.1111/nph.14057

Taylor, A.E., Zeglin, L.H., Dooley, S., Myrold, D.D., Bottomley, P.J., 2010. Evidence for different contributions of archaea and bacteria to the ammonia-oxidizing potential of diverse Oregon soils. Applied and Environmental Microbiology 76, 7691–8. doi:10.1128/AEM.01324-10

Tubiello, F.N., Salvatore, M., Rossi, S., Ferrara, A., Fitton, N., Smith, P., 2013. The FAOSTAT database of greenhouse gas emissions from agriculture. Environmental Research Letters 8, 015009. doi:10.1088/1748-9326/8/1/015009

Vinten, A.J.A., Whitmore, A.P., Bloem, J., Howard, R., Wright, F., 2002. Factors affecting N immobilisation/mineralisation kinetics for cellulose-, glucose- and straw-amended sandy soils. Biology and Fertility of Soils 36, 190–199. doi:10.1007/s00374-002-0524-y

Weiske, A., Benckiser, G., Herbert, T., Ottow, J.C.G., 2001. Influence of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) in comparison to dicyandiamide (DCD) on nitrous oxide emissions, carbon dioxide fluxes and methane oxidation during 3 years of repeated application in field experiments. Biology and Fertility of Soils 34, 109–117. doi:10.1007/s003740100386

Welten, B.G., Ledgard, S.F., Luo, J., 2014. Administration of dicyandiamide to dairy cows via drinking water reduces nitrogen losses from grazed pastures, in: Journal of Agricultural Science. Cambridge University Press, pp. S150–S158. doi:10.1017/S0021859613000634

Wu, S. fu, Wu, L. huan, Shi, Q. wei, Wang, Z. qiang, Chen, X. you, Li, Y. shan, 2007. Effects of a new nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on nitrate and potassium leaching in two soils. Journal of Environmental Sciences 19, 841–847. doi:10.1016/S1001-0742(07)60140-5

Xu, X., Thornton, P.E., Post, W.M., 2013. A global analysis of soil microbial biomass carbon, nitrogen and phosphorus in terrestrial ecosystems. Global Ecology and Biogeography 22, 737–749. doi:10.1111/geb.12029

Zaman, M., Saggar, S., Blennerhassett, J.D., Singh, J., 2009. Effect of urease and nitrification inhibitors on N transformation, gaseous emissions of ammonia and nitrous oxide, pasture yield and N uptake in grazed pasture system. Soil Biology and Biochemistry 41, 1270–1280. doi:10.1016/j.soilbio.2009.03.011

Chapter 5: Potential of biological nitrification inhibition by *Brachiaria humidicola* to mitigate nitrous oxide emissions following sheep urine application

Yan Ma[1,*], Alice F. Charteris[2], Nadine Loick[2], Laura M. Cardenas[2], Zhipeng Sha[3], Davey L. Jones[1,4], David R. Chadwick[1,5]

[1] *School of Natural Science, Bangor University, Bangor, Gwynedd LL57 2UW, UK*

[2] *Rothamsted Research, North Wyke, Okehampton, Devon, EX20 2SB, UK*

[3] *College of Resources and Environmental Sciences, China Agricultural University, Beijing 100193, P.R.China*

[4] *SoilsWest, UWA School of Agriculture and Environment, The University of Western Australia, Perth, WA 6009, Australia*

[5] *Interdisciplinary Research Centre for Agriculture Green Development in Yangtze River Basin, Southwest University, Chongqing, China*

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Abstract

Brachiaria humidicola (Bh) has the ability to produce biological nitrification inhibitors (NIs) in the shoot and root tissues and release from the root to the soil. To compare the effects of growing Bh with *Brachiaria ruziziensis* (Br, which is not able to produce NIs) on soil nitrogen (N) dynamics, N gases and carbon dioxide (CO_2) emissions following sheep urine application, a laboratory incubation was conducted in a He/O_2 continuous flow Denitrification System (DENIS). The treatments were as follow: 1) Bh with water application (Bh + W); 2) Bh with sheep urine (Bh + U); 3) Br with water application (Br + W); 4) Br with sheep urine (Br + U). Results showed that soil NO_3^- concentration increased significantly in the soil with sheep urine application after the incubation. Soil nitrous oxide (N_2O) and nitric oxide (NO) emissions increased immediately after the sheep urine application and peaked twice during the incubation, whilst dinitrogen (N_2) emissions peaked at the moment when the urine was deposited on the soil. Sheep urine addition did not affect the AOA, *nirS* and *nosZ* gene copies, but significantly increased the AOB gene copies. Even though no significant differences were observed in the total cumulative N_2O and NO emissions between the Bh + U and Br + U treatment at the end of the incubation, during the first peak of N_2O cumulative emissions were significantly lower from the Bh + U treatment (0.054 kg N ha^{-1}) compared with the Br + U treatment (0.111 kg N ha^{-1}). We conclude that there is potential for using Bh grass in sheep-grazed pastures to mitigate nitrification rates and N_2O emissions even for a highly nitrifying soil.

Keywords: *Brachiaria humidicola*, *Brachiaria ruziziensis*, nitrogen gas, carbon dioxide, nitrifier, denitrifier.

5.1. Introduction

Nitrification and denitrification are key processes of the soil nitrogen (N) cycle. Nitrification is a two-step microbially mediated process carried out by chemo-autotrophic nitrifying bacteria, first oxidising ammonium (NH_4^+) to nitrite (NO_2^-) which is further oxidised to nitrate (NO_3^-) (Firestone and Davidson, 1989). During the nitrification and subsequent denitrification, other gaseous forms of N are

produced and lost from agricultural soils, such as nitrous oxide (N_2O), nitric oxide (NO) and dinitrogen (N_2). Nitrous oxide has been attributed to nitrification, denitrification and nitrifier denitrification processes depending on the soil environmental conditions, such as water-filled pore space (WFPS), O_2 availability, soil pH and temperature (Bateman and Baggs, 2005; Lai et al., 2019; Loick et al., 2016; Wrage et al., 2005). Some studies present NO emitted from soils during nitrification process (Caranto and Lancaster, 2017; Kang et al., 2020; Wang et al., 2016). However denitrification can also be a major source of NO from soils at high water content and/or under the presence of a carbon (C) source (Ji et al., 2020; Loick et al., 2016; Wu et al., 2017), whilst N_2 is the final product of denitrification (Knowles, 1982).

Synthetic nitrification inhibitors (NIs) have been widely used to inhibit soil nitrification, e.g. dicyandiamide (DCD), 3,4-dimethylpyrazole phosphate (DMPP) (Chadwick et al., 2018; Chen et al., 2014; Weiske et al., 2001). Following concerns of synthetic NIs passing into human food chains (Lin et al., 2015; Study et al., 2014; Welten et al., 2016), there has been increasing interest in the role of biological NIs to reduce N_2O emissions and NO_3^- leaching. Some grass species (de Cerqueira Luz et al., 2014; Gopalakrishnan et al., 2009; Subbarao et al., 2008) and crop plants (Huérfano et al., 2016; Subbarao et al., 2013; Sun et al., 2016) have the ability to release compounds from their roots to suppress the nitrifier activity which is termed biological nitrification inhibition (BNI) (Subbarao et al., 2006a). *Brachiaria humidicola* (Bh), a typical tropical pasture grass used for grazing livestock, has been reported to release biological NIs from its roots. Active inhibitory compounds have been isolated from the root tissues (e.g. methyl-p-coumarate and methyl ferulate) (Gopalakrishnan et al., 2007), root exudates (e.g. brachialactone) (Subbarao et al., 2009), and shoot tissues (e.g. linoleic acid and linolenic acid) (Subbarao et al., 2008) of Bh.

Previous studies have focused on the effects of pure inhibitory compounds identified from the pasture grass or the root exudates of Bh on soil NH_4^+ transformation and N_2O emissions (Gopalakrishnan et al., 2009; Meena et al., 2014; Subbarao et al., 2008). Whilst experiments have been conducted to explore nitrification inhibition and N_2O emissions from soil planted with *Brachiaria* grasses, including pasture that receive bovine urine deposition (Byrnes et al., 2017; Simon et al., 2020), there is still a lack of understanding about the residual effects of growing Bh on soil nitrification and

other gaseous N forms other than N_2O, e.g. NO and N_2, and particularly after sheep urine application. Also, little is known about the residual effect of growing Bh on soil nitrifiers and denitrifiers.

There is strong evidence that other *Brachiaria* species, e.g. *Brachiaria ruziziensis* (Br), are not capable of inhibiting nitrification in the rhizosphere (Fernandes et al., 2011). Thus, this *Brachiaria* species was selected to compare with Bh (which has the ability to release biological NIs from the roots) to: 1) explore the residual effect of Bh and Br on soil NH_4^+ and NO_3^- concentrations; and 2) quantify the N_2O, NO, N_2 and CO_2 emissions and identify the processes responsible for their production (i.e. nitrification or denitrification) in soil sown with these two *Brachiaria* varieties. Based on current research, we hypothesised that i) soil under Bh retains soil NH_4^+-N, and results in lower NO_3^--N concentrations than soil under Br, ii) Bh results in lower N_2O, NO and N_2 emissions than soil under Br due to the higher BNI capacity of Bh, iii) soil applied with sheep urine retains significantly higher soil NH_4^+, NO_3^- concentrations and results in greater N_2O, NO, N_2 and CO_2 emissions than soil applied with water.

5.2. Materials and methods

5.2.1. Soil sampling and physicochemical analysis

A sandy clay loam textured Eutric Cambisol was collected from a typical sheep-grazed grassland in North Wales (53°24'N, 4°02'W). The soil had not been previously grown with Bh and Br. Square intact turves of soil (30×30 cm, depth of 10 cm) were collected from 3 spatially discrete points (at least 10m apart), which were retained as 3 replicates. Soil was sieved (2 mm) to remove roots and stones before analysis for a range of chemical properties: 19.4% moisture content (105 °C, 24 h), 6.7% organic matter (450 °C, 16 h) (Ball, 1964), 2.7% total C and 0.25% total N (CHN2000 Analyzer), pH of 5.9, 1.7 mg N kg^{-1} dry soil as NH_4^+-N (Mulvaney, 1996) and 30.4 mg N kg^{-1} dry soil as NO_3^--N (Miranda et al., 2001).

5.2.2. Cultivation of Brachiaria humidicola and Brachiaria ruziziensis

To investigate the residual effect of Bh and Br on soil nitrification, greenhouse gas emissions (GHG, N_2O and CO_2), NO and N_2 emissions after sheep urine application, two varieties of *Brachiaria* were sown separately in pots containing the field soil. Seeds of Bh and Br were germinated on wetted tissue paper in an incubator (20 °C). 1.7 kg field fresh soil were added to each pot (diameter: 15 cm; depth: 15 cm) at the same bulk density as the soil at the field site (1.6 g cm^{-3}) (Marsden et al., 2016a), and 10 geminated seeds were placed onto the soil surface before covering with 100 g soil. There were 12 pots in total, 6 pots were grown with Bh and 6 pots with Br. To stimulate grass growth and promote the release of the inhibitory compounds (Subbarao et al., 2007c), the plants were cut to 2 cm above the soil level on d 33 and d 75, and the equivalent of 25 kg N ha^{-1} as $(NH_4)_2SO_4$ was added to each pot 3 days after each cut. 50 mL of tap water was added to each pot twice per week to maintain plant growth prior to the incubation experiment. The incubation experiment (described below) was conducted on d 150 after sowing.

5.2.3. Experimental setup

The 23-d incubation experiment was conducted in the Denitrification System (DENIS) at Rothamsted Research (North Wyke) (Cárdenas et al., 2003), using the top (0-7.5 cm) of the intact (12 cm deep) soils including plants (obtained from section 5.2.2). The soil cores were placed into 12 stainless vessels (diameter: 14.1 cm) and sealed with stainless steel lids fitted with double 'O' rings. The incubation experiment comprised 4 treatments with 3 replicates: 1) Bh with water application (Bh + W); 2) Bh with sheep urine (Bh + U); 3) Br with water application (Br + W); 4) Br with sheep urine (Br + U). The sheep urine used in this experiment had been collected from 6 Welsh Mountain ewes that had been grazing a permanent pasture at the same site the soil was collected from. The urine had been frozen immediately after collection to avoid N losses during storage. The sheep urine was defrosted the day before application to the soil cores, and the individual urine samples (n=6) were pooled and mixed to generate one urine source (total C, 25.3 g L^{-1}; total N, 11.7 g L^{-1}, of which 670 mg N kg^{-1} dry soil were added in the treatments).

The incubation experiment followed a similar approach to previous experiments using this DENIS system (Loick et al., 2016; Wu et al., 2017). Briefly, to remove the native N_2 from the soil cores and the headspace, the soil cores were flushed from the base at a flow rate of 30 mL min^{-1} for 48 hours using a mixture of He: O_2 (80: 20), with the outlet flow from each chamber directed to a number of gas detectors. Once the N_2, N_2O and NO concentrations had reached very low levels, the airflow was decreased to 12 mL min^{-1} to measure the baseline emissions before being switched from the flow through the base to a flow over the soil surface. The sheep urine and water amendments were contained in sealed stainless steel vessels above the lid of each incubation vessel. In previous protocols these amendment vessels are usually flushed with He/O_2 (80:20) to remove N_2 (Cárdenas et al., 2003). However, in this experiment, the vessels containing the urine and water were not flushed with He/O_2, to avoid the N losses (via NH_3 volatilisation) from the sheep urine. After the urine and water had attained room temperature, the amendments were applied to the soil by opening the ball-valve connecting the 2 vessels. At the start of the soil incubation, the soil moisture content was increased to 65% WFPS, taking the volume of the urine or water amendments into account. The temperature of the vessels was maintained at 15 °C during the flushing phase and the 23-d incubation period after the urine and water applications.

5.2.4. Soil sampling and analysis

At the start and end of the incubation period, fresh soil samples were collected for analysis. Soil moisture content was measured after oven drying (105 °C, 24 h), and the soil organic matter was determined by loss on ignition of dried soil in a muffle furnace (450 °C, 16 h) (Ball, 1964). Total soil C and N concentrations were determined on milled oven dried soil samples using a CHN2000 Analyzer (Leco Corp., St. Joseph, MI). Soil pH and electrical conductivity (EC) were measured on fresh soil using standard electrodes (1:2.5 (w/v) soil-to-distilled water). Extractable NH_4^+-N and NO_3^--N were analysed in the filtrates after extracting 5 g of fresh soil with 25 ml K_2SO_4 (0.5 M) using the colorimetric methods of Mulvaney (1996) and Miranda et al., (2001), and total dissolved C and N were analysed with the Multi N/C 2100 (AnalytikJena, Jena, Germany). Data were expressed on a per kg dry soil basis.

At the same time, 5 g fresh soil from each vessel were collected and stored at -80°C prior to DNA extraction. Soil (0.25 g) was extracted by the the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After extraction, the purity and concentration of extracted soil DNA were determined by the Nanodrop spectrophotometer ND-1000 (Labtech, UK). Polymerase chain reaction (PCR) was carried out on real-time quantitative PCR (QPCR) using the QuantStudioTM 6 flex real-time PCR system (Thermo Fisher Scientific, UK). The 20 µL reaction mixture comprised 10 µL TB Green Premix Ex Taq (TaKaRa, Tokyo, Japan), 0.3 µL of each primer, 0.4 µL ROX Reference dye, 7 µL of sterilized deionised water and 2 µL template DNA. The primers for quantifying nitrification and denitrification function genes were the same as those used in previous studies (see more details in the appendix 4, Table 1) (Robinson et al., 2014; Zulkarnaen et al., 2019).

5.2.5. Gas sampling and analysis

The airflow from each vessel was automatically directed to a valve that directed the sample to different gas detectors, resulting in one sample being analysed every 8 minutes from each of the 12 vessels. Thus, one measurement was made every 1.5 hours from each vessel. The N_2O and CO_2 concentrations were determined using a gas chromatograph (GC, Pekin Elmer Clarus 500, Beaconsfield, UK) equipped with an electron capture detector (ECD), and a second GC with a helium ionization detector (HID, VICI AG International, Schenkon, Switzerland) was used to analyse N_2 concentrations. For NO concentrations, a chemiluminescence analyser was used (Sievers NOA280i, GE Instruments, Colorado, USA). The gas flow rate through each vessel was measured daily to calculate the volume of gas required for the flux calculation. The gaseous fluxes were corrected for the surface area and flow rate through the vessels and are presented in the unit of kg N or C ha^{-1} d^{-1}. Cumulative gaseous fluxes were calculated by the area under the curve after linear interpolation between sampling points using the Genstat 19th edition (VSN International Ltd) (Meijide et al., 2010).

5.2.6. Statistical analysis

One-way analysis of variance (ANOVA) followed by the LSD test at 5% confidence was used to determine the effect of Bh and Br on soil NH_4^+ and NO_3^- concentrations, cumulative gas emissions (N_2O, NO, N_2 and CO_2) and gene abundance (AOA, AOB, *nirK*, *nirS*, *nosZ*) at the start (d 0) and end (d 23) of the incubation respectively. All statistical analyses were performed in SPSS Statistics 25.0 (IBM Inc., Armonk, NY).

5.3. Results

5.3.1. Soil ammonium and nitrate concentrations

At the start of the incubation, there were no significant differences between all the treatments (Bh + W, Bh + U, Br + W, Br + U) for the soil NH_4^+ and NO_3^- concentrations, with average concentrations of 3.1 (ranging from 2.7 to 3.3 mg kg^{-1} soil) and 2.7 (ranging from 1.8 to 3.7 mg kg^{-1} soil) mg kg^{-1} soil, respectively (Table 5.1). In the Bh + W and Br + W treatments, after the 23 d incubation the NH_4^+ concentration decreased (Bh + W, 3.3 to 1.3 mg kg^{-1} soil; Br + W, 3.1 to 0.15 mg kg^{-1} soil) and NO_3^- increased (Bh + W, 3.7 to 16.0 mg kg^{-1} soil; Br + W, 2.8 to 17.3 mg kg^{-1} soil). 23 days after the sheep urine application, there was a small increase in the NH_4^+ concentration in the urine treatments (Bh + U, from 2.7 to 3.2 mg kg^{-1} soil; Br + U, from 3.3 to 3.6 mg kg^{-1} soil) and a large increase in the NO_3^- concentration in the same treatments (Bh + U, from 1.8 to 235.7 mg kg^{-1} soil; Br + U, from 2.6 to 213.9 mg kg^{-1} soil) (Fig. 5.1).

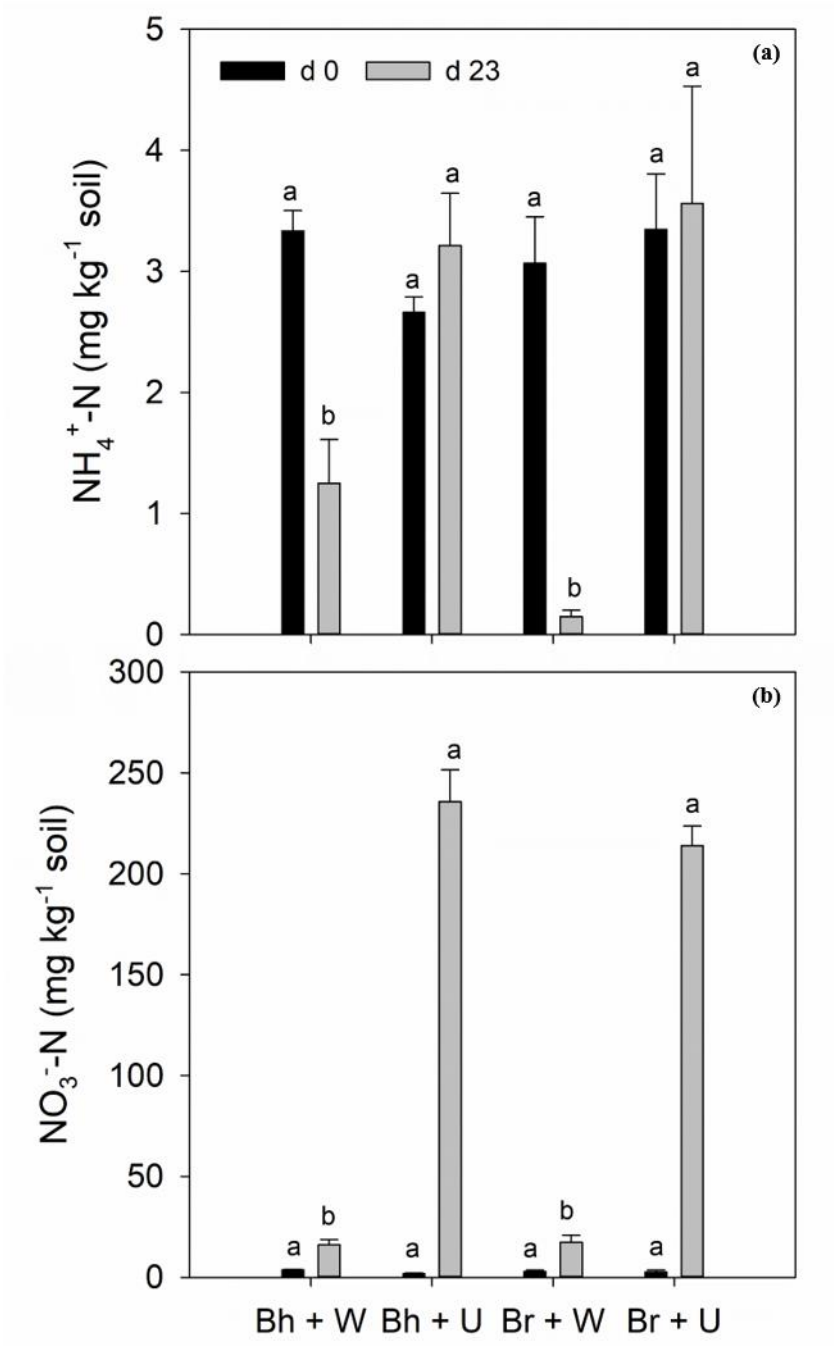

Fig. 5.1 Soil NH_4^+-N (panel a) and NO_3^--N (panel b) concentrations before urine application (d 0) and at the end of the incubation period (d 23). Error bars represent standard error of the mean (n=3). Different letters indicate significant differences between treatments at $P<0.05$ by LSD.

Table 5.1 Soil characteristics before urine application (d 0) and after the incubation (d 23).

Soil property	Bh + W		Bh + U		Br + W		Br + U	
	d 0	d 23	d 0	d 23	d 0	d 23	d 0	d 23
Moisture content (%)	30.3 ± 0.23^{a}	27.7 ± 0.78^{B}	30.6 ± 0.11^{a}	30.1 ± 0.54^{A}	29.4 ± 0.60^{a}	29.4 ± 0.79^{AB}	30.2 ± 0.36^{a}	28.0 ± 0.34^{AB}
Organic matter (%)	6.5 ± 0.15^{a}	6.4 ± 0.06^{AB}	6.4 ± 0.21^{a}	6.6 ± 0.05^{A}	6.3 ± 0.05^{a}	6.3 ± 0.07^{B}	6.3 ± 0.13^{a}	6.5 ± 0.03^{A}
pH	6.6 ± 0.03^{a}	6.0 ± 0.02^{A}	6.6 ± 0.04^{a}	5.3 ± 0.05^{B}	6.3 ± 0.08^{b}	6.0 ± 0.05^{A}	6.5 ± 0.04^{ab}	5.2 ± 0.04^{B}
Electrical conductivity ($\mu S\ cm^{-1}$)	116.8 ± 16.7^{a}	147.8 ± 6.84^{B}	109.3 ± 1.84^{a}	802.3 ± 21.8^{A}	111.0 ± 4.63^{a}	158.3 ± 11.0^{B}	104.5 ± 6.02^{a}	755.3 ± 22.0^{A}
Total carbon ($g\ kg^{-1}$ soil)	21.4 ± 0.43^{a}	23.3 ± 0.50^{A}	23.2 ± 1.00^{a}	24.9 ± 1.79^{A}	23.5 ± 0.49^{a}	24.1 ± 0.06^{A}	23.0 ± 0.49^{a}	25.1 ± 0.81^{A}
Total nitrogen ($g\ kg^{-1}$ soil)	2.6 ± 0.04^{b}	2.8 ± 0.09^{B}	2.7 ± 0.05^{ab}	3.1 ± 0.04^{AB}	2.8 ± 0.10^{a}	2.8 ± 0.08^{B}	2.7 ± 0.02^{ab}	3.2 ± 0.14^{A}
NH_4^+-N (mg N kg^{-1} soil)	3.3 ± 0.17^{a}	1.3 ± 0.36^{B}	2.7 ± 0.13^{a}	3.2 ± 0.43^{A}	3.1 ± 0.39^{a}	0.15 ± 0.05^{B}	3.3 ± 0.46^{a}	3.6 ± 0.97^{A}
NO_3^--N (mg N kg^{-1} soil)	3.7 ± 0.20^{a}	16.0 ± 2.61^{B}	1.8 ± 0.41^{a}	235.7 ± 15.8^{A}	2.8 ± 0.65^{a}	17.3 ± 3.48^{B}	2.6 ± 0.99^{a}	213.9 ± 9.63^{A}

Values represent means ± standard error. Different letters indicate the significant differences between treatments at d 0 (lowercase) and d 23 (capital) respectively (n=3, $P<0.05$).

5.3.2. Gas emissions

Nitrous oxide: N_2O emissions increased immediately after the sheep urine application, with maximum fluxes of 0.12 and 0.22 kg N ha^{-1} d^{-1} in the Bh + U and Br + U treatments, respectively (Fig. 5.2a). These fluxes decreased rapidly within the following 23 h and then reached another peak after d 13, with what seem to be broad peaks lasting up to 9 days (d 10 to 19). Fluxes, however, remained high until the end of the incubation. N_2O emissions in the Bh + W and Br + W treatments were much lower than that in the treatments with sheep urine application, with average fluxes of 0.009 and 0.006 kg N ha^{-1} d^{-1}, respectively. The cumulative N_2O emission for the first peak in the Br + U treatment (0.11 kg N ha^{-1}) was significantly higher than that in the Bh + U (0.05 kg ha^{-1}) treatment, although no significant differences were observed in the cumulative N_2O emissions for the entire 23-d incubation between the Bh + U and Br + U treatments (Table 5.2). The cumulative N_2O emissions in the Bh + W and Br + W treatments were significantly lower than that from both urine treatments during both the first peak period and the whole incubation period.

Nitric oxide: the pattern of NO emissions was similar to the N_2O emissions for all treatments during the 23-d incubation, with the exception that the maximum NO fluxes in the sheep urine application treatments occurred during the second peak on d 14-16 (Fig. 5.2b). The first peak of NO emissions appeared 7.0 h and 10.6 h after the urine application in the Bh + U and Br + U treatments, respectively, which was a little later than the peak time of maximum N_2O emissions (3.6 and 5.3 h, respectively) reaching values up to 3 g N ha^{-1} d^{-1}. Cumulative NO emissions in the treatments with the sheep urine application including the two peaks (Bh + U, 0.114 kg N ha^{-1}; Br + U, 0.103 kg N ha^{-1}) were significantly higher than those in the water only treatments (Bh + U, 0.007 kg N ha^{-1}; Br + U, 0.003 kg N ha^{-1}). Nevertheless, no significant differences in NO emissions were observed between the Bh + U and Br + U treatments, or the Bh + W and Br + W treatments during the first peak period or in the whole incubation period. The second NO peak was broader than the initial one (reached up to ~8 g N ha^{-1} d^{-1}) and had not reached background values at the end of the incubation, but clearly showed fluxes were decreasing from d 16 onwards.

Dinitrogen: N_2 emissions increased immediately after the urine or water application and then decreased rapidly, remaining stable until the end of the incubation (Fig. 5.2c). Dinitrogen was the main gaseous N form detected during the incubation, with the cumulative N_2 emissions being significantly greater than the total for N_2O and NO emissions. Cumulative N_2 detected in the Bh + W, Bh + U, Br + W, Br + U treatments were 81.3, 56.3, 42.2 and 61.5 kg N ha^{-1}, respectively. No significant differences were observed in the cumulative N_2 emissions between the Bh + W, Bh + U, Br + W and Br + U treatments during the first peak or entire incubation period.

Carbon dioxide: in the Bh + U and Br + U treatments, the CO_2 emissions increased rapidly and peaked at 10.8 h after the urine application (similar to the NO peak in the urine treatments), with the maximum fluxes of 207.2 and 198.9 kg C ha^{-1} d^{-1}, respectively (Fig. 5.2d). The CO_2 emissions decreased afterwards and remained stable (less than ca. 30 kg C ha^{-1} h^{-1}) from d 3.5 to end of the incubation in the Bh + U and Br + U treatments. The cumulative CO_2 emissions in the water only treatments were significantly lower than that in the urine treatments, following the series: Br + W < Bh + W < Br + U < Bh + U, with the cumulative fluxes of 333.5, 428.5, 654.6, 768.5 kg C ha^{-1}, respectively (Table 5.2).

Table 5.2 Cumulative emissions of NO, N_2O, N_2 in kg N ha^{-1} and CO_2 in kg C ha^{-1} after 23 d incubation and during the first peak period.

Gas	Bh + W	Bh + U	Br + W	Br + U
N_2O (23 d)	0.216 ± 0.026 b	1.73 ± 0.316 a	0.128 ± 0.068 b	1.72 ± 0.324 a
N_2O (first peak)	0.003 ± 0.000 c	0.054 ± 0.010 b	0.004 ± 0.001 c	0.111 ± 0.017 a
NO (23 d)	0.007 ± 0.001 b	0.114 ± 0.009 a	0.003 ± 0.001 b	0.103 ± 0.015 a
NO (first peak)	0.0003 ± 0.0001 b	0.0015 ± 0.0001 ab	0.0003 ± 0.0001 b	0.0025 ± 0.0007 a
N_2 (23 d)	81.31 ± 30.46 a	56.25 ± 22.36 a	42.21 ± 16.22 a	61.53 ± 9.84 a
N_2 (first peak)	19.09 ± 8.30 a	19.13 ± 9.48 a	13.70 ± 5.72 a	23.57 ± 8.74 a
CO_2 (23 d)	422.0 ± 10.5 c	761.9 ± 15.7 a	328.5 ± 13.4 d	649.0 ± 7.4 b
CO_2 (first peak)	97.83 ± 3.34 b	350.0 ± 10.28 a	84.56 ± 3.26 b	328.6 ± 12.59 a

Values represent means ± standard error. Different letters indicate a significant difference between treatments (n=3, P<0.05).

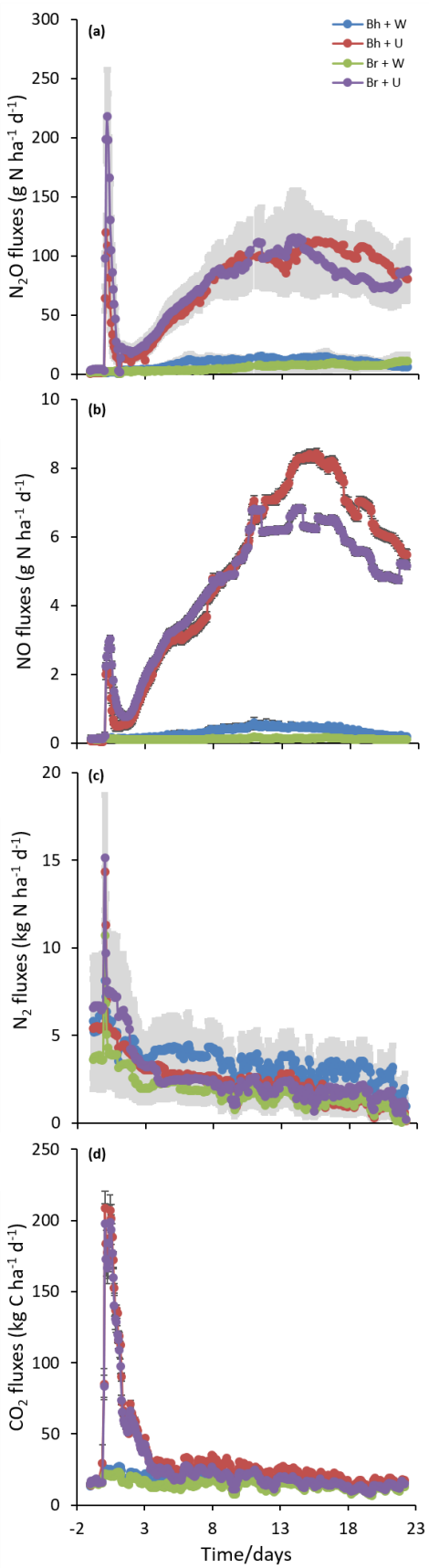

Fig. 5.2 Gaseous emissions of N_2O (panel a), NO (panel b), N_2 (panel c) and CO_2 (panel d) during the incubation.

5.3.3. Nitrifiers and denitrifiers gene copies

At the start of the incubation (d 0), there were no significant differences in the AOA, AOB, *nirK*, *nirS* and *nosZ* gene copies between the different treatments (Fig. 5.3). After the incubation (d 23), no significant differences were observed in the AOA, *nirS* and *nosZ* gene abundance between the treatments with the sheep urine application and without urine application (Fig. 5.3a, d, e). The sheep urine application increased the soil AOB and *nirK* gene copies at the end of the incubation (Fig. 5.3b, c). The AOB gene copies in the Bh + U treatment (7.7×10^6 copies g^{-1} soil) were significantly higher than that in the Br + U treatment (4.7×10^6 copies g^{-1} soil). The *nirK* gene copies in the Br + W (2.1×10^4 copies g^{-1} soil) was significantly lower than other treatments, but no significant differences were observed in the *nirK* gene copies between the Bh + W, Bh + U and Br + U treatments (3.3×10^4, 5.0×10^4, 3.7×10^4 copies g^{-1} soil, respectively).

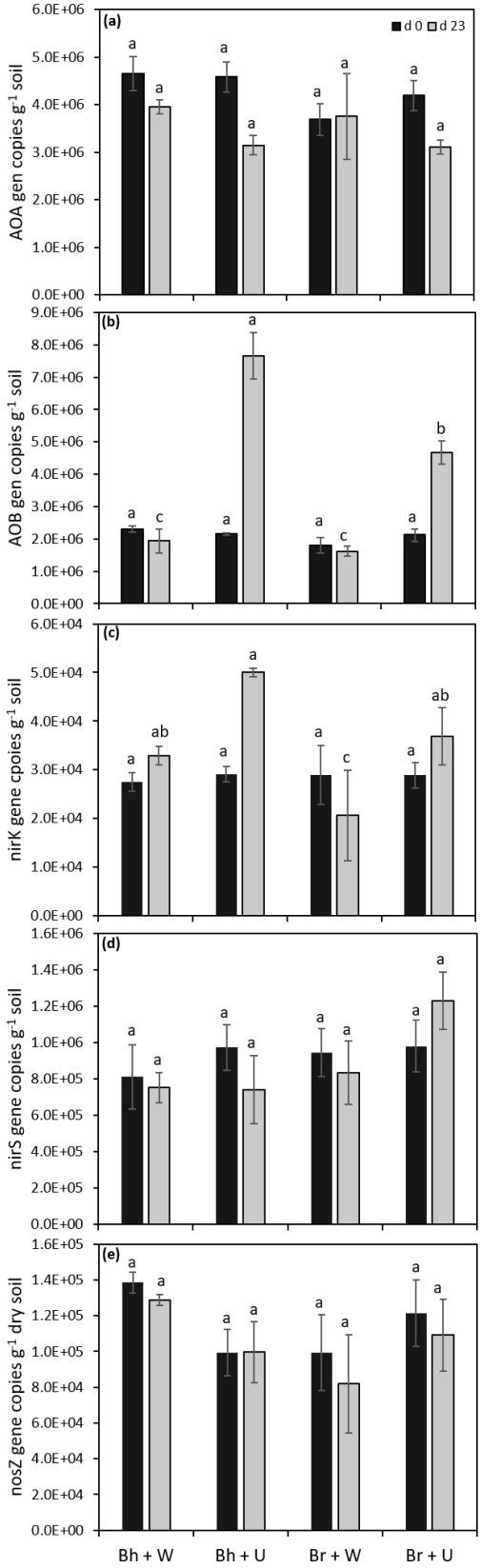

Fig. 5.3 AOA (panel a), AOB (panel b), *nirK* (panel c), *nirS* (panel d) and *nosZ* (panel e) gene abundance at d 0 and d 23. Error bars represent standard error of the mean (n=3). Different letters indicate significant differences between treatments at $P<0.05$ by LSD.

5.4. Discussion

5.4.1. Effect of Bh and Br on soil NH_4^+-N and NO_3^--N concentrations

The decrease of NH_4^+ and increase of NO_3^- in the treatments without sheep urine application was caused by the nitrification of residual soil NH_4^+ promoted by the relatively low soil moisture (65% WFPS). In the treatments with sheep urine application, the slight increase of NH_4^+ and marked increase in NO_3^- (over 200 mg N kg soil^{-1}) were caused by the hydrolysis of urea and further nitrification of the NH_4^+ from the urine-N applied (Byrnes et al., 2017). After the incubation, soil with Bh retained relatively higher NH_4^+ and lower NO_3^-concentrations than soil with Br (Fig. 5.1), which may be related to the biological NIs released from its root to suppress the transformation of NH_4^+ to NO_3^- (Gopalakrishnan et al., 2009; Nuñez et al., 2018; Subbarao et al., 2007a). Biological NIs released from the Bh grasses are more likely to block both the ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) enzymatic pathways, which play a critical role in the oxidation of NH_4^+ to NO_2^- in *Nitrosomonas* spp. (Subbarao et al., 2009, 2008).

5.4.2. Effect of Bh and Br on soil gaseous N

Nitrous oxide and NO are known products of both the nitrification and denitrification processes, which dominate under different optimal soil environment conditions such as soil moisture (Loick et al., 2016; Wu et al., 2017), pH (Robinson et al., 2014), temperature (Lai et al., 2019), O_2 availability (Senbayram et al., 2019; Zhu et al., 2013) and C availability (Miller et al., 2008; O'Neill et al., 2020). At the beginning of the incubation experiment, the initial soil water content was set as 65% WFPS which would have favoured nitrification of the NH_4^+ from the hydrolysed urea in the urine treatments causing the initial observed N_2O and NO emission peaks (first smaller peak). In addition, the initial CO_2 peak coincided with those of N_2O and NO, providing evidence of aerobic respiration (Lee et al., 2011). It is likely that the N_2 peak that appeared at the moment of sheep urine application, and before the N_2O and NO peaks, was because atmospheric N_2 was introduced into the system with the application of the urine and water amendments (the amendment vessels were not flushed with He/O_2), as N_2 is not

produced during nitrification (which we believe was the dominant soil process during this early part of the incubation). The second peak of N_2O and NO emissions may have resulted from partial denitrification of the NO_3^- produced, following the removal of O_2 by rapidly respiring micro-organisms. There is also support by the microbiology data for our assumption that denitrification occurred during the second peak of N_2O and NO, as there was an increase in *nirK* gene copies. The lack of change in *nosZ* agrees with the absence of reduction of N_2O to N_2. As a consequence, the N_2 emissions reported correspond to background values during the second peak of N_2O and NO emissions.

Soil grown with Bh is assumed to have lower cumulative N_2O, NO and N_2 emissions than that with Br due to the high BNI capacity in Bh (Gopalakrishnan et al., 2007; Subbarao et al., 2008). In this study, the cumulative N_2O in the Bh + U treatment during the first peak was significantly lower than that in the Br + U treatment, which may be due to the nitrification inhibition caused by the biological NIs released from the Bh as previous studies reported (Meena et al., 2014; Subbarao et al., 2007a, 2006a). In addition, N_2O emissions factors (EFs) from sheep urine in the soil grown with Bh and Br were 0.41% and 0.43%, respectively, which is consistent with reports from López-Aizpún et al. (2020) (with mean value of 0.39%, range from 0.04% to 1.80%). However, there was no significant difference in the cumulative N_2O and NO emissions during the whole soil incubation between the Bh + U treatment and Br + U treatment. The lack of effect later in the incubation corresponds to an absence of inhibition during denitrification, a fact that has been investigated before. A recent study by Simon et al. (2020) suggested that a possible effect of these *Brachiaria* grasses might be due to lower soil nitrate levels under these grasses, so the effect on denitrification is of an indirect nature due to a direct effect on nitrification. It is also possible that a reason for the short-lived effect of the Bh may have been the death of the grasses in the DENIS system (there were no lights present in the incubation vessels). The residual BNIs in the soil may inhibit the nitrification temporarily, but may not last for long enough after the death of the grasses.

Nitrification inhibitors, such as DCD and DMPP, have been confirmed to inhibit the AOA and/or AOB genes copies, which play an important role in controlling the nitrification rates and dominate at different conditions (Chen et al., 2014; Li et al., 2019; Shi et al., 2016). Nitrification inhibitors have also been shown to inhibit denitrifying microbes, *nirS* and/or *nirK* and/or *nosZ* and/ *narG* (Li et al.,

2019; Shi et al., 2017; Zhou et al., 2018). The biological NI, 1,9-decanediol (identified from rice), has also been shown to suppress the nitrification through impeding both AOA and AOB, when applied at high concentrations (≥500 mg kg^{-1}) (Lu et al., 2019). In this study, the controls, Bh and Br (plus water), did not influence the AOA, *nirS* and *nosZ* gene copies, but soil with Bh (with high BNI capacity) with sheep urine application significantly increased the AOB gene copies (responsible for the oxidation of NH_4^+) compared with Br (Fig. 5.2), which supports our suggestion that nitrification was a dominant soil process. This may be because biological NIs inhibit nitrification rates by reducing the cell-specific activity of AOA and/or AOB, rather than affecting ammonia oxidiser populations, as well as non-target soil microorganisms or functions (Kong et al., 2016). In addition, because of the need to retain air-tight seals throughout the incubation for the measurement of soil derived N_2 emissions, we were unable to collect soil samples during the incubation period. A greater number of time points to explore the dynamics of soil NH_4^+ and NO_3^-, as well as gene copies data during the incubation would have helped to explain the sources of gaseous N from soil grown with these two grasses, and nitrification inhibition mechanism of Bh.

To the best of our knowledge, this is the first time that NO and N_2 emissions have been measured alongside N_2O emissions from soil sown with Bh and Br. Even though there was no significant differences in the cumulative gaseous N_2O and NO emissions between the Bh and Br treatments over the entire experimental incubation period, measurements indicated nitrification inhibition during the initial peak of emission in the Bh + U treatment, suggesting a potential mitigation strategy for sheep grazed pastures in the future.

5.5. Conclusion

In this highly nitrifying soil, N_2O emissions dominated rather than the NO emissions, from the soil sown with Bh and Br after the sheep urine application. We suggest that nitrification generated the initial NO, N_2O and CO_2 peaks, based on the initial soil moisture content (65%WFPS) and CO_2 peaks. Afterwards it appears that incomplete denitrification may generate broad peaks for both NO and N_2O, as evidence by O_2 consumption by rapidly respiring micro-organisms and the increasing denitrifier

(*nirK* gene copies). Even though no significant differences were observed in the cumulative N_2O and NO emissions between the Bh + U and Br + U treatments over the entire 23-d incubation period, Bh inhibited N_2O emissions during the first peak compared with Br. This indicates that there is potential for future breeding programmes to introduce BNI traits into temperate grasses for ruminant grazing to reduce nitrification rates and mitigate N_2O emissions during nitrification process.

5.6. Acknowledgment

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5.7. References

Ball, D.F., 1964. Loss-on-ignition as an estimate of organic matter and organic carbon in non-calcareous soils. Journal of Soil Science 15, 84–92. doi:10.1111/j.1365-2389.1964.tb00247.x

Bateman, E.J., Baggs, E.M., 2005. Contributions of nitrification and denitrification to N2O emissions from soils at different water-filled pore space. Biology and Fertility of Soils 41, 379–388. doi:10.1007/s00374-005-0858-3

Byrnes, R.C., Nùñez, J., Arenas, L., Rao, I., Trujillo, C., Alvarez, C., Arango, J., Rasche, F., Chirinda, N., 2017. Biological nitrification inhibition by Brachiaria grasses mitigates soil nitrous oxide emissions from bovine urine patches. Soil Biology and Biochemistry 107, 156–163. doi:10.1016/j.soilbio.2016.12.029

Caranto, J.D., Lancaster, K.M., 2017. Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. Proceedings of the National Academy of Sciences of the United States of America 114, 8217–8222. doi:10.1073/pnas.1704504114

Cárdenas, L.M., Hawkins, J.M.B., Chadwick, D., Scholefield, D., 2003. Biogenic gas emissions from soils measured using a new automated laboratory incubation system. Soil Biology and Biochemistry 35, 867–870. doi:10.1016/S0038-0717(03)00092-0

Chadwick, D.R., Cardenas, L.M., Dhanoa, M.S., Donovan, N., Misselbrook, T., Williams, J.R., Thorman, R.E., McGeough, K.L., Watson, C.J., Bell, M., Anthony, S.G., Rees, R.M., 2018. The contribution of cattle urine and dung to nitrous oxide emissions: Quantification of country specific emission factors and implications for national inventories. Science of the Total Environment 635, 607–617. doi:10.1016/j.scitotenv.2018.04.152

Chen, Q., Qi, L., Bi, Q., Dai, P., Sun, D., Sun, C., Liu, W., Lu, L., Ni, W., Lin, X., 2014. Comparative effects of 3,4-dimethylpyrazole phosphate (DMPP) and dicyandiamide (DCD) on ammonia-oxidizing bacteria and archaea in a vegetable soil. Applied Microbiology and Biotechnology 99, 477–487. doi:10.1007/s00253-014-6026-7

de Cerqueira Luz, P.H., Romualdo, L.M., da Silva, N.R., Florindo, J.B., Bruno, O.M., de Fátima da

Silva, F., Herling, V.R., 2014. Brachiaria species identification using imaging techniques based on fractal descriptors. Computers and Electronics in Agriculture 103, 48–54. doi:10.1016/j.compag.2014.02.005

Fernandes, A.M., Andrade, G.J.M. de, Souza, E. de F.C. de, Rosolem, C.A., 2011. Brachiaria species affecting soil nitrification. Revista Brasileira de Ciência Do Solo 35, 1699–1706. doi:10.1590/s0100-06832011000500024

Firestone, M.K., Davidson, E.A., 1989. Microbiologial Basis of NO and N2O production and consumption in soil. Exchange of Trace Gases between Terrestrial Ecosystems and the Atmosphere 7–21. doi:10.1017/CBO9781107415324.004

Gopalakrishnan, S., Subbarao, G. V., Nakahara, K., Yoshihashi, T., Ito, O., Maeda, I., Ono, H., Yoshida, M., 2007. Nitrification inhibitors from the root tissues of Brachiaria humidicola, a tropical grass. Journal of Agricultural and Food Chemistry 55, 1385–1388. doi:10.1021/jf062593o

Gopalakrishnan, S., Watanabe, T., Pearse, S.J., Ito, O., Hossain, Z.A.K.M., Subbarao, G. V, 2009. Biological nitrification inhibition by Brachiaria humidicola roots varies with soil type and inhibits nitrifying bacteria, but not other major soil microorganisms. Soil Science and Plant Nutrition 55, 725–733. doi:10.1111/j.1747-0765.2009.00398.x

Huérfano, X., Fuertes-Mendizábal, T., Fernández-Diez, K., Estavillo, J.M., González-Murua, C., Menéndez, S., 2016. The new nitrification inhibitor 3,4-dimethylpyrazole succinic (DMPSA) as an alternative to DMPP for reducing N2O emissions from wheat crops under humid Mediterranean conditions. European Journal of Agronomy 80, 78–87. doi:10.1016/j.eja.2016.07.001

Ji, C., Li, S., Geng, Y., Yuan, Y., Zhi, J., Yu, K., Han, Z., Wu, S., Liu, S., Zou, J., 2020. Decreased N2O and NO emissions associated with stimulated denitrification following biochar amendment in subtropical tea plantations. Geoderma 365, 114223. doi:10.1016/j.geoderma.2020.114223

Kang, R., Yu, L., Dörsch, P., Mulder, J., 2020. Nitrification is the primary source for NO in N-saturated subtropical forest soils: Results from in situ 15N labeling. Rapid Communications in Mass Spectrometry 34, 1–7. doi:10.1002/rcm.8700

Knowles, R., 1982. Denitrification. Microbiological Reviews 46, 43–70. doi:10.1128/CMR.5.4.356.Updated

Kong, X., Duan, Y., Schramm, A., Eriksen, J., Petersen, S.O., 2016. 3,4-Dimethylpyrazole phosphate (DMPP) reduces activity of ammonia oxidizers without adverse effects on non-target soil microorganisms and functions. Applied Soil Ecology 105, 67–75. doi:10.1016/j.apsoil.2016.03.018

Lai, T. V., Farquharson, R., Denton, M.D., 2019. High soil temperatures alter the rates of nitrification, denitrification and associated N2O emissions. Journal of Soils and Sediments 19, 2176–2189. doi:10.1007/s11368-018-02238-7

Lee, S.-I., Lim, S.-S., Lee, K.-S., Kwak, J.-H., Jung, J.-W., Ro, H.-M., Choi, W.-J., 2011. Kinetic Responses of Soil Carbon Dioxide Emission to Increasing Urea Application Rate. Korean Journal of Environmental Agriculture 30, 99–104. doi:10.5338/kjea.2011.30.2.99

Li, J., Shi, Y., Luo, J., Li, Y., Wang, L., Lindsey, S., 2019. Effects of 3,4-dimethylpyrazole phosphate (DMPP) on the abundance of ammonia oxidizers and denitrifiers in two different intensive vegetable cultivation soils. Journal of Soils and Sediments 19, 1250–1259. doi:10.1007/s11368-018-2155-4

Lin, X., Hasi, W.L.J., Lou, X.T., Han, S.Q.G.W., Lin, D.Y., Lu, Z.W., 2015. Direct and quantitative detection of dicyandiamide (DCD) in milk using surface-enhanced Raman spectroscopy. Analytical Methods 7, 3869–3875. doi:10.1039/c5ay00313j

Loick, N., Dixon, E.R., Abalos, D., Vallejo, A., Matthews, G.P., J.McGeough, K., Well, R., Watson, C., L.Laughlin, R.J., Cardenas, L.M., 2016. Denitrification as a source of nitric oxide emissions from incubated soil cores from a UK grassland soil. Soil Biology and Biochemistry 95, 1–7.

doi:10.1016/j.soilbio.2015.12.009

López-Aizpún, M., Horrocks, C.A., Charteris, A.F., Marsden, K.A., Ciganda, V.S., Evans, J.R., Chadwick, D.R., Cárdenas, L.M., 2020. Meta-analysis of global livestock urine-derived nitrous oxide emissions from agricultural soils. Global Change Biology 26, 2002–2013. doi:10.1111/gcb.15012

Lu, Y., Zhang, X., Jiang, J., Kronzucker, H.J., Shen, W., Shi, W., 2019. Effects of the biological nitrification inhibitor 1,9-decanediol on nitrification and ammonia oxidizers in three agricultural soils. Soil Biology and Biochemistry 129, 48–59. doi:10.1016/j.soilbio.2018.11.008

Marsden, K.A., Jones, D.L., Chadwick, D.R., 2016. The urine patch diffusional area: An important N2O source? Soil Biology and Biochemistry 92, 161–170. doi:10.1016/j.soilbio.2015.10.011

Meena, H.M., Sachdev, M.S., Manjaiah, K.M., Dotaniya, M.L., 2014. Nitrification Inhibition Potential of Brachiaria humidicola. National Academy Science Letters 37, 113–116. doi:10.1007/s40009-013-0216-1

Meijide, A., Cardenas, L.M., Bol, R., Bergstermann, A., Goulding, K., Well, R., Vallejo, A., Scholefield, D., 2010. Dual isotope and isotopomer measurements for the understanding of N2O production and consumption during denitrification in an arable soil. European Journal of Soil Science 61, 364–374. doi:10.1111/j.1365-2389.2010.01233.x

Miller, M.N., Zebarth, B.J., Dandie, C.E., Burton, D.L., Goyer, C., Trevors, J.T., 2008. Crop residue influence on denitrification, N2O emissions and denitrifier community abundance in soil. Soil Biology and Biochemistry 40, 2553–2562. doi:10.1016/j.soilbio.2008.06.024

Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A Rapid, Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite. Nitric Oxide 5, 62–71. doi:10.1006/NIOX.2000.0319

Mulvaney, R.L., 1996. Nitrogen—Inorganic Forms, in: Methods of Soil Analysis Part 3—Chemical Methods. Soil Science Society of America, American Society of Agronomy, pp. 1123–1184. doi:10.2136/sssabookser5.3.c38

Nuñez, J., Arevalo, A., Karwat, H., Egenolf, K., Miles, J., Chirinda, N., Cadisch, G., Rasche, F., Rao, I., Subbarao, G., Arango, J., 2018. Biological nitrification inhibition activity in a soil-grown biparental population of the forage grass, Brachiaria humidicola. Plant and Soil 426, 401–411. doi:10.1007/s11104-018-3626-5

O'Neill, R.M., Girkin, N.T., Krol, D.J., Wall, D.P., Brennan, F.P., Lanigan, G.J., Renou-Wilson, F., Müller, C., Richards, K.G., 2020. The effect of carbon availability on N2O emissions is moderated by soil phosphorus. Soil Biology and Biochemistry 142, 107726. doi:10.1016/j.soilbio.2020.107726

Robinson, A., Di, H.J., Cameron, K.C., Podolyan, A., He, J., 2014. The effect of soil pH and dicyandiamide (DCD) on N2O emissions and ammonia oxidiser abundance in a stimulated grazed pasture soil. Journal of Soils and Sediments 14, 1434–1444. doi:10.1007/s11368-014-0888-2

Senbayram, M., Budai, A., Bol, R., Chadwick, D., Marton, L., Gündogan, R., Wu, D., 2019. Soil NO 3− level and O 2 availability are key factors in controlling N 2 O reduction to N 2 following long-term liming of an acidic sandy soil. Soil Biology and Biochemistry. doi:10.1016/j.soilbio.2019.02.009

Shi, X., Hu, H., He, J., Chen, D., Suter, H.C., 2016. Effects of 3,4-dimethylpyrazole phosphate (DMPP) on nitrification and the abundance and community composition of soil ammonia oxidizers in three land uses. Biology and Fertility of Soils 52, 927–939. doi:10.1007/s00374-016-1131-7

Shi, X., Hu, H.W., Kelly, K., Chen, D., He, J.Z., Suter, H., 2017. Response of ammonia oxidizers and denitrifiers to repeated applications of a nitrification inhibitor and a urease inhibitor in two pasture soils. Journal of Soils and Sediments 17, 974–984. doi:10.1007/s11368-016-1588-x

Simon, P.L., Dieckow, J., Zanatta, J.A., Ramalho, B., Ribeiro, R.H., van der Weerden, T., de Klein,

C.A.M., 2020. Does Brachiaria humidicola and dicyandiamide reduce nitrous oxide and ammonia emissions from cattle urine patches in the subtropics? Science of the Total Environment 720, 137692. doi:10.1016/j.scitotenv.2020.137692

Study, C., Lankan, S., Powder, M., 2014. DCD , a Potential Shield to Uplift Local Milk II, 20–25.

Subbarao, G. V., Ishikawa, T., Ito, O., Nakahara, K., Wang, H.Y., Berry, W.L., 2006. A bioluminescence assay to detect nitrification inhibitors released from plant roots: A case study with Brachiaria humidicola. Plant and Soil. doi:10.1007/s11104-006-9094-3

Subbarao, G. V., Nakahara, K., Hurtado, M.P., Ono, H., Moreta, D.E., Salcedo, A.F., Yoshihashi, A.T., Ishikawa, T., Ishitani, M., Ohnishi-Kameyama, M., Yoshida, M., Rondon, M., Rao, I.M., Lascano, C.E., Berry, W.L., Ito, O., 2009. Evidence for biological nitrification inhibition in Brachiaria pastures. Proceedings of the National Academy of Sciences 106, 17302–17307. doi:10.1073/pnas.0903694106

Subbarao, G. V., Rondon, M., Ito, O., Ishikawa, T., Rao, I.M., Nakahara, K., Lascano, C., Berry, W.L., 2007a. Biological nitrification inhibition (BNI) - Is it a widespread phenomenon? Plant and Soil. doi:10.1007/s11104-006-9159-3

Subbarao, G. V., Wang, H.Y., Ito, O., Nakahara, K., Berry, W.L., 2007b. NH4+ triggers the synthesis and release of biological nitrification inhibition compounds in Brachiaria humidicola roots. Plant and Soil 290, 245–257. doi:10.1007/s11104-006-9156-6

Subbarao, G. V, Nakahara, K., Ishikawa, T., 2008. Free fatty acids from the pasture grass Brachiaria humidicola and one of their methyl esters as inhibitors of nitrification 89–99. doi:10.1007/s11104-008-9682-5

Subbarao, G. V, Nakahara, K., Ishikawa, T., Ono, H., Yoshida, M., Yoshihashi, T., Zhu, Y., Zakir, H.A.K.M., Deshpande, S.P., Hash, C.T., Sahrawat, K.L., 2013. Biological nitrification inhibition (BNI) activity in sorghum and its characterization. Plant Soil 366, 243–259. doi:10.1007/s11104-012-1419-9

Sun, L., Lu, Y., Yu, F., Kronzucker, H.J., Shi, W., 2016. Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency. New Phytologist 212, 646–656. doi:10.1111/nph.14057

Wang, Y., Fang, H., Zhou, D., Han, H., Chen, J., 2016. Characterization of nitrous oxide and nitric oxide emissions from a full-scale biological aerated filter for secondary nitrification. Chemical Engineering Journal 299, 304–313. doi:10.1016/j.cej.2016.04.050

Weiske, A., Benckiser, G., Herbert, T., Ottow, J.C.G., 2001. Influence of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) in comparison to dicyandiamide (DCD) on nitrous oxide emissions, carbon dioxide fluxes and methane oxidation during 3 years of repeated application in field experiments. Biology and Fertility of Soils 34, 109–117. doi:10.1007/s003740100386

Welten, B.G., Ledgard, S.F., Balvert, S.F., Kear, M.J., Dexter, M.M., 2016. Effects of oral administration of dicyandiamide to lactating dairy cows on residues in milk and the efficacy of delivery via a supplementary feed source. Agriculture, Ecosystems and Environment 217, 111–118. doi:10.1016/j.agee.2015.10.013

Wrage, N., Van Groenigen, J.W., Oenema, O., Baggs, E.M., 2005. A novel dual-isotope labelling method for distinguishing between soil sources of N2O. Rapid Communications in Mass Spectrometry 19, 3298–3306. doi:10.1002/rcm.2191

Wu, D., Cárdenas, L.M., Calvet, S., Brüggemann, N., Loick, N., Liu, S., Bol, R., 2017. The effect of nitrification inhibitor on N2O, NO and N2emissions under different soil moisture levels in a permanent grassland soil. Soil Biology and Biochemistry 113, 153–160. doi:10.1016/j.soilbio.2017.06.007

Zhou, Z.F., Zhang, Z.Y., Wang, M.X., Liu, Y.M., Dai, J.S., 2018. Effect of the nitrification inhibitor (3, 4-dimethylpyrazole phosphate) on the activities and abundances of ammonia-oxidizers and

denitrifiers in a phenanthrene polluted and waterlogged soil. Ecotoxicology and Environmental Safety 161, 474–481. doi:10.1016/j.ecoenv.2018.06.030

Zhu, X., Burger, M., Doane, T.A., Horwath, W.R., 2013. Ammonia oxidation pathways and nitrifier denitrification are significant sources of N2O and NO under low oxygen availability. Proceedings of the National Academy of Sciences of the United States of America 110, 6328–6333. doi:10.1073/pnas.1219993110

Zulkarnaen, N., Zhang, Y., Zhang, P., Liu, Y., Cheng, Y., Zhao, J., Zhang, J., 2019. Abundance of AOA, AOB, nir S, nir K, and nos Z in red soil of China under different land use . IOP Conference Series: Earth and Environmental Science 393, 012007. doi:10.1088/1755-1315/393/1/012007

Chapter 6: Labile carbon sources stimulate soil nitrous oxide emissions during denitrification

Yan Ma[1,*], Dave R. Chadwick[1,2], Davey L. Jones[1,3], David Schofield[4], Jane Hawkins[4], Jerry C. Dlamini[4]，Laura M. Cardenas[4]

[1] *School of Natural Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW, UK*

[2] *Interdisciplinary Research Centre for Agriculture Green Development in Yangtze River Basin, Southwest University, Chongqing, China*

[3] *SoilsWest, UWA School of Agriculture and Environment, The University of Western Australia, Perth, WA 6009, Australia*

[4] *Sustainable Agriculture Sciences Department, North Wyke, Okehampton, Devon, EX20 2SB, UK*

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Note: The experiments presented in this Chapter were performed in 2001-2002 by research staff at North Wyke. The data from these experiments have not been processed or published previously. Because of the relevance of the relationship between lability of C compounds and subsequent denitrification fluxes (as N_2O and N_2) to my previous PhD Chapters, I was provided with the raw data by my co-supervisor, Dr Laura Cardenas. I am responsible for calculation of the fluxes from the raw data, subsequent processing of these data, statistical analyses and interpretation of the results presented in this Chapter.

Abstract

Animal manures are important sources of nutrients for crop production, but they are also associated with emissions of nitrogenous gases, e.g. nitrous oxide (N_2O), nitric oxide (NO) and dinitrogen (N_2) after application to the soil. The relative proportions of these emitted gases depend on the dominance of nitrification and denitrification in the soil, with denitrification rates being controlled by the lability of carbon (C) substrates. Six incubations were conducted in a He/O_2 flow denitrification system (DENIS) by researchers at Rothamsted Research (North Wyke) in 2001-2002, to determine the effects of different C compounds, identified from fresh and aged cattle slurry, on soil N_2O and N_2 during the denitrification process (in the presence of nitrate (NO_3^-)). The treatments in this study were as follow: 1) NO_3^-; 2) NO_3^- + glucose; 3) NO_3^- + C source (vanillin, cellulose, glucosamine, butyric acid, fresh cattle slurry and aged cattle slurry; 4) NO_3^- + NH_4 (to match the NH_4^+ content of the slurry). I obtained the raw GC results from each incubation and each treatment and calculated the gas fluxes. Results show that the reactivity order of individual C sources on denitrification can be established as: glucose > glucosamine > butyric acid > vanillin > aged slurry > cellulose > fresh slurry, indicated by the higher total N_2O-N + N_2-N emissions. In addition, the N_2O/N_2 ratio was significantly higher in the NO_3^- + NH_4^+ treatments in the Inc-FSl and Inc-ASl, also with in the glucosamine treatment, due to the additional N application. We conclude that labile C compounds inputs may stimulate N_2O production and emission during denitrification, and may result in higher N_2O/N_2 ratio.

Keywords: carbon quality, nitrous oxide, dinitrogen, cattle slurry, denitrification

6.1. Introduction

Nitrous oxide (N_2O) emissions, one of the greenhouse gases (GHG) with a global warming potential 310 times greater than that of carbon dioxide (CO_2) on a 100-year time horizon (UNFCCC, 2020), have been reported from multiple sectors, including industry, energy, agriculture, waste water, land use, land-use change and forestry (Skiba et al., 2012). The agricultural sector is the largest anthropogenic source of N_2O emissions in the UK (75%), and these losses are associated with direct

emissions from soil following nitrogen (N) fertiliser and manure applications, and indirect emissions from nitrate (NO_3^-) leaching (de Bastos et al., 2020; López-Aizpún et al., 2020; Trolove et al., 2019) and N deposition (Britton et al., 2019). N_2O is generated from both the nitrification and denitrification processes (He et al., 2020) which are favoured in different soil conditions, such as soil moisture, soil texture, pH, nutrient availability and form (Robinson et al., 2014; Wu et al., 2017; Xu et al., 2018). Nitrification is a microbially mediated process, in which ammonium (NH_4^+) is firstly oxidised to nitrite (NO_2^-) and further oxidised to NO_3^- (Firestone and Davidson, 1989). Subsequent denitrification refers to the dissimilatory reduction of one or both of the ionic nitrogen oxides (NO_3^- and NO_2^-) to the gaseous oxides, nitric oxide (NO) and N_2O, which may be further reduced to dinitrogen (N_2) (Knowles, 1982).

Growing populations, economies and individual incomes, as well as other demographic factors such as urbanization contribute to the increasing livestock food demand (Bai et al., 2018; Enahoro et al., 2018) and subsequent manure generation, e.g. in China (Chadwick et al., 2015, 2020). The total annual production of livestock manures in the UK was estimated to *ca*. 95.1 million tonnes in 2018, with 84% arising from the housing of cattle (farmyard manure and slurry) (Defra, 2019), compared with 83.4 million tonnes in 2010 (with 80% arising from cattle) (Smith and Williams, 2016). In cattle manures, protein contributes to no more than 20% of the dry matter content; in contrast, the fibre content (cellulose, hemicellulose and lignin) is the highest, accounting for more than half of the dry matter (Chen et al., 2003). Yamamoto et al. (2008) extracted vanillin, protocatechuic acid, vanillic acid and syringic acid from livestock excreta. Volatile fatty acids have also been measured in animal slurries, among which acetic acid is predominant, followed by propionic, n-butyric, iso-butyric, iso-valeric and n-valeric (Bastami et al., 2018; Cooper and Cornforth, 1978).

Animal manures are valuable sources of macro- and micronutrients for crop production (ADHB, 2019; Moral et al., 2009). In 2018, 68% of farms used organic manures on at least one field on the farm, among which cattle manure from beef and dairy farms (cattle farmyard manure, 51%; cattle slurry, 17%) represented the largest volume of manure type generated in the UK (Defra, 2019). However, manure, and especially liquid slurry can be detrimental to the environment as they are important sources of ammonia (NH_3) (Bourdin et al., 2014; Ramanantenasoa et al., 2019; Sommer et al., 2019), GHG and N_2 emissions (Chadwick et al., 2011; Rodhe et al., 2015; Sokolov et al., 2019) during their storage or

following application to the field. They are also sources of point and diffuse pollution of water courses (Trolove et al., 2019; Zanon et al., 2020).

Research has shown that amendments of labile carbon (C) compounds (e.g. glucose, sucrose, glycerol and mannitol, ethanol and acetate) (Adouani et al., 2010; Murray et al., 2004; Senbayram et al., 2012) and plant residues (such as straw, alfalfa) (Beauchamp, 1985; Senbayram et al., 2012) affect denitrification rates and/or denitrifying microorganism (Henderson et al., 2010; Schipper et al., 2011). Less is known about the effects of specific labile organic compounds identified from cattle slurry on soil denitrification rates, e.g. glucosamine, vanillin, cellulose, butyric acid. Furthermore, as slurry ages (e.g. during storage) the organic matter content, including these C compounds, biodegrades, resulting in a modified C composition and the loss of NH_3 and GHG emissions (Moset et al., 2012; Rodhe et al., 2009).

In Chapter 4, I highlighted that at high rates of biological nitrification inhibitor (NI) application, e.g. >635 mg kg^{-1} dry soil, the apparent 'inhibitory' effect observed (via measurements of soil NO_3^- concentration) may be the result of the application of available C affecting two key soil N processes: i) this additional available C may have stimulated microbial immobilisation of NH_4^+ and/or NO_3^-, and ii) the additional available C promoted denitrification. Both processes would have resulted in a reduction in the pool of NO_3^- in the soil. Therefore, in this Chapter I explore the effects of a series of C compounds with a range of lability on denitrification products.

Specifically, the aims of this study were to determine the effects of different C compounds (previously identified in animal slurry), cattle slurry (fresh and aged) and NH_4^+ application on soil N_2O and N_2 emissions during the denitrification process. We hypothesised that, 1) the addition of easily decomposed C compounds would increase the N_2O and N_2 emissions from soil via denitrification; 2) soils receiving cattle slurry may result in relatively lower N_2O and N_2 emissions compared with soil with easily decomposed C compounds; 3) N_2 from soils receiving slurry may be the predominant gas due to complete anaerobic denitrification.

6.2. Materials and methods

I was provided with the raw data of six incubations by my co-supervisor, Dr Laura Cardenas, which included daily N_2O and N_2 emissions from each chamber, soil NH_4^+ and NO_3^- concentrations in the unit of mg N L^{-1}. The details of the six incubations are presented in the appendix 4. I was responsible for the 1) calculation of the average daily N_2O and N_2 emissions from each treatment, cumulative N_2O and N_2 emissions and soil NH_4^+ and NO_3^- concentrations in the unit of mg N kg^{-1} dry soil, 2) subsequent processing of these data, 3) statistical analyses, and 4) interpretation of the results presented in this Chapter. Gas concentrations were corrected for surface area and the flow rate through each vessel (measured daily by means of glass bubble meter), and fluxes calculated in the units of kg N or C ha^{-1} d^{-1}. The cumulative gas flux was calculated using Genstat (the 19th edition, VSNI, UK) using the Trapezoidal rule (Meijide et al., 2010).

6.2.1. Statistical analysis

The effect of C source treatments (C compounds and cattle slurries) on soil cumulative N_2O and N_2 emissions, NH_4^+ and NO_3^- concentrations in each incubation was carried out by one-way analysis of variance (ANOVA) followed by the LSD test at 5% confidence. One-way ANOVA was carried out to compare the initial soil NH_4^+ and NO_3^- concentrations from each incubation, respectively. One-way ANOVA was also performed to compare the effects of NO_3^-/NO_3^- + glucose/NO_3^- + C sources on soil cumulative N_2O and N_2, soil NH_4^+ and NO_3^- concentration from different incubations after the incubation. All the statistical analysis was performed in the SPSS Statistics 25.0 (IBM Inc., Armonk, NY).

6.3. Results

6.3.1. N_2O and N_2 emissions

Figure 6.1 shows the N_2O and N_2 emissions in the NO_3^- only (Fig. 6.1a, d), NO_3^- + glucose (Fig. 6.1b, e) and NO_3^- + C source/NH_4^+ treatments (Fig. 6.1c, f) during the incubations. The N_2O emissions

increased rapidly after amendments in all cases, but after a few days decreased to background levels. In the case of the NO_3^- only treatment this took about 4 days, whilst in the NO_3^- + glucose treatment it took 2 days. In the NO_3^- + C source/NH_4^+ treatments, N_2O emissions were more variable with levels reaching background between 3-10 days. The maximum N_2O fluxes in the NO_3^- only treatment ranged from 2.2-4.8 kg N ha^{-1} d^{-1}. The maximum fluxes of N_2O in the NO_3^- + glucose, NO_3^- + glucosamine, NO_3^- + butyric acid, NO_3^- + vanillin treatments were higher than that in the NO_3^- only treatment, with maximum fluxes of 18.3 (ranging from 14.0-24.2 kg N ha^{-1} d^{-1}), 19.6, 9.1 and 7.2 kg N ha^{-1} d^{-1}, respectively. The N_2O emissions in the NO_3^- + cellulose treatments, the NO_3^- + fresh and aged slurry treatments were similar to the NO_3^- only treatment. The maximum N_2O emissions for all the treatments occurred between d 0 and 2.

The N_2 emissions showed a similar trend to the N_2O emissions during the incubation, but levels reached background levels much later than for N_2O, at about 8 days after the application of the treatments. The appearance of the N_2 peak was slightly later than that of the N_2O in all treatments, on day 3 for NO_3^- only treatment, day 2 for the other treatments. The maximum values of N_2 fluxes in the NO_3^- + glucose in the Inc-Van, Inc-Cel, Inc-Glu and Inc-But were frequently higher than that in the NO_3^- only treatment from each incubation. Except for the maximum values of N_2 fluxes in the NO_3^- + glucosamine and NO_3^- + butyric acid treatment which were higher than that in the NO_3^- only treatment, the maximum fluxes of N_2 in other NO_3^- + C sources and NH_4^+ treatments were similar to the NO_3^- only treatment.

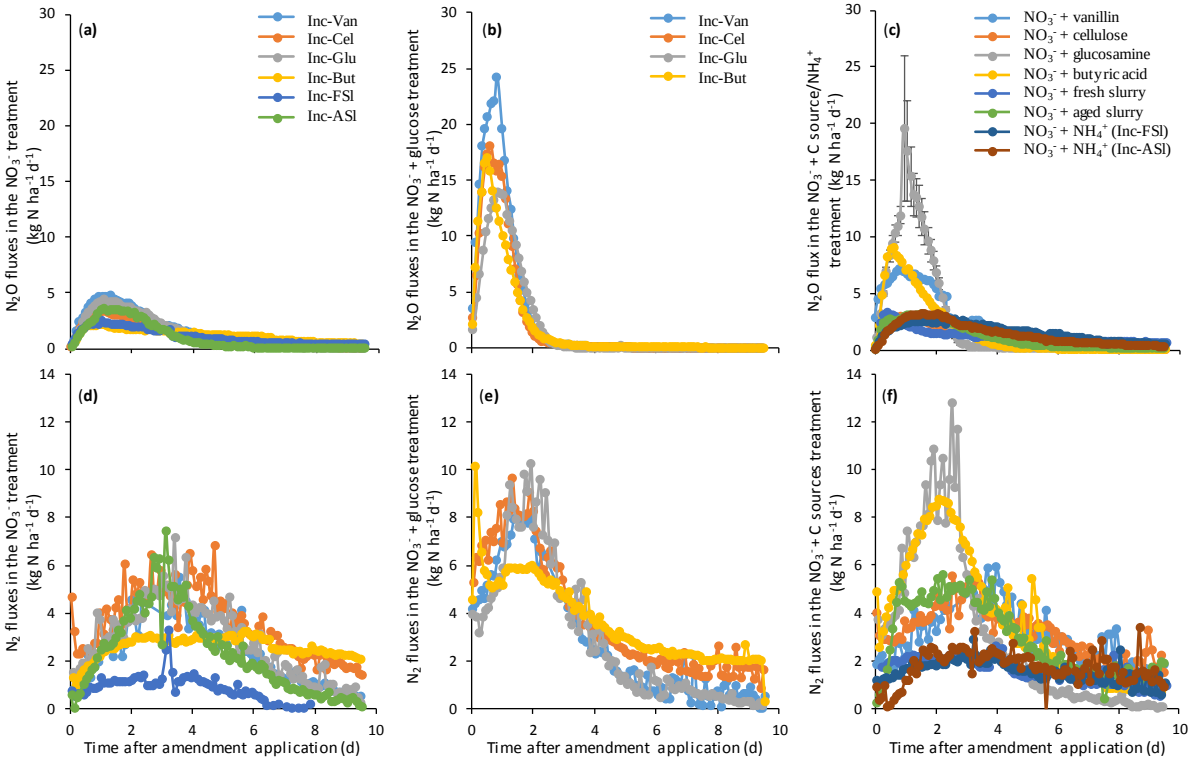

Fig. 6.1 N_2O and N_2 emissions in the NO_3^- only (panels a, d), NO_3^- + glucose (panels b, e) and NO_3^- + NH_4^+ or C sources (vanillin, cellulose, glucosamine, butyric acid, fresh slurry and aged slurry) (panels c, f) treatments during the incubation. Error bars are omitted for clarity of presentation, except the error bar of N_2O emissions in the NO_3^- + glucosamine treatment to show the size. Note the different y-axis scales for the N_2O and N_2 graphs.

6.3.2. Cumulative N_2O emissions

The cumulative N_2O fluxes in the NO_3^- only, NO_3^- + glucose, and NO_3^- + NH_4^+ or C sources treatments are shown in Fig. 6.2a and Table 6.1. In the Inc-Van, Inc-Cel, Inc-Glu and Inc-But, the glucose application significantly increased the cumulative N_2O compared with the NO_3^- only treatment. There was no significant difference between the NO_3^- + vanillin treatment and the NO_3^- only treatment in the Inc-Van, and also between the NO_3^- + cellulose treatment and the NO_3^- treatment in the Inc-Cel. The cumulative N_2O in the NO_3^- + glucosamine treatment was significantly higher than that in the NO_3^- + glucose treatment in the Inc-Glu. The NO_3^- + butyric acid resulted in a cumulative N_2O flux of 16.2 kg N ha^{-1} in the Inc-But, which was significantly greater than that in the NO_3^- treatment, however, no significant differences were observed between the NO_3^- + butyric acid and NO_3^- + glucose treatment.

In the Inc-FSl, the cumulative N_2O flux in the NO_3^- + NH_4^+ treatment (15.1 kg N ha^{-1}) was significantly higher than that in the NO_3^- only and NO_3^- + fresh slurry treatments. Nevertheless, there was no significant differences in the cumulative N_2O flux between the NO_3^-, NO_3^- + NH_4^+ and NO_3^- + aged slurry treatments in the Inc-ASl.

Results showed that there were no significant differences in the cumulative N_2O emission between the NO_3^- only treatments in the Inc-Van, Inc-Cel, Inc-Glu, Inc-But, Inc-FSl and Inc-ASl, with the average cumulative N_2O flux of 11.6 kg N ha^{-1} (ranging from 9.6-15.0 kg N ha^{-1}). In addition, there was no significant difference in the cumulative N_2O between the NO_3^- + glucose treatments in the Inc-Van, Inc-Cel, Inc-Glu and Inc-But, ranging from 19.1-27.3 kg N ha^{-1}. Thus, we were able to explore the effects of different C sources on N_2O and N_2 emissions, using data from the NO_3^- + C sources treatments in the Inc-Van, Inc-Cel, Inc-Glu, Inc-But, Inc-FSl and Inc-ASl. The cumulative N_2O in the NO_3^- + glucosamine treatment was significantly higher than other NO_3^- + C sources treatments, except for the NO_3^- + vanillin treatment. There were no significant differences in the cumulative N_2O between the NO_3^- + vanillin and NO_3^- + butyric acid. In addition, no significant differences were observed in the cumulative N_2O between the NO_3^- + butyric acid, NO_3^- + cellulose, NO_3^- + fresh slurry and NO_3^- + aged slurry treatments.

6.3.3. Cumulative N_2 emissions

Figure 6.2b presents the cumulative N_2 in the treatments from the six incubations. In the Inc-Van, Inc-Cel, Inc-But and Inc-FSl, there were no significant differences in the cumulative N_2 between the NO_3^-, NO_3^- + glucose and NO_3^- + C source treatments (Table 6.1). The glucose application significantly increased the cumulative N_2 emissions compared with the NO_3^- only and NO_3^- + glucosamine treatments in the Inc-Glu. In the Inc-ASl, the cumulative N_2 emission from the NO_3^- + aged slurry treatment was much higher than that in the NO_3^- + NH_4^+ treatment, however, the aged slurry or the NH_4^+ application did not affect the cumulative N_2 emissions compared with the NO_3^- only treatment.

The average cumulative N_2 flux in all the NO_3^- only treatments was 23.3 kg N ha^{-1}, ranging from 15.0-30.9 kg N ha^{-1}. The NO_3^- + glucose treatments in the Inc-Van, Inc-Cel, Inc-Glu and Inc-But gave

averages of 33.4 kg N-N_2 ha^{-1} (ranging from 27.7-37.0 kg N ha^{-1}). The effect of NO_3^- + C sources on cumulative N_2 emissions was compared between all the incubations. The results showed that only the cumulative N_2 emission from the NO_3^- + butyric acid treatment (34.9 kg N ha^{-1}) was significantly higher than that in the NO_3^- + fresh slurry treatment (15.7 kg N ha^{-1}). No significant differences were observed in cumulative N_2 emission between the NO_3^- + butyric acid treatment and NO_3^- + vanillin/cellulose/glucosamine/aged slurry treatments, or between the NO_3^- + fresh slurry treatment and NO_3^- + vanillin/cellulose/glucosamine/aged slurry treatments.

Table 6.1 Cumulative N_2O and N_2 emissions in the NO_3^-, NO_3^- + glucose, NO_3^- + C sources (vanillin, cellulose, glucosamine, butyric acid, fresh slurry, aged slurry) treatments after the incubation, in kg N ha^{-1}. Lowercases indicate the significant differences between the NO_3^-, NO_3^- + glucose/NH_4^+ and NO_3^- + C source treatments at $P<0.05$ by LSD in the Inc-Van, Inc-Cel, Inc-Glu, Inc-But, Inc-FSl and Inc-ASl, respectively. Capital letters indicate the significant differences between the NO_3^-/NO_3^- + glucose/ NO_3^- + C source treatments from different incubations (n=4).

Incubation / Treatment	Inc-Van	Inc-Cel	Inc-Glu	Inc-But	Inc-FSl	Inc-ASl
			N_2O			
NO_3^-	15.0±1.9b^{A}	10.7±3.1b^{A}	13.2±2.0c^{A}	10.7±0.7b^{A}	10.5±1.3b^{A}	9.6±0.9a^{A}
NO_3^- + glucose or NH_4^+	27.3±2.0a^{A}	21.0±2.5a^{A}	19.3±1.5b^{A}	19.1±4.8a^{A}	15.1±1.0a	13.7±3.8a
NO_3^- + C	21.0±3.3ab^{AB}	9.5±1.3b^{C}	25.2±1.5a^{A}	16.2±2.7a^{BC}	11.4±1.2b^{C}	11.3±1.6a^{C}
			N_2			
NO_3^-	22.3±3.1a^{BC}	30.9±3.2a^{A}	26.3±0.9b^{AB}	23.4±2.9a^{AB}	15.0±0.6a^{C}	22.0±2.4ab^{BC}
NO_3^- + glucose or NH_4^+	27.7±1.5a^{A}	37.0±4.1a^{A}	34.1±0.6a^{A}	34.6±3.4a^{A}	14.3±1.8a	16.3±3.5b
NO_3^- + C	28.4±3.5a^{AB}	29.6±7.1a^{AB}	28.2±2.4b^{AB}	34.9±6.1a^{A}	15.7±1.8a^{B}	27.7±2.8a^{AB}
			$N_2O + N_2$			
NO_3^-	37.3±3.6c^{AB}	41.6±4.1b^{A}	39.5±2.1b^{AB}	34.1±2.4b^{ABC}	25.5±1.3a^{C}	31.6±2.4a^{BC}
NO_3^- + glucose or NH_4^+	55.0±1.7a^{AB}	58.0±1.8a^{A}	53.4±1.5a^{AB}	52.9±0.3a^{B}	29.4±2.5a	30.0±6.3a
NO_3^- + C	49.4±5.7ab^{A}	39.1±7.0b^{BC}	53.4±3.7a^{A}	51.1±5.4a^{A}	27.1±1.7a^{C}	39.0±3.2a^{BC}

6.3.4. Cumulative total N_2O-N + N_2-N emissions

The NO_3^- + glucose treatment had significantly greater cumulative total N_2O-N + N_2-N emissions compared with the NO_3^- only treatment in the Inc-Van, Inc-Cel, Inc-Glu and Inc-But (Table 6.1). The cumulative total N_2O-N + N_2-N emissions in the NO_3^- + vanillin/glucosamine/butyric acid (49.4/53.4/51.2 kg N ha^{-1}) treatments were significantly higher than the NO_3^- only treatment in the Inc-

Van, Inc-Glu and Inc-But, however there were no significant differences between the NO_3^- only and NO_3^- + cellulose treatment (39.2 kg N ha^{-1}) in the Inc-Cel. In addition, no significant differences were observed in the cumulative N_2O-N + N_2-N between the NO_3^-, NO_3^- + NH_4^+ and NO_3^- + cattle slurry treatment in the Inc-FSl and Inc-ASl. The cumulative N_2O-N + N_2-N in the NO_3^- + C sources treatments were ranked as: NO_3^- + vanillin/glucosamine/butyric acid > NO_3^- + cellulose/ aged slurry/fresh slurry.

6.3.5. N_2O/N_2

Table 6.2 presents the cumulative N_2O-N, N_2-N and total N_2O-N + N_2-N as a percentage of N applied, and the N_2O/N_2 ratio in all incubations. The NO_3^- + glucosamine, NO_3^- + butyric acid, NO_3^- + glucose treatments showed the highest percentage of N_2O-N, N_2-N and N_2O-N + N_2-N, reaching 34.0%, 46.5% and 73.4%, respectively. When taking the N supplied by the glucosamine into account, the highest percentage of N_2O-N to N applied was observed in the NO_3^- + glucose treatment. The proportions of N_2 and total N_2O-N + N_2-N to N applied in the Inc-FSl and Inc-ASl were relatively lower compared with that in the Inc-Van, Inc-Cel, Inc-Glu and Inc-But. The N_2O/N_2 ratio in the NO_3^- + NH_4^+ (Inc-FSl and Inc-ASl) and NO_3^- + glucosamine treatments were relatively high compared to other treatments, being 1.66, 1.32 and 1.40, respectively. The lowest N_2O/N_2 ratio was observed in the NO_3^- + cellulose treatment, with the ratio of 0.50.

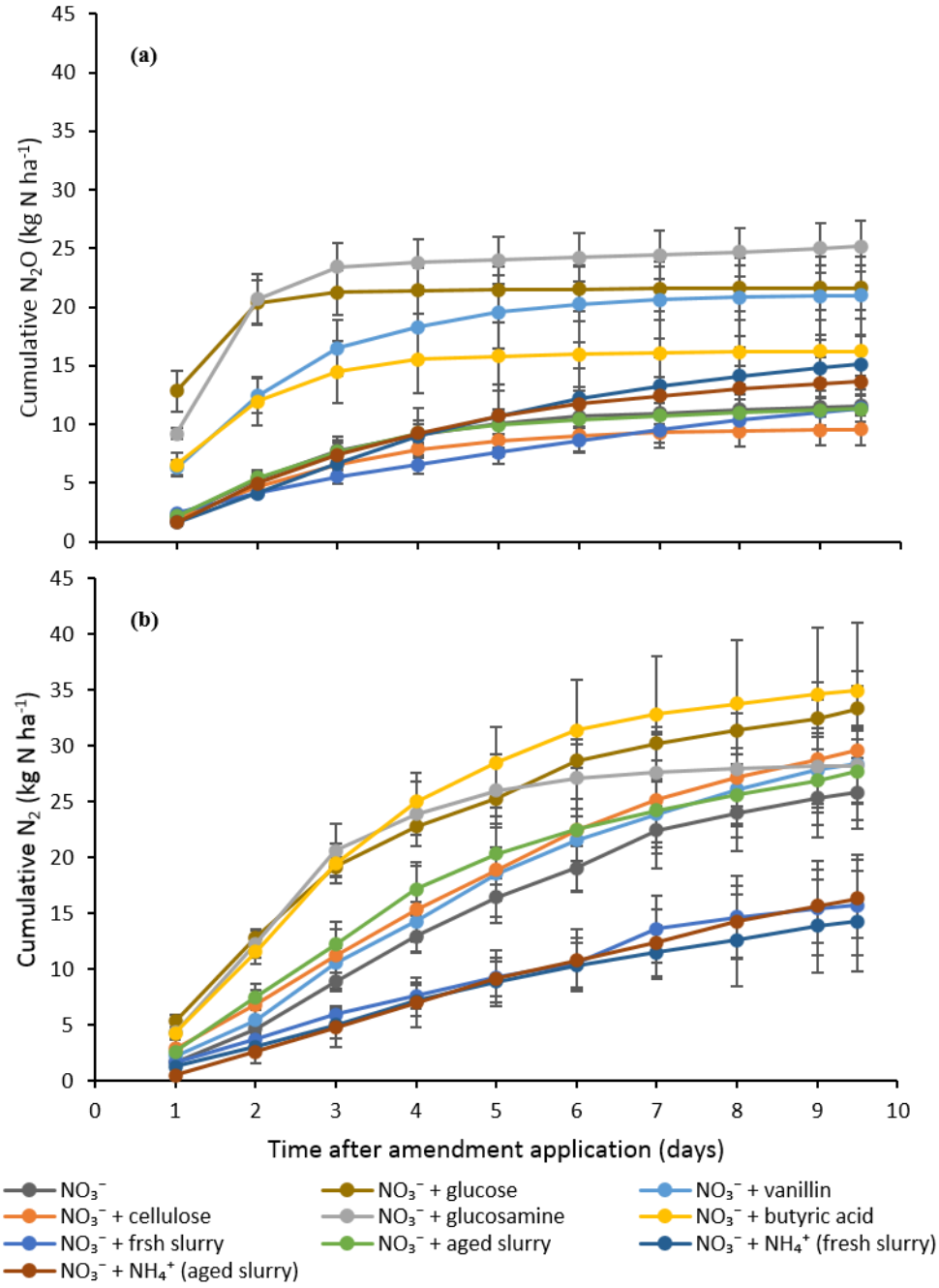

Fig. 6.2 Cumulative N_2O (panel a) and N_2 (panel b) fluxes after the application of NO_3^-, NO_3^- + glucose, NO_3^- + NH_4^+ or C sources (vanillin, cellulose, glucosamine, butyric acid, fresh slurry and aged slurry). Error bars represent the standard error of the mean (n=4).

Table 6.2 Total fluxes of N_2O and N_2 in kg N ha^{-1}.

Treatment	Total emitted N_2O-N	Total N_2O-N emitted / N applied (%)	Total N_2-N emitted	Total N_2-N emitted / N applied (%)	Total emitted N_2O-N+N_2-N	Total N emitted /N applied (%)	N_2O /N_2*
NO_3^-	11.6±0.8	15.5	23.3±2.2	31.1	34.9±2.4	46.6	0.78
NO_3^- + glucose	21.7±1.9	28.9	33.4±2.0	44.5	55.1±1.1	73.4	1.02
NO_3^- + vanillin	21.0±3.3	28.0	28.4±3.5	37.9	49.4±5.7	65.9	1.16
NO_3^- + cellulose	9.5±1.3	12.7	29.6±7.1	39.5	39.1±7.0	52.2	0.50
NO_3^- + glucosamine	25.2±1.5	34.0/16.3*	28.2±2.4	37.6/18.2*	53.4±3.7	71.6/34.5*	1.40
NO_3^- + butyric acid	16.2±2.7	21.6	34.9±6.1	46.5	51.1±5.4	68.1	0.73
NO_3^- + fresh slurry	11.4±1.2	11.2	15.7±1.8	15.4	27.1±1.7	26.6	1.14
NO_3^- + aged slurry	11.3±1.6	12.0	27.7±2.8	29.5	39.0±3.2	41.5	0.64
NO_3^- + NH_4^+ (Inc-FSl)	15.1±1.0	19.9	14.3±1.8	18.8	29.4±2.5	38.7	1.66
NO_3^- + NH_4^+ (Inc-ASl)	13.7±3.8	17.1	16.3±3.5	20.4	30.0±6.3	37.5	1.32

Total N applied in the NO_3^- + C compound treatment: 75 kg N ha^{-1} (*taking account of the N in the glucosamine (80 kg ha^{-1})).
Total N applied in the NO_3^- + fresh slurry treatment: 102 kg N ha^{-1} (37 kg N ha^{-1} was supplied by the fresh slurry application).
Total N applied in the NO_3^- + aged slurry treatment: 94 kg N ha^{-1} (19 kg N ha^{-1} was supplied by the aged slurry application).
Total N applied in the NO_3^- + NH_4^+ (fresh) treatment: 76 kg N ha^{-1} (1.1 kg N ha^{-1} was supplied by the NH_4^+ application).
Total N applied in the NO_3^- + NH_4^+ (aged) treatment: 80 kg N ha^{-1} (4.7 kg N ha^{-1} was supplied by the NH_4^+ application).
*the mole fraction ratio

6.3.6. Soil ammonium and nitrate concentrations

Before the soil incubations, the soil N in the form of NH_4^+ concentrations in all incubations, ranged from 2.0 to 7.1 mg N kg^{-1} dry soil (Table 6.3). The soil NO_3^- concentrations were much lower, ranging from 0.05 to 0.26 mg N kg^{-1} dry soil. After the incubations, in the Inc-Van, Inc-cellulose and Inc-FSl, no significant differences were observed in the soil NH_4^+-N and NO_3^--N concentrations between the NO_3^- only, NO_3^- + glucose/NH_4^+ and NO_3^- + C source treatments. Glucosamine application resulted in significantly higher soil NH_4^+-N and NO_3^--N concentrations compared with the NO_3^- only and NO_3^- + glucose. However, soil NO_3^--N concentration in the NO_3^- + glucose and NO_3^- + butyric acid was significantly lower than the NO_3^- treatment. There were no significant differences in the NH_4^+-N concentration between the NO_3^-, NO_3^- + glucose and NO_3^- + butyric acid treatments. (There are no soil NH_4^+-N and NO_3^--N concentrations from the Inc-AFl due to the missing data). In general, soil inorganic N was mostly in the form of NH_4^+-N, ranging from 4.9 to 49.3 mg N kg^{-1} dry soil; and much less in the NO_3^--N form, which ranged from 0.3 to 6.2 mg kg^{-1} N dry soil.

There were no significant differences in the NH_4^+-N concentration between the NO_3^- treatments from all the incubations, and between the NO_3^- + glucose treatments in the Inc-Van, Inc-Cel, Inc-Glu

and Inc-But. The resulting averages for NH_4^+-N in the NO_3^- treatments from all the incubations, and NO_3^- + glucose treatments from Inc-Van, Inc-Cel, Inc-Glu and Inc-But were 15.5 and 19.0 mg N kg^{-1} dry soil, ranging from 10.1 to 21.5, and 11.5 to 23.9 mg kg^{-1} N dry soil (Table 6.3). The averages NO_3^--N values in the NO_3^- and NO_3^- + glucose treatments from all incubations were 3.4 and 0.7 mg N kg^{-1} dry soil, respectively. The NH_4^+-N concentrations in the NO_3^- + glucose and NO_3^- + C sources treatments followed the order:

NO_3^- + glucosamine > NO_3^- + glucose/cellulose > NO_3^- + fresh slurry > NO_3^- + butyric acid, (values were: 49.3>19.0/20.2>11.6>4.9 mg N kg^{-1} dry soil, Fig. 6.3a). No significant differences were observed in the NH_4^+-N concentration between the NO_3^- + vanillin (15.7 mg N kg^{-1} dry soil) and NO_3^- + glucose/cellulose, and also between the NO_3^- + vanillin and NO_3^- + fresh slurry. Soil NO_3^- concentration in the NO_3^- + fresh slurry treatment (5.8 mg N kg^{-1} dry soil) was significantly higher than that in the NO_3^- + glucose/vanillin/butyric acid/cellulose (0.7/1.4/1.8/2.5 mg N kg^{-1} dry soil, respectively), except for the NO_3^- + glucosamine treatment (3.8 mg N kg^{-1} dry soil) (Fig. 6.3b). It was also higher than the initial soil NO_3^- content.

Table 6.3 Soil NH_4^+ and NO_3^- concentrations before and after the incubation, in mg N kg^{-1} dry soil. Error bars represent standard error of the mean (n=3 before incubation, n=4 after the incubation). Different letters indicate significant differences in the soil initial NH_4^+ and NO_3^- concentration between each incubation at $P<0.05$ by LSD. After the incubation, different letters indicate the significant differences between the NO_3^-, NO_3^- + glucose/NH_4^+ and NO_3^- + C source treatment in the NH_4^+ and NO_3^- concentration, respectively.

Incubation	Inc-Van	Inc-Cel	Inc-Glu	Inc-But	Inc-FSl	Inc-ASl
	Initial soil NH_4^+ and NO_3^- concentrations					
NH_4^+	3.6±0.43bc	2.0±0.19c	2.9±0.52c	5.1±1.2ab	5.0±0.79ab	7.1±0.03a
NO_3^-	0.26±0.06a	0.11±0.04bc	0.15±0.02abc	0.21±0.05ab	0.05±0.00c	0.13±0.04bc
Treatment	Soil NH_4^+ concentration after the incubation					
NO_3^-	11.2±1.8a	21.5±3.3a	19.3±6.1b	10.1±3.9a	15.5±9.8a	/
NO_3^- +glucose or NH_4^+	11.5±1.6a	18.9±1.5a	23.9±9.3b	21.7±9.5a	11.6±1.6a	/
NO_3^- + C source	15.7±2.7a	20.2±2.1a	49.3±1.4a	4.9±1.1a	11.6±2.7a	/
Treatment	Soil NO_3^- concentration after the incubation					
NO_3^-	1.7±0.6a	3.9±1.7a	0.8±0.1b	6.2±1.3a	4.4±1.6a	/
NO_3^- +glucose or NH_4^+	0.4±0.0a	1.3±0.5a	0.3±0.0b	0.9±0.5b	4.1±0.6a	/
NO_3^- + C source	1.4±0.7a	2.5±1.6a	3.8±1.2a	1.8±0.5b	5.8±0.9a	/

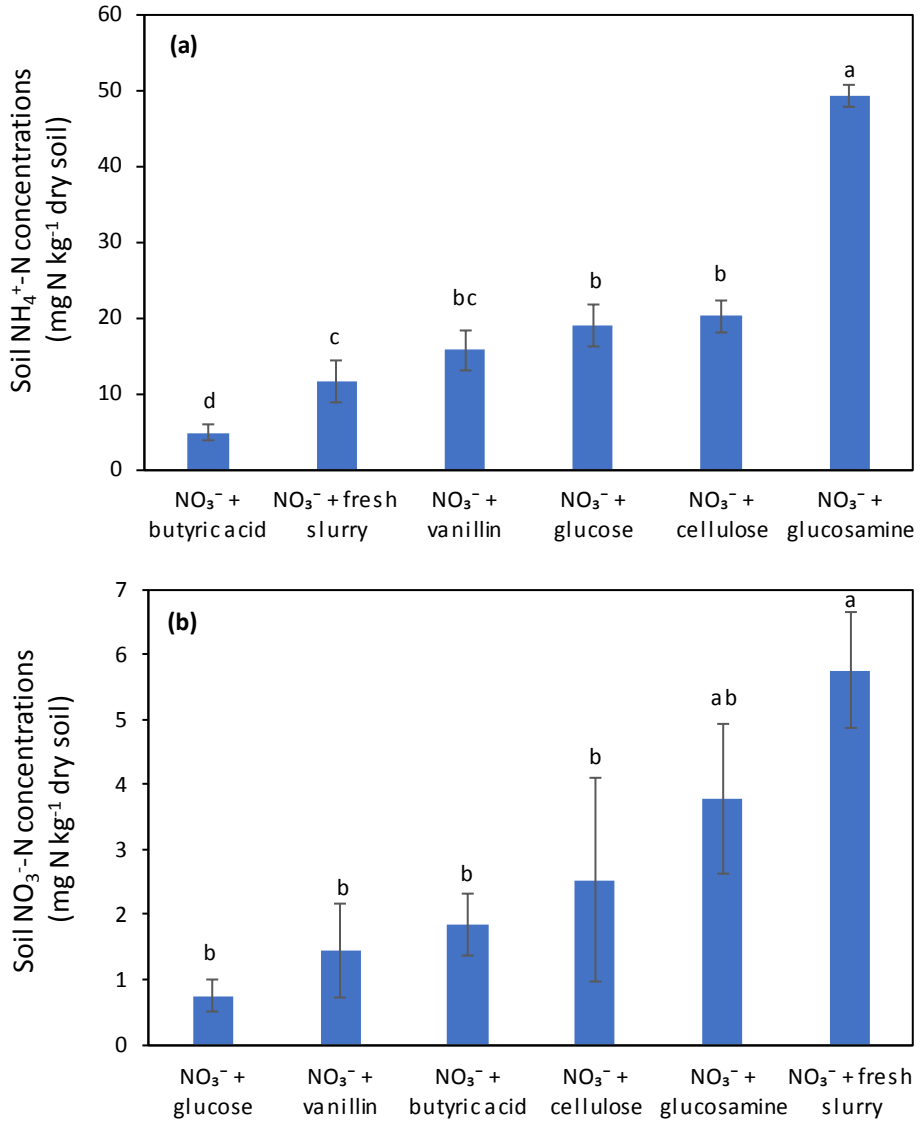

Fig. 6.3 Soil NH_4^+ (panel a) and NO_3^- (panel b) concentrations in the NO_3^- + glucose (positive control) and NO_3^- + C sources (vanillin, cellulose, glucosamine, butyric acid and fresh slurry) treatments at the end of the incubations. Error bars represent the standard error of the mean (n=4). Different letters indicate the significant differences between the treatments at $P<0.05$ by LSD. Note different y-axis units between the NH_4^+-N and NO_3^+-N panels.

6.4. Discussion

6.4.1. Effects of C compounds on soil denitrification

Glucose, vanillin and butyric acid application significantly increased soil total denitrification process, as indicated by the higher total N_2O-N + N_2-N emissions and relatively lower soil NO_3^- concentrations after the incubation (Table 6.1, Fig. 6.3). During denitrification, NO_3^- is denitrified in the sequence $NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ by corresponding reductase enzymes (Zumft, 1997), resulting in NO_3^- consumption, NO and N_2O emissions during partial denitrification, and N_2 emissions due to total denitrification (Knowles, 1982). In this study, glucosamine application increased the N_2O emissions and total N_2O + N_2 emissions, more than the other C compounds except for glucose. In addition, the final soil NO_3^--N and NH_4^+-N concentrations with glucosamine application were higher than other C compounds, probably due to also being a N source (-NH_2, which we estimate the N input via the glucosamine and KNO_3 was 80 and 75 kg N ha^{-1} respectively) (Currey et al., 2010). This extra N would have also stimulated the observed emissions, although the NH_4^+ provided with glucosamine needed to be nitrified before becoming available for denitrification. Denitrification has shown to be stimulated by a series of C substrates, including plant residues such as alfalfa, red clover, soybean and barley straw (DeCatanzaro and Beauchamp, 1985; Gillam et al., 2008; Henderson et al., 2010), and also organic compounds e.g. glucose, mannitol, sucrose, methanol, ethanol and cellulose (Henderson et al., 2010; Lescure et al., 1992; Srinandan et al., 2012). Our results showed however that cellulose application had no effect on denitrification rate, which is likely due to the low microbial availability of this C source.

Organic C is a substrate for the growth of denitrifying bacteria, a source of energy and an electron donor, which is one of the most important factors to affect soil denitrifying activity (Schipper et al., 2011; Tiedje, 1988). Based on the same amount of NO_3^- concentration application, the availability of C compounds or rapid decomposition of soluble C in soils after application may affect denitrification (Miller et al., 2012, 2008). More readily decomposed compounds, such as glucose and glucosamine have been found to stimulate denitrification more than complex carbon compounds, such as cellulose and lignin (DeCatanzaro and Beauchamp, 1985), which may be one of the explanations for the

variability in the effect on soil denitrification. In addition, the physical properties of organic compounds, such as solubility may affect the availability of C to the denitrifying microorganisms under anaerobic conditions (Miller et al., 2008). Among the C compounds applied in this study, glucose, vanillin, glucosamine and butyric acid are soluble either in water, ethanol, acetic acid or benzoyl, however, cellulose is insoluble in any of the solvents described before (data from the handbook of chemistry and physics). In addition, potential enzyme activities involved in breakdown of more complex forms of C or nutrient acquisition decreased slightly or remained unchanged with N amendments (NH_4^+ or NO_3^-) (Currey et al., 2010). This may also result in the relatively lower N_2O emissions in the NO_3^- + cellulose treatment compared with other C compounds treatments. The total N emitted from all the C compounds treatments suggest that the ranking of potential for N losses can be established as:

glucose > glucosamine > butyric acid > vanillin > cellulose.

6.4.2. Effect of cattle slurry on soil denitrification

In this study, the application of fresh or aged cattle slurry + NO_3^- had no influence on the N_2O or N_2 emissions compared with the NO_3^- only treatment (Table 6.1). The relatively lower total N emissions, combined with the higher soil NO_3^- concentration in the NO_3^- + fresh cattle slurry compared with that in the NO_3^- + C compounds treatments (except for the NO_3^- + cellulose treatment), indicates significantly lower denitrification rates in the cattle slurry application treatments. Nitrous oxide emissions from the denitrification process and CO_2 evolution in manure-amended soil was closely related to both water-soluble C and volatile fatty acids concentrations in the manures (Paul and Beauchamp, 1989). Volatile fatty acids measured in stored cow slurry were less than those from pig slurry (Cooper and Cornforth, 1978). In addition, previous studies show that the fibre content (cellulose, hemicellulose and lignin) is greatest in cattle slurry compared to swine and poultry, accounting for more than half of the dry matter (Chen et al., 2003). Indeed, a great proportion of slurry-derived N was probably lost through the NH_3 volatilisation process, before even penetrating the upper soil layers, which subsequently would have decreased the soil N pool available to both nitrifying and denitrifying microorganisms (Bourdin et al., 2014). These may explain why the fresh cattle slurry + NO_3^- application

did not affect the soil N_2O or N_2 emissions compared with the NO_3^- only treatment, and why the cumulative N_2O and N_2 was relatively lower than that in the NO_3^- + C compounds treatments, indicating that C availability in the cattle slurry limited denitrification of the added NO_3^- (Firestone and Davidson, 1989; Gillam et al., 2008).

We would expect that the aged slurry would generate more emissions compared to the control and fresh slurry, based on the fact that the intractable C in the fresh slurry undergoes changes due to microbial activity during ageing which mineralises C and N and which become available (Bastami et al., 2018; Bertora et al., 2008; De Vries et al., 2012). Although this did not happen for N_2O, it did for N_2, the relatively higher N_2 emissions observed in the NO_3^- + aged slurry treatment than NO_3^- + fresh slurry (Table 6.1). Ammonium application significantly increased the cumulative N_2O emissions compared with the NO_3^- and NO_3^- + fresh slurry treatments, which may result in N_2O emissions directly from nitrification and/or denitrification of the NO_3^- produced from nitrification.

The soil initial NH_4^+ (≤ 7.1 mg N kg^{-1} dry soil) and NO_3^- (≤ 0.26 mg N kg^{-1} dry soil) concentrations were relatively low before the applications of C and N. After the incubations, the soil NH_4^+ increase may have been the result of the mineralisation of soil organic matter stimulated by the C compounds application or compounds existing in the cattle slurry (Sutton-Grier et al., 2011), which is consistent with Dlamini et al. (2020). The slight increase of soil NO_3^- was most likely the result of the NO_3^- application itself, or produced from the mineralisation of soil organic matter (Zech et al., 1997) which was not denitrified. The significant increase in soil NO_3^- in the fresh slurry incubation treatments (NO_3^-, NO_3^- + NH_4^+ and NO_3^- + slurry) (ca. a factor of 10^4) indicates nitrification is likely to have occurred in these highly denitrifying conditions. Increases in the other incubations although smaller, particularly in NO_3^- and NO_3^- + C compound treatments supports this assumption. The smaller increases in the NO_3^- + glucose treatments could have been due to the larger consumption of NO_3^- due to removal by denitrification. Simultaneous or possible concurrent occurrence of denitrification and nitrification has been observed before (Owusu-Twum et al., 2017).

6.4.3. Effects of carbon sources on N_2O/N_2 ratio

Large denitrification rates might not result in higher N_2O emissions due to a change in the split between N_2O and N_2. Some authors use the ratio of N_2O/N_2 others $N_2O/(N_2+N_2O)$ to express this split. It has been reported that this ratio depends on C quality or the proportion of NO_3^- and C quality (in arable soils) (Weier et al., 1993b). Senbayram et al. (2012) reports higher $N_2O/(N_2+N_2O)$ at high NO_3^- concentrations due to inhibition of N_2O reduction. Scholefield et al. (1997) also reports high ratios N_2O/N_2 with high NO_3^- due to preference of microbes for this electron acceptor. At high soil moisture (Weier et al., 1993a) and with long term organic matter application (Senbayram et al., 2012) the ratio decreases. However, it seems this relationship changes (it does not apply) if NO_3^- is high. Cardenas et al. (2007) reports different N_2O/N_2 ratios from application of slurry to incubated soils depending on the pasture fed to the sheep that generated the excreted material. Values for the ratios were highest for the pasture that produced slurry with the lowest soil organic carbon, volatile fatty acids and carbohydrate contents indicating that lower available C promoted higher N_2O. The N_2O/N_2 ratio was >1, indicating that most emissions occurred as N_2O in these treatments, except for the NO_3^-, NO_3^- + cellulose, NO_3^- + butyric acid and NO_3^- + aged slurry (Table 6.2). Previous studies have shown that increasing soil NO_3^- concentration may result in an increased N_2O/N_2 ratio as a result of: 1) the inhibition of N_2O reductase activity; and 2) the greater affinity of NO_3^- relative to N_2O as terminal electron acceptor for their respective reductase enzymes (Firestone et al., 1979; Knowles, 1982; Ruser et al., 2001; Weier et al., 1993a). This may explain the relatively high N_2O/N_2 ratio in the NO_3^- + NH_4^+ (Inc-FSl and Inc-ASl) and NO_3^- + glucosamine treatments. Ammonium application and additional N supply by the glucosamine application (80 kg N ha^{-1}), resulted in a greater soil NO_3^- pool compared with the NO_3^- only, NO_3^- + C compound (except for the glucosamine) and NO_3^- + cattle slurry treatments. In this study, the N_2O/N_2 ratio in the easily degradable C compounds treatments (e.g. glucose, glucosamine) was significantly greater than that in the NO_3^- + cellulose treatment (similar N_2, but lower N_2O emissions), indicating the lower denitrification rates in the NO_3^- + cellulose treatment, which was also supported higher soil NO_3^- concentration after the incubation with cellulose application. The microbial preference for electron acceptors during the denitrification process follows the order: $O_2 > NO_3^- > N_2O$ (Firestone

and Davidson, 1989). Therefore, the low N_2O/N_2 ratio (<1) in the NO_3^- + aged slurry treatment (similar N_2O, but higher N_2 emissions compared with the fresh slurry treatment) suggests that the NO_3^- supply may not meet the demand for electron acceptors, thus inducing N_2O reduction and resulting in N_2 the primary product of denitrification. It also agrees with further reduction of N_2O to N_2 probably due to the dry matter added with the slurry that would have favour anaerobic conditions and restricted diffusion of N_2O out of the soil (Chadwick et al., 2000).

6.4.4. Implications for agriculture

The study of the reactivity of individual C compounds, although useful to determine the potential for N losses, needs to be related to the practical context, i.e. on what is expected to occur when slurry is applied to soil. There are strong interactions between C and N, but also between the various types of C (and N). The application of fresh slurry to soil intuitively suggests that there is potential for larger losses as nutrients are in their original forms and nothing has been lost. During ageing, compounds change becoming more available to microorganisms, but also nutrient losses would have occurred during storage, so less N is available. Taking this into consideration, it would be possible to use different aged slurries and assess their effect on emissions after application to soil; but also measuring the losses during storage so the full account of GHG emissions is taken (Chadwick et al., 2011).

In addition, we provide new data for the cumulative N_2O to N_2 ratio following manure spreading to land. In our study this ratio is close to 1:1 for fresh cattle slurry. This ratio is often a fixed value in models and N balances irrespective of manure type. For example, Nicholson et al (2013) use a ratio of 1:3 for all manure types in the MANNER-*NPK* decision support system. After storage, the N_2O/N_2 ratio was <1 in the aged slurry treatment, as a result of additional N_2 emissions compared with the fresh slurry treatment.

The significant higher soil N_2O and N_2 emissions in the labile C compounds treatments, may imply that the easily mineralised biological NIs identified from plants may stimulate soil NO_3^- consumption via denitrification, but appear as nitrification inhibition. Thus, this study reinforces the need to consider whether any observed effects on NO_3^- concentration following the addition of biological NIs are the

result of a direct inhibition of nitrification, or a potential indirect effect via their influence in other related soil N processes, as this has implications for developing effective mitigation strategies for N_2O emission and NO_3^- leaching.

6.5. Conclusions

The reactivity order of individual C sources on denitrification can be established as: glucose > glucosamine > butyric acid > vanillin > aged slurry > cellulose > fresh slurry, indicated by the higher total N_2O-N + N_2-N emissions. Fresh and aged cattle slurry applications did not affect the soil N_2O and N_2 emissions compared with the NO_3^- only treatments, with only the NH_4^+ application increasing N_2O emissions in the Inc-FSl. Nitrous oxide is the predominant denitrification product (compared with N_2) in the labile C compound and NH_4^+ application treatments, which may be because of the high NO_3^- concentrations and high C availability. We conclude that labile C compounds inputs would increase soil N_2O emissions and result in higher N_2O/N_2 ratio.

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6.7. References

ADHB, 2019. Nutrient Management Guide (RB209). Nutrient Management Guide (RB209).

Adouani, N., Lendormi, T., Limousy, L., Sire, O., 2010. Effect of the carbon source on N2O emissions during biological denitrification. Resources, Conservation and Recycling 54, 299–302. doi:10.1016/j.resconrec.2009.07.011

Bai, Z., Lee, M.R.F., Ma, L., Ledgard, S., Oenema, O., Velthof, G.L., Ma, W., Guo, M., Zhao, Z., Wei, S., Li, S., Liu, X., Havlík, P., Luo, J., Hu, C., Zhang, F., 2018. Global environmental costs of China's thirst for milk. Global Change Biology 24, 2198–2211. doi:10.1111/gcb.14047

Bastami, M. saufi, Davey, J., Dave, C., 2018. Methane inhibition from a slurry storage by addition of chemical and natural additives. J. Trop. Agric. and Fd. Sc. 46, 1–16.

Bertora, C., Alluvione, F., Zavattaro, L., van Groenigen, J.W., Velthof, G., Grignani, C., 2008. Pig slurry treatment modifies slurry composition, N2O, and CO2 emissions after soil incorporation. Soil Biology and Biochemistry 40, 1999–2006. doi:10.1016/j.soilbio.2008.03.021

Bourdin, F., Sakrabani, R., Kibblewhite, M.G., Lanigan, G.J., 2014. Effect of slurry dry matter content, application technique and timing on emissions of ammonia and greenhouse gas from cattle slurry

applied to grassland soils in Ireland. Agriculture, Ecosystems and Environment 188, 122–133. doi:10.1016/j.agee.2014.02.025

Britton, A.J., Gibbs, S., Fisher, J.M., Helliwell, R.C., 2019. Impacts of nitrogen deposition on carbon and nitrogen cycling in alpine Racomitrium heath in the UK and prospects for recovery. Environmental Pollution 254, 112986. doi:10.1016/j.envpol.2019.112986

Cardenas, L.M., Chadwick, D., Scholefield, D., Fychan, R., Marley, C.L., Jones, R., Bol, R., Well, R., Vallejo, A., 2007. The effect of diet manipulation on nitrous oxide and methane emissions from manure application to incubated grassland soils. Atmospheric Environment 41, 7096–7107. doi:10.1016/j.atmosenv.2007.04.055

Chadwick, D., Sommer, S., Thorman, R., Fangueiro, D., Cardenas, L., Amon, B., Misselbrook, T., 2011. Manure management: Implications for greenhouse gas emissions. Animal Feed Science and Technology 166–167, 514–531. doi:10.1016/j.anifeedsci.2011.04.036

Chadwick, D., Wei, J., Yan'an, T., Guanghui, Y., Qirong, S., Qing, C., 2015. Improving manure nutrient management towards sustainable agricultural intensification in China. Agriculture, Ecosystems and Environment 209, 34–46. doi:10.1016/j.agee.2015.03.025

Chadwick, D.R., Pain, B.F., Brookman, S.K.E., 2000. Nitrous Oxide and Methane Emissions following Application of Animal Manures to Grassland. Journal of Environmental Quality 29, 277–287. doi:10.2134/jeq2000.00472425002900010035x

Chadwick, D.R., Williams, J.R., Lu, Y., Ma, L., Bai, Z., Hou, Y., Chen, X., Misselbrook, T.H., 2020. Strategies to reduce nutrient pollution from manure management in China. Frontiers of Agricultural Science and Engineering 0, 0. doi:10.15302/j-fase-2019293

Chen, S., Liao, W., C, L., Wen, Z., Kincaid, R.L., Harrison, J.H., Elliott, D.C., Brown, M.D., Solanba, a. E., Stevens, D.J., 2003. Value-Added Chemicals from Animal Manure. Pacific Northwest National Laboratory PNNL-14495, 1–142. doi:http://dx.doi.org/10.2172/15009485.

Cooper, P., Cornforth, I.S., 1978. Volatile Fatty Acids in Stored Animal Slurry 19–27.

Currey, P.M., Johnson, D., Sheppard, L.J., Leith, I.D., Toberman, H., van der Wal, R., Dawson, L.A., Artz, R.R.E., 2010. Turnover of labile and recalcitrant soil carbon differ in response to nitrate and ammonium deposition in an ombrotrophic peatland. Global Change Biology 16, 2307–2321. doi:10.1111/j.1365-2486.2009.02082.x

de Bastos, D.F., Magiero, E.C., Tomazi, M., Schirmann, J., Veloso, M.G., de Faccio Carvalho, P.C., Bayer, C., 2020. A 3-year assessment of nitrous oxide emission factors for urine and dung of grazing sheep in a subtropical ecosystem. Journal of Soils and Sediments 20, 982–991. doi:10.1007/s11368-019-02456-7

De Vries, J.W., Vinken, T.M.W.J., Hamelin, L., De Boer, I.J.M., 2012. Comparing environmental consequences of anaerobic mono- and co-digestion of pig manure to produce bio-energy - A life cycle perspective. Bioresource Technology 125, 239–248. doi:10.1016/j.biortech.2012.08.124

DeCatanzaro, J.B., Beauchamp, E.G., 1985. The effect of some carbon substrates on denitrification rates and carbon utilization in soil 183–187.

Department of Environment & Rural Affairs, 2019. British Survey of Fertiliser Practice: Fertiliser use on farm for the 2018 crop year. 5.

Dlamini, J.C., Chadwick, D., Hawkins, J.M.B., Martinez, J., Scholefield, D., Ma, Y., Cárdenas, L.M., 2020. Evaluating the potential of different carbon sources to promote denitrification.

Enahoro, D., Lannerstad, M., Pfeifer, C., Dominguez-Salas, P., 2018. Contributions of livestock-derived foods to nutrient supply under changing demand in low- and middle-income countries. Global Food Security 19, 1–10. doi:10.1016/j.gfs.2018.08.002

Firestone, M.K., Davidson, E.A., 1989. Microbiologial Basis of NO and N2O production and consumption in soil. Exchange of Trace Gases between Terrestrial Ecosystems and the

Atmosphere 7–21. doi:10.1017/CBO9781107415324.004

Firestone, M.K., Smith, M.S., Firestone, R.B., Tiedje, J.M., 1979. The Influence of Nitrate, Nitrite, and Oxygen on the Composition of the Gaseous Products of Denitrification in Soil. Soil Science Society of America Journal 43, 1140–1144. doi:10.2136/sssaj1979.03615995004300060016x

Gillam, K.M., Zebarth, B.J., Burton, D.L., 2008. Nitrous oxide emissions from denitrification and the partitioning of gaseous losses as affected by nitrate and carbon addition and soil aeration 133–143.

He, T., Xie, D., Ni, J., Li, Zhu, Li, Zhenlun, 2020. Nitrous oxide produced directly from ammonium, nitrate and nitrite during nitrification and denitrification. Journal of Hazardous Materials 388, 122114. doi:10.1016/j.jhazmat.2020.122114

Henderson, S.L., Dandie, C.E., Patten, C.L., Zebarth, B.J., Burton, D.L., Trevors, J.T., Goyer, C., Al, H.E.T., Icrobiol, A.P.P.L.E.N.M., 2010. Changes in Denitrifier Abundance , Denitrification Gene mRNA Levels , Nitrous Oxide Emissions , and Denitrification in Anoxic Soil Microcosms Amended with Glucose and Plant Residues doi:10.1128/AEM.02993-09

Knowles, R., 1982. Denitrification. Microbiological Reviews 46, 43–70. doi:10.1128/CMR.5.4.356.Updated

Lescure, C., Menendez, L., Lensi, R., Chalamet, A., Pidello, A., 1992. Effect of addition of various carbon substrates on denitrification in a vertic Mollisol 125–129.

López-Aizpún, M., Horrocks, C.A., Charteris, A.F., Marsden, K.A., Ciganda, V.S., Evans, J.R., Chadwick, D.R., Cárdenas, L.M., 2020. Meta-analysis of global livestock urine-derived nitrous oxide emissions from agricultural soils. Global Change Biology 26, 2002–2013. doi:10.1111/gcb.15012

Meijide, A., Cardenas, L.M., Bol, R., Bergstermann, A., Goulding, K., Well, R., Vallejo, A., Scholefield, D., 2010. Dual isotope and isotopomer measurements for the understanding of N2O production and consumption during denitrification in an arable soil. European Journal of Soil Science 61, 364–374. doi:10.1111/j.1365-2389.2010.01233.x

Miller, J.J., Beasley, B.W., Drury, C.F., Zebarth, B.J., 2012. Denitrification during the growing season as influenced by long-term application of composted versus fresh feedlot manure. Canadian Journal of Soil Science 92, 865–882. doi:10.4141/CJSS2011-107

Miller, M.N., Zebarth, B.J., Dandie, C.E., Burton, D.L., Goyer, C., Trevors, J.T., 2008. Crop residue influence on denitrification, N2O emissions and denitrifier community abundance in soil. Soil Biology and Biochemistry 40, 2553–2562. doi:10.1016/j.soilbio.2008.06.024

Moral, R., Paredes, C., Bustamante, M.A., Marhuenda-Egea, F., Bernal, M.P., 2009. Utilisation of manure composts by high-value crops: Safety and environmental challenges. Bioresource Technology 100, 5454–5460. doi:10.1016/j.biortech.2008.12.007

Moset, V., Cambra-López, M., Estellés, F., Torres, A.G., Cerisuelo, A., 2012. Evolution of chemical composition and gas emissions from aged pig slurry during outdoor storage with and without prior solid separation. Biosystems Engineering 111, 2–10. doi:10.1016/j.biosystemseng.2011.10.001

Murray, P.J., Hatch, D.J., Dixon, E.R., Stevens, R.J., Laughlin, R.J., Jarvis, S.C., 2004. Denitrification potential in a grassland subsoil: Effect of carbon substrates. Soil Biology and Biochemistry 36, 545–547. doi:10.1016/j.soilbio.2003.10.020

Owusu-Twum, M.Y., Loick, N., Cardenas, L.M., Coutinho, J., Trindade, H., Fangueiro, D., 2017. Nitrogen dynamics in soils amended with slurry treated by acid or DMPP addition. Biology and Fertility of Soils 53, 339–347. doi:10.1007/s00374-017-1178-0

Paul, J.W., Beauchamp, E.G., 1989. soIL compare denitrification rates 61, 49–61.

Ramanantenasoa, M.M.J., Génermont, S., Gilliot, J.M., Bedos, C., Makowski, D., 2019. Meta-modeling methods for estimating ammonia volatilization from nitrogen fertilizer and manure applications. Journal of Environmental Management 236, 195–205. doi:10.1016/j.jenvman.2019.01.066

Robinson, A., Di, H.J., Cameron, K.C., Podolyan, A., He, J., 2014. The effect of soil pH and dicyandiamide (DCD) on N2O emissions and ammonia oxidiser abundance in a stimulated grazed pasture soil. Journal of Soils and Sediments 14, 1434–1444. doi:10.1007/s11368-014-0888-2

Rodhe, L., Ascue, J., Nordberg, Å., 2009. Emissions of greenhouse gases (methane and nitrous oxide) from cattle slurry storage in Northern Europe. IOP Conference Series: Earth and Environmental Science 8, 012019. doi:10.1088/1755-1315/8/1/012019

Rodhe, L.K.K., Ascue, J., Willén, A., Persson, B.V., Nordberg, Å., 2015. Greenhouse gas emissions from storage and field application of anaerobically digested and non-digested cattle slurry. Agriculture, Ecosystems and Environment 199, 358–368. doi:10.1016/j.agee.2014.10.004

Ruser, R., Flessa, H., Schilling, R., Beese, F., Munch, J.C., 2001. Effect of crop-specific field management and N fertilization on N2o emissions from a fine-loamy soil. Nutrient Cycling in Agroecosystems 59, 177–191. doi:10.1023/A:1017512205888

Schipper, L.A., Matiasek, M.G., Scow, K.M., Cameron, S., Bruesewitz, D.A., Mcdonald, I.R., 2011. Nitrate removal , communities of denitrifiers and adverse effects in different carbon substrates for use in denitrification beds So 5. doi:10.1016/j.watres.2011.08.007

Scholefield, D., Hawkins, J.M.B., Jackson, S.M., 1997. Use of a flowing helium atmosphere incubation, technique to measure the effects of denitrification controls applied to intact cores of a clay soil. Soil Biology and Biochemistry 29, 1337–1344. doi:10.1016/S0038-0717(97)00059-X

Senbayram, M., Chen, R., Budai, A., Bakken, L., Dittert, K., 2012. N 2O emission and the N 2O/(N 2O+N 2) product ratio of denitrification as controlled by available carbon substrates and nitrate concentrations. Agriculture, Ecosystems and Environment 147, 4–12. doi:10.1016/j.agee.2011.06.022

Senbayram, M, Chen, R., Budai, A., Bakken, L., Dittert, K., 2012. Agriculture , Ecosystems and Environment N 2 O emission and the N 2 O /(N 2 O + N 2) product ratio of denitrification as controlled by available carbon substrates and nitrate concentrations. "Agriculture, Ecosystems and Environment" 147, 4–12. doi:10.1016/j.agee.2011.06.022

Skiba, U., Jones, S.K., Dragosits, U., Drewer, J., Fowler, D., Rees, R.M., Pappa, V.A., Cardenas, L., Chadwick, D., Yamulki, S., Manning, A.J., 2012. UK emissions of the greenhouse gas nitrous oxide. Philosophical Transactions of the Royal Society B: Biological Sciences 367, 1175–1185. doi:10.1098/rstb.2011.0356

Smith, K.A., Williams, A.G., 2016. Production and management of cattle manure in the UK and implications for land application practice. Soil Use and Management 32, 73–82. doi:10.1111/sum.12247

Sokolov, V., VanderZaag, A., Habtewold, J., Dunfield, K., Wagner-Riddle, C., Venkiteswaran, J., Gordon, R., 2019. Greenhouse gas emissions from gradually-filled liquid dairy manure storages with different levels of inoculant. Nutrient Cycling in Agroecosystems 115, 455–467. doi:10.1007/s10705-019-10023-2

Sommer, S.G., Webb, J., Hutchings, N.D., 2019. New Emission Factors for Calculation of Ammonia Volatilization From European Livestock Manure Management Systems. Frontiers in Sustainable Food Systems 3, 1–9. doi:10.3389/fsufs.2019.00101

Srinandan, C.S., Glen, D., Srivastava, N., Bhusan, B., Nerurkar, A.S., 2012. Bioresource Technology Carbon sources influence the nitrate removal activity , community structure and biofilm architecture. BIORESOURCE TECHNOLOGY 117, 292–299. doi:10.1016/j.biortech.2012.04.079

Sutton-Grier, A.E., Keller, J.K., Koch, R., Gilmour, C., Megonigal, J.P., 2011. Electron donors and acceptors influence anaerobic soil organic matter mineralization in tidal marshes. Soil Biology and Biochemistry 43, 1576–1583. doi:10.1016/j.soilbio.2011.04.008

Tiedje, J.M., 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium.

Trolove, S., Thomas, S., van der Klei, G., Beare, M., Cichota, R., Meenken, E., 2019. Nitrate leaching losses during pasture renewal – Effects of treading, urine, forages and tillage. Science of the Total Environment 651, 1819–1829. doi:10.1016/j.scitotenv.2018.09.333

United Nations Framework Convention on Climate Change (UNFCCC), 2020. Global Warming Potentials (IPCC Second Assessment Report).

Weier, K.L., Doran, J.W., Power, J.F., Walters, D.T., 1993a. Denitrification and the Dinitrogen/Nitrous Oxide Ratio as Affected by Soil Water, Available Carbon, and Nitrate. Soil Science Society of America Journal 57, 66–72. doi:10.2136/sssaj1993.03615995005700010013x

Weier, K.L., Macrae, I.C., Myers, R.J.K., 1993b. Denitrification in a clay soil under pasture and annual crop: Estimation of potential losses using intact soil cores. Soil Biology and Biochemistry 25, 991–997. doi:10.1016/0038-0717(93)90145-2

Wu, D., Cárdenas, L.M., Calvet, S., Brüggemann, N., Loick, N., Liu, S., Bol, R., 2017. The effect of nitrification inhibitor on N2O, NO and N2emissions under different soil moisture levels in a permanent grassland soil. Soil Biology and Biochemistry 113, 153–160. doi:10.1016/j.soilbio.2017.06.007

Xu, Z., Dai, X., Chai, X., 2018. Effect of different carbon sources on denitrification performance, microbial community structure and denitrification genes. Science of the Total Environment 634, 195–204. doi:10.1016/j.scitotenv.2018.03.348

Yamamoto, M., Futamura, Y., Fujioka, K., Yamamoto, K., 2008. Novel Production Method for Plant Polyphenol from Livestock Excrement Using Subcritical Water Reaction. International Journal of Chemical Engineering 2008, 1–5. doi:10.1155/2008/603957

Zanon, J.A., Favaretto, N., Democh Goularte, G., Dieckow, J., Barth, G., 2020. Manure application at long-term in no-till: Effects on runoff, sediment and nutrients losses in high rainfall events. Agricultural Water Management 228. doi:10.1016/j.agwat.2019.105908

Zech, W., Senesi, N., Guggenberger, G., Kaiser, K., Lehmann, J., Miano, T.M., Miltner, A., Schroth, G., 1997. Factors controlling humification and mineralization of soil organic matter in the tropics. Geoderma 79, 117–161. doi:10.1016/S0016-7061(97)00040-2

Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. Microbiology and Molecular Biology Reviews : MMBR 61, 533–616. doi:10.1128/.61.4.533-616.1997

Chapter 7: Discussion and outlook

7.1. Introduction

Since the experimental chapters (Chapters 3-6) are presented in the forms of journal articles manuscripts, discussions have been conducted in each relevant chapter. In this Chapter, I return to the three objectives of the thesis: 1) to determine the efficacy of the biological nitrification inhibitors (NIs); 2) to assess the factors controlling the efficacy of biological NIs; 3) to clarify the mechanism of the biological NIs on soil nitrification inhibition. In addition, this chapter provides recommendations for future research.

7.2. Efficacy of biological NIs

Synthetic NIs, e.g. dicyandiamide (DCD) (Cameron and Di, 2002; Cardenas et al., 2016; Monaghan et al., 2013) and 3, 4-dimethylpyrazole phosphate (DMPP) (Gilsanz et al., 2016; Nair et al., 2020; Shi et al., 2016), have been well understood and widely used to reduce the soil nitrous oxide (N_2O) emissions and/or nitrate (NO_3^-) leaching in agriculture. In recent years, some pasture grasses (*Brachiaria humidicola*) and crops have been confirmed to have the ability to release biological NIs, with more newly identified biological NIs from *Brachiaria humidicola* (Gopalakrishnan et al., 2007; Subbarao et al., 2009, 2008), rice (Sun et al., 2016), sorghum (Subbarao et al., 2013; Zakir et al., 2008).

In Chapters 3 and 4, biological NIs identified from *Brachiaria humidicola* (linoleic acid, LA and linolenic acid, LN) and rice (1,9-decanediol) were applied (from synthetic production) to a highly nitrifying soil to compare their effects on soil nitrification and greenhouse gas (GHG) emissions (N_2O, and carbon dioxide (CO_2)) compared with the proven synthetic NI, DCD. Results in these two chapters showed that DCD retained relatively higher ammonium (NH_4^+) and lower NO_3^- concentrations in the soil (Fig. 3.1, 4.1) and reduced cumulative N_2O emissions (Fig. 3.3, 4.2) compared with biological NIs (LA, LN and 1,9-decaneidiol) applied at the same concentrations (12.7 and 127 mg kg^{-1} dry soil), which is consistent with the first hypothesis of the thesis that the effective dose of LA and LN to reduce soil

NO_3^- concentration was found to be much higher (≥635 mg kg^{-1} dry soil) than that of DCD (12.7-127 mg kg^{-1} dry soil).

Previous studies compared the effects of biological NIs (Lu et al., 2019; Nardi et al., 2013) or root exudates containing biological nitrification inhibition (BNI) activity (Gopalakrishnan et al., 2009; Meena et al., 2014; Souri and Neumann, 2010) and NIs (e.g. DCD, DMPP, Nitrapyrin, as a control) on soil nitrification, N_2O emissions and nitrifiers. Dicyandiamide applied at a concentration of 10-20 mg kg^{-1} dry soil showed similar inhibition of nitrification rates with 1,9-decanediol applied at 500 mg kg^{-1} dry soil (Lu et al., 2019) and methyl 3-(4-hydroxyphenyl) propionate (MHPP, identified from sorghum) applied at 350 mg C kg^{-1} dry soil (Nardi et al., 2013). Root exudates from *Brachiaria humidicola* applied at least 30 Allylthiourea (AT) unit g^{-1} dry soil (ATU, the inhibitory effect of 0.22 μM AT in an assay containing 18.9 mM of NH_4^+ is defined as one AT unit of activity) (Subbarao et al., 2006a), or extracted by higher concentration of ethyl alcohol (70%, BNI activity in freeze-dried shoot tissue extracted with 70% methanol was 215 ATU) (Subbarao et al., 2008) showed close or even higher nitrification inhibition rates compared with DCD applied with 10-50 mg kg^{-1} dry soil (Gopalakrishnan et al., 2009; Meena et al., 2014). The relatively lower mineralisation of NIs compared with biological NIs may be one of the explanations for the higher nitrification inhibition rates in NIs application than BNIs application, which is described in Section 7.3.1.

7.3. Factors controlling the efficacy of biological NIs

7.3.1. How biological NIs stability affect soil nitrification?

The mineralisation rates of LA, LN and DCD were determined by using the ^{14}C-labelling methods (Chapter 4). Linoleic acid and LN mineralised much more rapidly than DCD, with mineralisation rates of 47-56%, 37-61% and 2.7-5.5% (Fig. 4.4), respectively, after a 38-d incubation. Due to the high cost of the ^{14}C labelled 1,9-decanediol, the mineralisation rate of this compound was not explored in this study, instead, the cumulative CO_2 emissions were assessed when it was applied at a rate of 12.7 and 127 mg kg^{-1} dry soil. The emissions were significantly higher than those in the DCD treatments (applied at 12,7 and 127 mg kg^{-1} dry soil), indicating higher mineralisation rate of 1,9-decanediol than DCD (Fig.

3.3b). Combining the higher soil NH_4^+ and lower NO_3^- concentrations, and/or reduced soil N_2O emissions with the lower mineralisation rates in the DCD treatments compared with biological NIs, confirms the initial hypothesis that the rate of nitrification inhibition would decrease as the increasing mineralisation rate.

The efficacy of NIs is largely related to their mineralisation rate or hydrolysis rate, which are strongly influenced by temperature (Guardia et al., 2018; Kelliher et al., 2008; Marsden et al., 2016b) and soil aeration (Balaine et al., 2015). This is the first time the stability of biological NIs has been determined, and results showed that with increasing application rates of LN the mineralisation rate also increased. Further studies need to be conducted to explore the factors controlling mineralisation rates of the range of biological NIs that have been identified. The relatively higher mineralisation rate of biological NIs (LA, LN and 1,9-decanediol) compared to DCD may result in lower nitrification inhibition rates, because mineralisation of biological NIs may stimulate soil N immobilisation or denitrification (see detailed in section 7.4).

7.3.2. How biological NI concentrations affect soil nitrification?

Nitrification inhibitor concentrations have also been shown to be one of the factors limiting the efficacy of NIs (Brath et al., 2008). In previous studies, biological NIs were applied at a range of concentrations (0-1000 mg kg^{-1} dry soil) to explore their effects on soil nitrification and N_2O emissions (Lu et al., 2019; Nardi et al., 2013; Subbarao et al., 2008). In Chapters 3 and 4, BNI compounds (synthetic LA, LN and 1,9-decanediol) were applied at a concentration of 0, 12.7, 127, 635 and 1270 mg kg^{-1} dry soil. Results showed that LA and LN applied at higher concentrations reduced soil NO_3^- concentration (Fig. 4.1), and LA, 1,9-decanediol applied at rates lower than 127 mg kg^{-1} dry soil was infective at inhibiting soil nitrification (Fig. 3.1), which supports the hypothesis that nitrification inhibition would increase with increasing biological NI application rate. This agrees with previous studies that found that the nitrification inhibition rate increased as the LA and LN application rates (ranging from 0 to 1000 mg kg^{-1} dry soil, or 0.05-0.5% of NH_4^+ application) increased (Souri, 2016;

Subbarao et al., 2008). In addition, 1,9-decanediol showed significant nitrification inhibition when it was applied at rates ≥500 mg kg^{-1} dry soil (Lu et al., 2019).

Root exudates from grasses or crops that contain BNI activity have also been applied in previous studies to determine the potential of plants to inhibit soil nitrification (Souri, 2016; Subbarao et al., 2006a). In Chapter 5, soil grown with *Brachiaria humidicola* was used to compare the residual effect of grass with and without BNI activity. Results showed that soil grown with grass containing BNI activity (*Brachiaria humidicola*) retained higher NH_4^+ concentration and lower NO_3^- concentration than when grass had no BNI activity (*Brachiaria ruziziensis*). Additionally N_2O emissions were reduced during the first peak after the application of a N source (sheep urine application in this case) in the presence of BNI activity (Fig. 5.1, 5.2), which indicated that nitrification could be inhibited when biological NIs are applied under certain concentrations. In the study of Gopalakrishnan et al. (2009), root exudates from *Brachiaria humidicola* applied at a range of 10-40 ATU g^{-1} dry soil resulted in the inhibition of soil nitrification rates, and the inhibition rates increased with increasing concentrations (reaching 95% inhibition when applied >30 ATU g^{-1} dry soil). The inhibition activity of root exudates from *Brachiaria humidicola* is largely associated with BNI compounds identified, e.g. LA, LN (Subbarao et al., 2008), brachialactone (Subbarao et al., 2009), methyl p-coumarate and methyl ferulate (Gopalakrishnan et al., 2007), which block the ammonia monooxygenase (AMO) and/or hydroxylamine oxidoreductase (HAO) pathways. The presence of NH_4^+, low pH and aeration of the root environment were confirmed to stimulate the release of biological NIs from its roots (Subbarao et al., 2007c; X. Zhang et al., 2019).

7.4. Mechanism of biological NIs on soil nitrification

7.4.1 Do biological NIs act as a direct nitrification inhibitor of soil nitrification?

In Chapter 4, soil NO_3^- concentration significantly decreased as biological NIs (LA and LN identified from *Brachiaria humidicola*) concentration increased, however, no significant differences were observed in the soil NH_4^+ concentration with increasing BNIs concentrations (Fig. 4.1). 1,9-decanediol (identified from rice) application also resulted in decreased NO_3^- concentration, but the

inhibition of NO_3^- formation decreased as concentration increased (Chapter 3, Fig. 3.1). In Chapter 5, soil grown with *Brachiaria humidicola* (with ability to release biological NIs) retained higher soil NH_4^+ and lower NO_3^- concentrations compared with soil grown with *Brachiaria ruziziensis* (not able to release biological NIs) after sheep urine application. The cumulative N_2O emissions during first peak in the soil grown with *Brachiaria humidicola* was significantly inhibited compared with soil grown with *Brachiaria ruziziensis* after sheep urine application (Chapter 5, Table 5.2). This could be partly due to direct soil nitrification inhibition by the biological NIs, which inhibit the transformation of soil NH_4^+ to NO_3^- and reduce soil N_2O emissions during the nitrification. A second N_2O peak as a result of denitrification was not affected by the NIs application. In addition, the biological NIs did not affect NO emissions.

A useful approach to test the direct effect of biological NIs on nitrification rates, is via analysis of the nitrification gene abundance. During nitrification, NH_4^+ is first oxidised to nitrite (NO_2^-) catalysed by the AMO and HAO, which is then oxidised to NO_3^- (Firestone and Davidson, 1989). Most biological NIs have been confirmed to inhibit both the HAO and AMO enzymatic pathways, such as LA, LN and brachialactone identified from *Brachiaria humidicola* (Subbarao et al., 2009, 2008), sorgoleone and sakuranetin identified from sorghum (Subbarao et al., 2013). There are also some biological NIs that inhibit only the AMO, e.g. biological NIs identified from sorghum and rice (Sun et al., 2016; Zakir et al., 2008), or the HAO, such as wheat root exudates (no identified biological NIs) (Subbarao et al., 2007b). In contrast, synthetic NIs inhibit only the AMO pathway (Benckiser et al., 2013; Subbarao et al., 2013; Zakir et al., 2008).

The abundance, diversity and structure of ammonia oxidising bacteria (AOB) and archaea (AOA) responding to the application of NIs have been shown to be highly variable, and controlled by soil type (Gong et al., 2013; Liu et al., 2015), soil pH (Robinson et al., 2014), soil temperature (McGeough et al., 2016), and soil water content (Barrena et al., 2017). Lu et al. (2019) confirmed that the 1,9-decanediol application significantly inhibited the abundance of AOB and AOA in an acidic red soil, paddy soil and fluvoaquic soil, however, the community structure of AOA and AOB was significantly different in these three typical agriculture soils. Dicyandiamide was effective at inhibiting nitrification (soil retained high NH_4^+ concentration and low NO_3^- concentration) and reduced cumulative N_2O emission by inhibiting

AOB gene abundance rather than AOA (Chapter 3). No significant differences were observed in the AOA and AOB abundance after the LA and 1,9-decanediol (Chapter 3), and may be due to the lower biological NIs application rate (≤127 mg NI kg^{-1} dry soil), which is consistent with previous studies (Lu et al., 2019; Nardi et al., 2013). A relatively high dose (350 µg C g^{-1} soil) of BNI compound (identified from sorghum, 3-4-hydroxyphenyl propionate (MHPP)) application significantly reduced the abundance of AOB and AOA (Nardi et al., 2013).

7.4.2 Do biological NIs act as C sources to stimulate N immobilisation?

Nitrification inhibitors suppress soil nitrification, resulting in higher soil NH_4^+ and lower NO_3^- concentrations (Lu et al., 2019; Subbarao et al., 2008; Xu et al., 2019). However, only significantly reduced soil NO_3^- concentrations were observed in this study after the applications of LA, LN and 1,9-decanediol, but there was no increased/retention in the soil NH_4^+ concentration, which disproves the initial hypothesis that biological NIs application would retain higher soil NH_4^+ and lower NO_3^- concentration. In addition, low nitrification rates sometimes have been attributed to a decline in NH_4^+ availability rather than to the toxicity to nitrifiers (Schimel et al., 1996). Labile carbon (C) rich substrates have previously been shown to increase net N immobilisation in soil (G. Chen et al., 2003; Magill and Aber, 2000; Vinten et al., 2002). In Chapter 4, the nitrification inhibition mechanism of the biological NIs seemed to differ from DCD. In the case of LA and LN, an indirect effect under low soil N conditions could be due to the addition of labile C promoting microbial immobilisation of soil NH_4^+ and/or NO_3^- (under high C/N ratios), and/or denitrification losses (Chapter 4). We did not measure soil microbial immobilisation of NH_4^+ or NO_3^- using ^{15}N-labelling technique directly, but we determined the linear relationship between the predicted microbial N immobilisation (predicted value, using the standard C:N ratio of the soil microbial biomass of 8:1 to evaluate the microbial N demand needed to assimilate the C-rich substrates) using the ^{14}C-labelling method and observed N immobilisation (observed value, ($NH_4^+ + NO_3^-$) in control minus ($NH_4^+ + NO_3^-$) in treatment) (see details in section 4.2.4). This provides evidence to support the initial hypothesis that the reduced NO_3^- concentration after application of the biological NIs, LA and LN, could have been partly due to soil microbial NH_4^+ and/or

NO_3^- immobilisation. This is also supported by the significantly higher mineralisation rates of ^{14}C-labelled LA and LN compared to DCD (Fig. 4.5). In addition, mineralisation rate was positively linearly correlated to cumulative CO_2 emissions (Chapter 4).

The study of Nardi et al. (2013), suggested that the Biological NI, MHPP, would limit NH_4^+ supply (NH_4^+ immobilisation) and indirectly reduced soil nitrification, due to the BNI compound acting as a C source for soil microorganisms, favouring heterotrophs that are better competitors for NH_4^+ than autotrophs. Nevertheless, it has been argued that the influence of MHPP on NH_4^+ immobilisation is of minor importance and the MHPP shows a more direct effect on ammonia oxidisers (Nardi et al., 2013). This was also supported by Lu et al. (2019), who indicated that the high doses of 1,9-decanediol application suppress soil nitrification by inhibiting AOB and AOA but not NH_4^+ availability.

7.4.3 Do biological NIs act as C sources to stimulate denitrification?

Denitrification refers to the dissimilatory reduction of $NO_3^-\rightarrow NO_2^-\rightarrow NO\rightarrow N_2O\rightarrow N_2$ by corresponding reductases (Knowles, 1982; Zumft, 1997). In Chapter 4, higher concentration of LA and LN application significantly increased soil N_2O emissions compared with the control without biological NIs application, which may result from their role as C sources (significant higher mineralisation rate of LA and LN) stimulating soil denitrification of residual NO_3^- and/or NO_3^- produced via soil nitrification. Soil C availability has been confirmed to be one of the most important factors controlling denitrification rates, due to its role as a substrate for the growth of denitrifying bacteria, a source of energy and an electron donor (Henderson et al., 2010; Schipper et al., 2011; Tiedje, 1988). The effects of C sources on soil denitrification have been explored in Chapter 6, the study confirmed that labile C compounds stimulated soil N_2O emissions and NO_3^- consumption during the denitrification process, e.g. glucose and glucosamine compared with relatively stable and complex C compounds such as cellulose (Table 6.2, 6.5), resulted in greater N_2O emissions, and a higher N_2O/N_2 ratio. This provides evidence for the initial hypothesis that biological NIs may act as a C source to stimulate soil denitrification, especially at high application rates. Even though the effect of the biological NIs, LA, LN and 1,9-decanediol, were not explored in this DENIS study. To our knowledge, this is the first time to mention that biological NI

may act as C source to stimulate soil denitrification. Further research needs to be conducted to confirm the effects of easily mineralised biological NIs on denitrification.

7.5. Conclusions

Biological NIs, such as LA, LN and 1,9-decanediol, were able to reduce soil NO_3^- concentration after application to a highly nitrifying soil with or without chemical N fertiliser applications. However, the required doses of LA, LN and 1,9-decanediol to inhibit soil nitrification were significantly higher than the application rates of the proven synthetic NI, DCD. The efficacy of biological NIs were largely related to the initial biological NI concentration and stability in soil, which increased as the increasing of BNI concentration and decreasing mineralisation rates. The apparent reduction of soil NO_3^- concentration after the application of biological NIs may result from biological NIs 1) directly inhibiting thee nitrification process; 2) providing a C source to stimulate soil NH_4^+ and/or NO_3^- immobilisation; 3) providing a C source to promote soil denitrification. The synthetic NI, DCD, was confirmed to suppress the transformation of soil NH_4^+ to NO_3^-, and inhibit soil N_2O emissions by impeding AOB but not AOA directly in a highly nitrifying soil.

7.6. Recommendations for future studies

This thesis has identified that biological NIs can appear to result in direct nitrification inhibition, but at high concentrations they may also provide a suitable C source that stimulates microbial immobilisation and/or denitrification, both of which result in a reduction in soil NO_3^-. However, the thesis has also highlighted some important gaps in our knowledge, which are summarised in the following section, and in Fig 7.1.

1. The study in Chapter 4 provided evidence to support the possibility that biological NIs may indirectly inhibit soil nitrification by N immobilisation, using the ^{14}C-labelling method and indirect calculation. But we did not directly measure soil microbial immobilisation of NH_4^+ or NO_3^- using ^{15}N-labelling technique. Further studies could be conducted to measure the effects

of biological NIs on direct soil microbial immobilisation to provide more evidence for the mechanism of biological NIs on soil nitrification.

2. In Chapter 3, we only explored the effects of BNI compounds applied at doses ≤127 mg BNI kg^{-1} dry soil on soil nitrifiers and denitrifiers. However, the effects of higher concentration of BNI compounds application on soil nitrifiers and denitrifiers were not determined. In addition, BNI compounds were not included in the series of experiments to determine the effect of C lability on soil denitrification. Thus, further studies could be conducted to explore the effects of biological NIs on soil denitrification and denitrifier populations, to verify the possible mechanism of being a C source to stimulate denitrification.
3. In this study, we did not include glucose as a reference C source alongside the biological NIs treatments to compare their effects on soil nitrification, immobilisation and denitrification. In addition, biological NIs were applied at the same mass but not the same amount C content in this study. Including glucose as C reference at the same level of C addition or adding the same number of moles, to compare with LA, LN and 1,9-decanediol in the future, may result in a better understanding of the efficacy and mechanism of biological NIs.
4. Due to the high cost of ^{14}C-labelling 1,9-decanediol molecule, the mineralisation rate of 1,9-decanediol was not determined in this study, further studies could be conducted to explore the stability of 1,9-decanediol (by measuring its disappearance in soil, or measurements rates of $^{14}CO_2$ emissions, after ^{14}C labelling this biological NI, which may improve our understanding of the factors affecting the efficacy of 1,9-decanediol.
5. This study focused on soil N_2O, NO, N_2 and CO_2 emissions during nitrification and denitrification processes. Ammonia (NH_3) emissions were not included in this study. However, previous studies showed that the NIs application retains higher soil NH_4^+ concentrations, thus increasing NH_3 emissions (Lam et al., 2017; Sánchez-Rodríguez et al., 2018; Soares et al., 2012). Attention should be paid to NH_3 emissions when biological NIs are applied in future studies.
6. In this study, we only explored the effects, efficacy and mechanism of the BNI compounds, LA, LN and 1,9-decanediol, and the root exudates of *Brachiaria humidicola* on soil nitrification. In

recent years, more and more plants have been confirmed to have the ability to suppress the soil nitrification, and more and more BNI compounds have been identified. A greater understanding of the efficacy of these newly identified biological NIs could support more sustainable N use, improve NUE and reduce N losses in the future.

7. Biological NIs, LA, LN and 1,9-decanediol, and synthetic NI, DCD differ in their water solubilities. Future research, identifying the effects of leaching on the efficacy of NIs will improve our understanding of their fate in the soil and guide better management of NIs in agricultural systems.

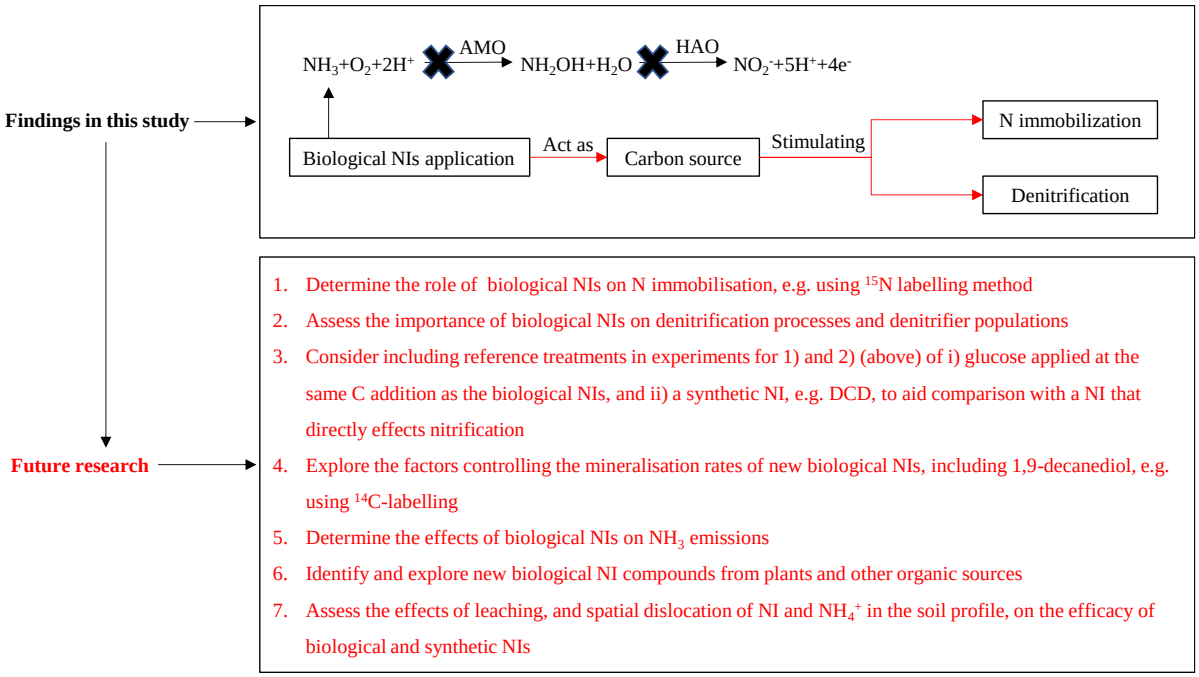

Fig. 7.1 Summary of the thesis. Note: red arrows represent new knowledge developed in the thesis.

7.7. References

Balaine, N., Clough, T.J., Kelliher, F.M., van Koten, C., 2015. Soil aeration affects the degradation rate of the nitrification inhibitor dicyandiamide. Soil Research 53, 137. doi:10.1071/SR14162

Barrena, I., Menéndez, S., Correa-Galeote, D., Vega-Mas, I., Bedmar, E.J., González-Murua, C., Estavillo, J.M., 2017. Soil water content modulates the effect of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on nitrifying and denitrifying bacteria. Geoderma 303, 1–8. doi:10.1016/j.geoderma.2017.04.022

Benckiser, G., Christ, E., Herbert, T., Weiske, A., Blome, J., Hardt, M., 2013. The nitrification inhibitor 3,4-dimethylpyrazole-phosphat (DMPP) - quantification and effects on soil metabolism. Plant and Soil 371, 257–266. doi:10.1007/s11104-013-1664-6

Brath, G., Von Tucher, S., Schmidhalter, U., 2008. Effectiveness of 3,4-Dimethylpyrazole Phosphate as Nitriflcation Inhibitor in Soil as Influenced by Inhibitor Concentration, Application Form, and Soil Matric Potential1 1 Project supported by the Federal Ministry of Education and Research, Bonn, Germany. Pedosphere 18, 378–385. doi:10.1016/S1002-0160(08)60028-4

Cameron, K.C., Di, H.J., 2002. The use of a nitrification inhibitor, dicyandiamide (DCD), to decrease nitrate leaching and nitrous oxide emissions in a simulated grazed and irrigated grassland. Soil Use and Management 18, 395–403. doi:10.1079/SUM2002151

Cardenas, L.M., Misselbrook, T.M., Hodgson, C., Donovan, N., Gilhespy, S., Smith, K.A., Dhanoa, M.S., Chadwick, D., 2016. Effect of the application of cattle urine with or without the nitrification inhibitor DCD, and dung on greenhouse gas emissions from a UK grassland soil. Agriculture, Ecosystems and Environment 235, 229–241. doi:10.1016/j.agee.2016.10.025

Chen, G., Zhu, H., Zhang, Y., 2003. Soil microbial activities and carbon and nitrogen fixation. Research in Microbiology 154, 393–398. doi:10.1016/S0923-2508(03)00082-2

Firestone, M.K., Davidson, E.A., 1989. Microbiologial Basis of NO and N2O production and consumption in soil. Exchange of Trace Gases between Terrestrial Ecosystems and the Atmosphere 7–21. doi:10.1017/CBO9781107415324.004

Gilsanz, C., Báez, D., Misselbrook, T.H., Dhanoa, M.S., Cárdenas, L.M., 2016. Development of emission factors and efficiency of two nitrification inhibitors, DCD and DMPP. Agriculture, Ecosystems and Environment 216, 1–8. doi:10.1016/j.agee.2015.09.030

Gong, P., Zhang, L.L., Wu, Z.J., Chen, Z.H., Chen, L.J., 2013. Responses of Ammonia-Oxidizing Bacteria and Archaea in Two Agricultural Soils to Nitrification Inhibitors DCD and DMPP: A Pot Experiment. Pedosphere 23, 729–739. doi:10.1016/S1002-0160(13)60065-X

Gopalakrishnan, S., Subbarao, G. V., Nakahara, K., Yoshihashi, T., Ito, O., Maeda, I., Ono, H., Yoshida, M., 2007. Nitrification inhibitors from the root tissues of Brachiaria humidicola, a tropical grass. Journal of Agricultural and Food Chemistry 55, 1385–1388. doi:10.1021/jf062593o

Gopalakrishnan, S., Watanabe, T., Pearse, S.J., Ito, O., Hossain, Z.A.K.M., Subbarao, G. V, 2009. Biological nitrification inhibition by Brachiaria humidicola roots varies with soil type and inhibits nitrifying bacteria, but not other major soil microorganisms. Soil Science and Plant Nutrition 55, 725–733. doi:10.1111/j.1747-0765.2009.00398.x

Guardia, G., Marsden, K.A., Vallejo, A., Jones, D.L., Chadwick, D.R., 2018. Determining the influence of environmental and edaphic factors on the fate of the nitrification inhibitors DCD and DMPP in soil. Science of the Total Environment 624, 1202–1212. doi:10.1016/j.scitotenv.2017.12.250

Henderson, S.L., Dandie, C.E., Patten, C.L., Zebarth, B.J., Burton, D.L., Trevors, J.T., Goyer, C., Al, H.E.T., Icrobiol, A.P.P.L.E.N.M., 2010. Changes in Denitrifier Abundance , Denitrification Gene mRNA Levels , Nitrous Oxide Emissions , and Denitrification in Anoxic Soil Microcosms Amended with Glucose and Plant Residues doi:10.1128/AEM.02993-09

Kelliher, F.M., Clough, T.J., Clark, H., Rys, G., Sedcole, J.R., 2008. The temperature dependence of dicyandiamide (DCD) degradation in soils: A data synthesis. Soil Biology and Biochemistry 40, 1878–1882. doi:10.1016/j.soilbio.2008.03.013

Knowles, R., 1982. Denitrification. Microbiological Reviews 46, 43–70. doi:10.1128/CMR.5.4.356.Updated

Liu, R., Hayden, H., Suter, H., He, J., Chen, D., 2015. The effect of nitrification inhibitors in reducing nitrification and the ammonia oxidizer population in three contrasting soils. Journal of Soils and Sediments 15, 1113–1118. doi:10.1007/s11368-015-1086-6

Lu, Y., Zhang, X., Jiang, J., Kronzucker, H.J., Shen, W., Shi, W., 2019. Effects of the biological nitrification inhibitor 1,9-decanediol on nitrification and ammonia oxidizers in three agricultural soils. Soil Biology and Biochemistry 129, 48–59. doi:10.1016/j.soilbio.2018.11.008

Magill, A.H., Aber, J.D., 2000. Variation in soil net mineralization rates with dissolved organic carbon

additions. Soil Biology and Biochemistry 32, 597–601. doi:10.1016/S0038-0717(99)00186-8

Marsden, K.A., Marín-Martínez, A.J., Vallejo, A., Hill, P.W., Jones, D.L., Chadwick, D.R., 2016. The mobility of nitrification inhibitors under simulated ruminant urine deposition and rainfall: a comparison between DCD and DMPP. Biology and Fertility of Soils 52, 491–503. doi:10.1007/s00374-016-1092-x

McGeough, K.L., Watson, C.J., Müller, C., Laughlin, R.J., Chadwick, D.R., 2016. Evidence that the efficacy of the nitrification inhibitor dicyandiamide (DCD) is affected by soil properties in UK soils. Soil Biology and Biochemistry 94, 222–232. doi:10.1016/j.soilbio.2015.11.017

Meena, H.M., Sachdev, M.S., Manjaiah, K.M., Dotaniya, M.L., 2014. Nitrification Inhibition Potential of Brachiaria humidicola. National Academy Science Letters 37, 113–116. doi:10.1007/s40009-013-0216-1

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

Nair, D., Baral, K.R., Abalos, D., Strobel, B.W., Petersen, S.O., 2020. Nitrate leaching and nitrous oxide emissions from maize after grass-clover on a coarse sandy soil: Mitigation potentials of 3,4-dimethylpyrazole phosphate (DMPP). Journal of Environmental Management 260. doi:10.1016/j.jenvman.2020.110165

Nardi, P., Akutsu, M., Pariasca-Tanaka, J., Wissuwa, M., 2013. Effect of methyl 3-4-hydroxyphenyl propionate, a Sorghum root exudate, on N dynamic, potential nitrification activity and abundance of ammonia-oxidizing bacteria and archaea. Plant and Soil 367, 627–637. doi:10.1007/s11104-012-1494-y

Robinson, A., Di, H.J., Cameron, K.C., Podolyan, A., He, J., 2014. The effect of soil pH and dicyandiamide (DCD) on N2O emissions and ammonia oxidiser abundance in a stimulated grazed pasture soil. Journal of Soils and Sediments 14, 1434–1444. doi:10.1007/s11368-014-0888-2

Schimel, J.P., Cleve, K. Van, Cates, R.G., Clausen, T.P., Reichardt, P.B., 1996. Effects of balsam poplar (Populus balsamifera) tannins and low molecular weight phenolics on microbial activity in taiga floodplain soil: implications for changes in N cycling during succession. Canadian Journal of Botany 74, 84–90. doi:10.1139/b96-012

Schipper, L.A., Matiasek, M.G., Scow, K.M., Cameron, S., Bruesewitz, D.A., Mcdonald, I.R., 2011. Nitrate removal , communities of denitrifiers and adverse effects in different carbon substrates for use in denitrification beds So 5. doi:10.1016/j.watres.2011.08.007

Shi, X., Hu, H., He, J., Chen, D., Suter, H.C., 2016. Effects of 3,4-dimethylpyrazole phosphate (DMPP) on nitrification and the abundance and community composition of soil ammonia oxidizers in three land uses. Biology and Fertility of Soils 52, 927–939. doi:10.1007/s00374-016-1131-7

Souri, M.K., 2016. Plants adaptation to control nitrification process in tropical region; case study with Acrocomia totai and Brachiaria humidicola plants. Open Agriculture 1, 144–150. doi:10.1515/opag-2016-0019

Souri, M.K., Neumann, G., 2010. Nitrification Inhibition Properties in Root Exudates of Brachiaria humidicola Plants 5, 13–25.

Subbarao, G. V., Ishikawa, T., Ito, O., Nakahara, K., Wang, H.Y., Berry, W.L., 2006. A bioluminescence assay to detect nitrification inhibitors released from plant roots: A case study with Brachiaria humidicola. Plant and Soil. doi:10.1007/s11104-006-9094-3

Subbarao, G. V., Nakahara, K., Hurtado, M.P., Ono, H., Moreta, D.E., Salcedo, A.F., Yoshihashi, A.T., Ishikawa, T., Ishitani, M., Ohnishi-Kameyama, M., Yoshida, M., Rondon, M., Rao, I.M., Lascano, C.E., Berry, W.L., Ito, O., 2009. Evidence for biological nitrification inhibition in Brachiaria pastures. Proceedings of the National Academy of Sciences 106, 17302–17307.

doi:10.1073/pnas.0903694106

Subbarao, G. V., Tomohiro, B., Masahiro, K., Osamu, I., Samejima, H., Wang, H.Y., Pearse, S.J., Gopalakrishnan, S., Nakahara, K., Zakir Hossain, A.K.M., Tsujimoto, H., Berry, W.L., 2007a. Can biological nitrification inhibition (BNI) genes from perennial Leymus racemosus (Triticeae) combat nitrification in wheat farming? Plant and Soil. doi:10.1007/s11104-007-9360-z

Subbarao, G. V., Wang, H.Y., Ito, O., Nakahara, K., Berry, W.L., 2007b. NH4+ triggers the synthesis and release of biological nitrification inhibition compounds in Brachiaria humidicola roots. Plant and Soil 290, 245–257. doi:10.1007/s11104-006-9156-6

Subbarao, G. V, Nakahara, K., Ishikawa, T., 2008. Free fatty acids from the pasture grass Brachiaria humidicola and one of their methyl esters as inhibitors of nitrification 89–99. doi:10.1007/s11104-008-9682-5

Subbarao, G. V, Nakahara, K., Ishikawa, T., Ono, H., Yoshida, M., Yoshihashi, T., Zhu, Y., Zakir, H.A.K.M., Deshpande, S.P., Hash, C.T., Sahrawat, K.L., 2013. Biological nitrification inhibition (BNI) activity in sorghum and its characterization. Plant Soil 366, 243–259. doi:10.1007/s11104-012-1419-9

Sun, L., Lu, Y., Yu, F., Kronzucker, H.J., Shi, W., 2016. Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency. New Phytologist 212, 646–656. doi:10.1111/nph.14057

Tiedje, J.M., 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium.

Vinten, A.J.A., Whitmore, A.P., Bloem, J., Howard, R., Wright, F., 2002. Factors affecting N immobilisation/mineralisation kinetics for cellulose-, glucose- and straw-amended sandy soils. Biology and Fertility of Soils 36, 190–199. doi:10.1007/s00374-002-0524-y

Xu, J., Zhu, T., Xue, W., Ni, D., Sun, Y., Yang, J., Xu, L., Chen, X., Li, H., Liu, M., 2019. Influences of nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) and application method on nitrogen dynamics at the centimeter-scale. European Journal of Soil Biology 90, 44–50. doi:10.1016/j.ejsobi.2018.12.004

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

Zhang, X., Lu, Y., Yang, T., Kronzucker, H.J., Shi, W., 2019. Factors influencing the release of the biological nitrification inhibitor 1,9-decanediol from rice (Oryza sativa L.) roots. Plant and Soil 436, 253–265. doi:10.1007/s11104-019-03933-1

Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. Microbiology and Molecular Biology Reviews : MMBR 61, 533–616. doi:10.1128/.61.4.533-616.1997

Appendix 1: Supplementary material for Chapter 3

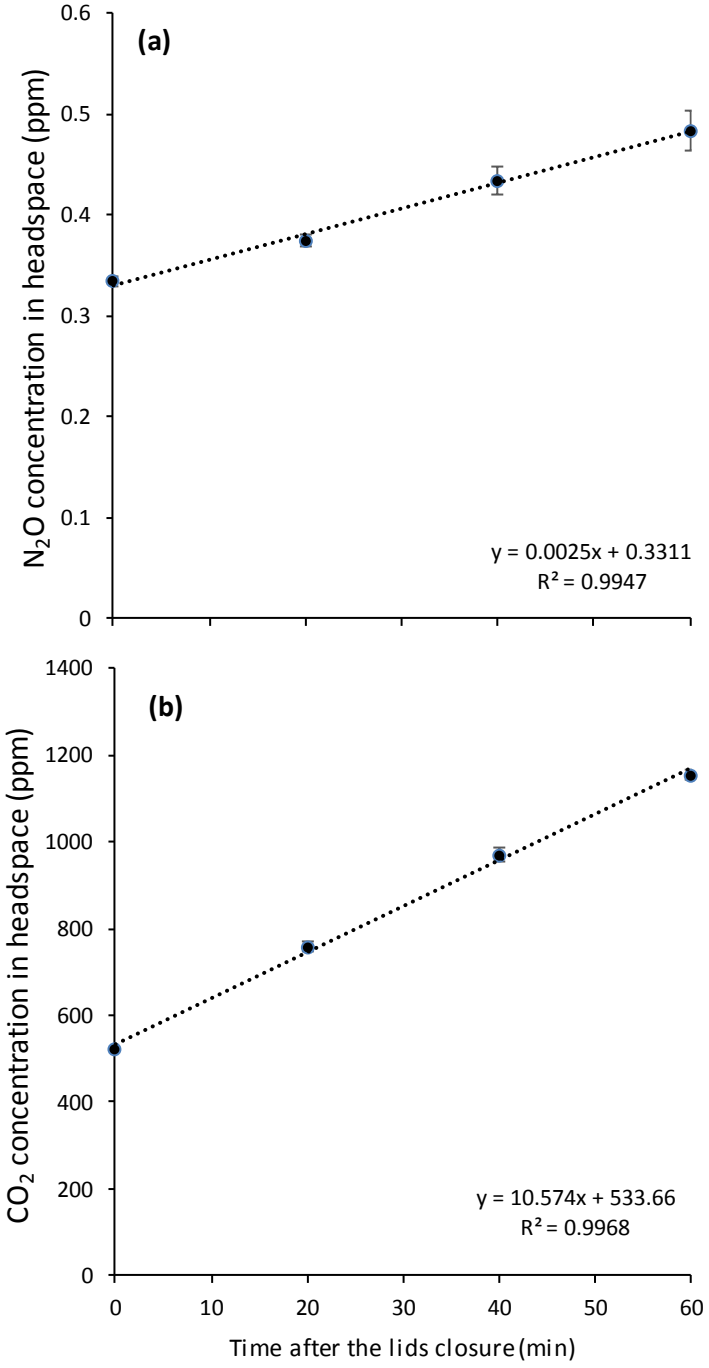

Fig. 1 N_2O (panels, a) and CO_2 (panels, b) concentration in headspace over 1 hour.

Appendix 2: Supplementary material for Chapter 4

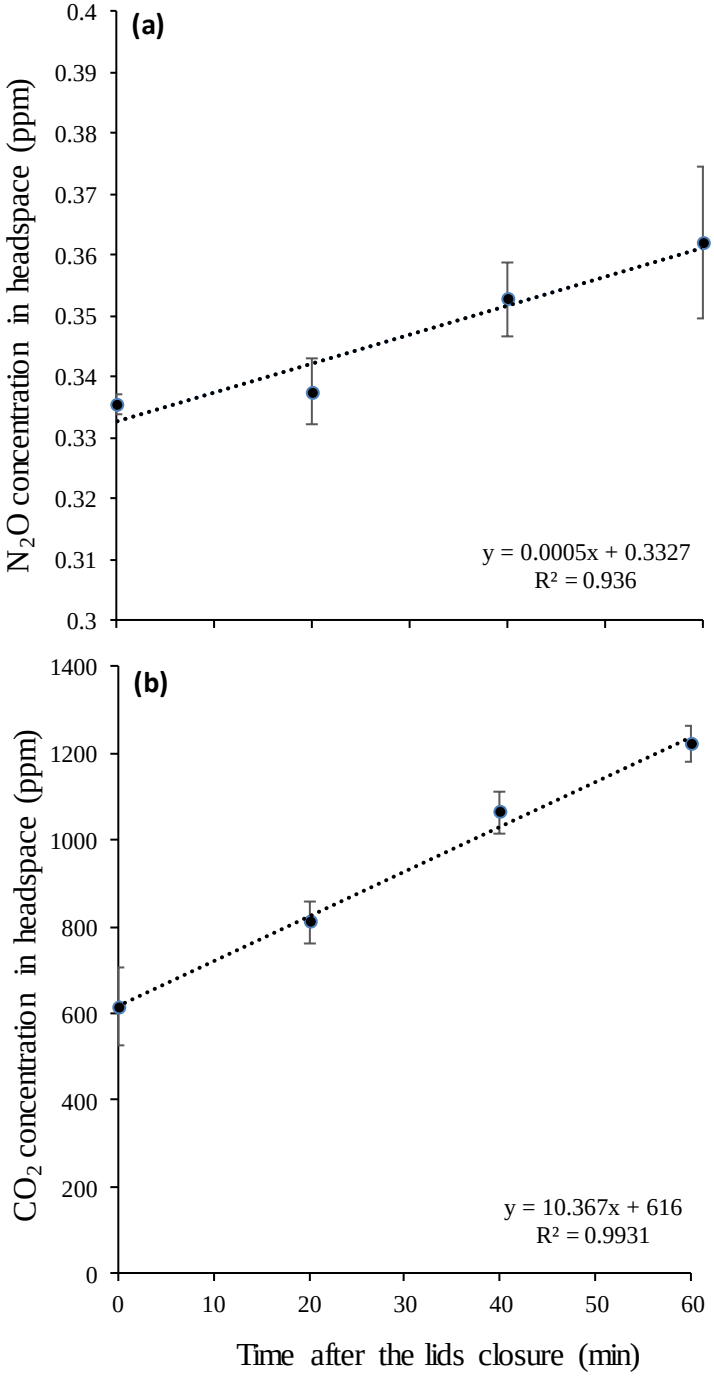

Fig. 1 N_2O (panels, a) and CO_2 (panels, b) concentration in headspace over 1 hour.

Appendix 3: Supplementary material for Chapter 5

Fig. 1 The experiment setup of the Denitrification system (DENIS)

Table 1 Primer sets used for the real-time PCR

Targeting gene	Primer set	Sequence (5'-3')	Reference
AOA	Arch-amoAF	STAATGGTCTGGCTTAGACG	(Robinson et al., 2014)
	Arch-amoAR	GCGGCCATCCATCTGTATGT	
AOB	amoA-1F	GGGGTTTCTACTGGTGGT	(Robinson et al., 2014)
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC	
nirK	FlaCu	ATCATGGTSCTGCCGCG	(Zulkarnaen et al., 2019)
	R3Cu	GCCTCGATCAGRTTGTGGTT	
nirS	cd3aF	GTSAACGTSAAGGARACSGG	(Zulkarnaen et al., 2019)
	R3cd	GASTTCGGRTGSGTCTTGA	
nosZ	2F	CGCRACGGCAASAAGGTSMSSGT	(Zulkarnaen et al., 2019)
	2R	CAKRTGCAKSGCRTGGCAGAA	

References

Robinson, A., Di, H.J., Cameron, K.C., Podolyan, A., He, J., 2014. The effect of soil pH and dicyandiamide (DCD) on N2O emissions and ammonia oxidiser abundance in a stimulated grazed pasture soil. Journal of Soils and Sediments 14, 1434–1444. doi:10.1007/s11368-014-0888-2

Zulkarnaen, N., Zhang, Y., Zhang, P., Liu, Y., Cheng, Y., Zhao, J., Zhang, J., 2019. Abundance of AOA, AOB, nir S, nir K, and nos Z in red soil of China under different land use . IOP Conference Series: Earth and Environmental Science 393, 012007. doi:10.1088/1755-1315/393/1/012007

Appendix 4: Supplementary material for Chapter 6

Details of six denitrification incubations used in Chapter 6

The 6 incubations referred to in this Chapter were conducted by Dr Laura Cardenas and other researchers at Rothamsted Research (North Wyke) in 2001-2002. I was not involved in the experimentation, but was responsible for taking the raw data and calculating fluxes, cumulative fluxes, the statistical analyses and interpretation of the results. Below, I summarise the experimental details (for completeness).

1. Soil site and sampling regime

Soil samples used in this study were collected from an experimental site at Rothamsted Research (North Wyke, 50°46´N, 3°54´E). The site was a plot belonging to the Rowden experiment (Blackwell et al., 2018), which had not received inorganic N fertiliser for at least 60 years and did not have artificial drainage. The climate at this site is cool temperate with an average 30-year annual temperature of 10.1 °C and an annual average total rainfall of 1040 mm (Orr et al., 2016). The soil is a clayey pelostagnogley of the Hallsworth series (Clayden et al., 1984), or a FAO dystric gleysol (FAO, 2006).

Intact soil cores (0-10cm depth) were collected using stainless steel rings (diameter: 14.3 cm). Soil cores were carefully dug up and vegetation was trimmed off. The base of each soil core was pared carefully to avoid smearing using a sharp blade, until it was level with the edge of the corer. The top 10 cm was characterised by 36.6% clay, 47.7% silt, 13.9% fine sand and 1.8% coarse sand in the inorganic fraction (Harrod and Hogan, 2008; Schnürer et al., 1985; Scholefield et al., 1997), with the soil organic C content of 5.3% and a soil pH of 5.7. The intact soil samples were watered twice with 100 ml deionised water each prior to incubation and left to drain for two hours to ensure they were at maximum soil water holding capacity (~90% water filled pore space, WFPS).

2. Carbon sources and experimental design

To explore the effect of C quality on N_2O and N_2 emissions, a series of incubations were conducted in the Denitrification System (DENIS) (Cárdenas et al., 2003). In each incubation, the intact soil cores were engineered to fit tightly within 12 incubation vessels (143 mm diameter, 120 mm height). The vessels were then sealed with stainless steel lids that incorporated two 'O' rings. In order to purge the N_2 from the soil atmosphere, headspace and all gas lines, a mixture of He: O_2 (80: 20, each of 99.999% purity) was passed through the inlet at the base of the vessel at flow rates of about 100 ml min^{-1} per vessel, with the outlet from each vessel passing through a 16-port Valco valve (flow-through mode) to N_2O and N_2 detectors. Once baseline N_2 concentrations were achieved in the airflow from all vessels, the He + O_2 mixture was directed to the vessel via the lid of the vessel, in a flow-over mode. The flow rate of the mixture was decreased to about 38 ml min^{-1} with 16% O_2. After replacement of the atmosphere within the soil cores, the corresponding amendments (C and N sources) were added from a He/O_2 flushed vessel fitted to the lid of the intact soil cores, by turning a ball valve. During the incubation, the temperature of the vessels was kept constant at 15ºC.

Six incubations were conducted in this study: 1) Inc-Van; 2) Inc-Cel; 3) Inc-Glu, 4) Inc-But; 5) Inc-FSl; 6) Inc-ASl (vanillin, cellulose, glucosamine, butyric acid, fresh cattle slurry and aged cattle slurry were applied as C source in each incubation respectively) (Table 1). The treatments in each incubation were 1) NO_3^-; 2) NO_3^- + glucose; 3) NO_3^- + C source (Inc-Van, Inc-Cel, Inc-Glu, Inc-But)/NH_4^+ (Inc-FSl, Inc-ASl). C was added at an equivalent rate of 396 kg C ha^{-1}. NO_3^- was also added to each vessel, at 75 kg N ha^{-1} in a volume of 50 ml deionised water, to provide a source of N for the denitrifiers. Four incubations (Inc-Van, Inc-Cel, Inc-Glu and Inc-But) were carried out to assess the specific C compounds (vanillin, cellulose, glucosamine and butyric acid) on soil N_2O and N_2 emissions during denitrification. Each of these 4 incubations contained a NO_3^- only treatment (as a reference or zero control) and a NO_3^- + glucose treatment (as a positive control). The NO_3^- + glucose treatment would also provide information on the denitrification potential of the soil (Dendooven and Anderson, 1994). Two further incubations (Inc-FSl and Inc-ASl) were carried out to assess the effect of cattle slurry on denitrification losses. Inc-FSl and Inc-ASl also had a NO_3^- only treatment, but the NO_3^- + glucose treatment was replaced with NO_3^- + NH_4^+ as slurries retain inorganic N in the NH_4^+-N form but not the NO_3^--N due to the anaerobic condition (Bastami et al., 2018). The same amount of NH_4^+ as that

contained in the cattle slurries was applied, in order to account for the NH_4^+ content present in the slurry that could affect the production of N_2O (either during nitrification or following subsequent denitrification) (Table 2 shows the N and C rates in kg ha^{-1}). Each treatment had 4 replicates, so the inclusion of the NO_3^- only and NO_3^- + glucose treatments, resulted in sufficient vessels (12 in total) in the DENIS system for one of the C sources in each incubation.

Table 1 Treatments in the six incubations (n=4).

Incubation	Incubation name	Treatments
1	Inc-Van	NO_3^-, NO_3^- + glucose, NO_3^- + vanillin
2	Inc-Cel	NO_3^-, NO_3^- + glucose, NO_3^- + cellulose
3	Inc-Glu	NO_3^-, NO_3^- + glucose, NO_3^- + glucosamine
4	Inc-But	NO_3^-, NO_3^- + glucose, NO_3^- + butyric acid
5	Inc-FSl	NO_3^-, NO_3^- + NH_4^+, NO_3^- + fresh cattle slurry
6	Inc-ASl	NO_3^-, NO_3^- + NH_4^+, NO_3^- + aged cattle slurry

Table 2 Rates of N and C applied in the NO_3^- + glucose, and NO_3^- + NH_4^+ treatments.

Treatment	NO_3^- + glucose treatment, nitrate added, kg N ha^{-1}	NO_3^- + glucose treatment, C added, kg C ha^{-1}	NO_3^- + NH_4^+ treatment, nitrate added, kg N ha^{-1}	NO_3^- + NH_4^+ treatment, ammonium added, kg N ha^{-1}
Inc-Van	75	396	na	na
Inc-Cel	75	396	na	na
Inc-Glu	75	396	na	na
Inc-But	75	396	na	na
Inc-FSl	na	na	75	1.1
Inc-ASl	na	na	75	4.7

na: not applicable

Cattle slurry was obtained from the reception pit (fresh) and the slurry tank of a commercial beef farm (aged). The cattle slurry was sieved through a 670 μm mesh to ensure that it went through the ball valve connecting the amendment vessel to the incubation vessel. After sieving the slurry, approximately 50 ml were frozen at -20 °C and freeze-dried for 24 hours before being ground in a pestle and mortar in preparation for total C and N analyses in an elemental analyser (Carlo Erba). The slurry NH_4^+ and NO_3^- concentrations were extracted (fresh slurry: 2M KCl, 1:5 (w/v)) and the filtrate was analysed using a colorimetric technique in a Skalar Sans plus segmented-flow analyser linked to a model 1050d diluting auto-sampler (Kamphake et al., 1967; Searle, 1984). These analyses were completed prior to application to the soil in order to provide the same C: N loading as in the C compound experiments. Sieved fresh and aged cattle slurry has 4.6% and 3.2% dry matter, 37.7% and 39.0% total C, 3.5% and 1.9% total N,

0.1% and 0.46% NH_4^+ in dry basis, respectively. According to the result of the analysis, 35.3 g sieved fresh slurry and 53.7 g sieved aged slurry were applied to each vessel, which supplied 0.61 g C (equivalent to 396 kg C ha^{-1}), 1.6 mg NH_4^+-N and 56.8 mg total N for the fresh slurry experiment, and 0.61 g C (equivalent to 396 kg C ha^{-1}), 7.9 mg NH_4^+-N and 32.6 mg total N per vessel for the aged slurry experiment, respectively.

3. Gas measurements and soil analysis

During the pre-flush period and experimental incubations, the N_2O and N_2 concentrations were analysed automatically every 8 minutes, which result in a measurement every 1.5 hour for each vessel (12 vessels in total). N_2O and N_2 were quantified by Electron Capture Detection (ECD) and He Ionisation Detection (HID), respectively (Cárdenas et al., 2003). The separation of N_2O and N_2 was achieved by a stainless steel packed column (2 m long, 4 mm bore) filled with 'Porapak Q' (80-100 mesh) using N_2 as the carrier gas and a PLOT column (30 m long, 0.53 mm i.d.) with He as the carrier gas, respectively (Cárdenas et al., 2003). Gas concentrations were corrected for surface area and the flow rate through each vessel (measured daily by means of glass bubble meter), and fluxes calculated in the units of kg N or C ha^{-1} d^{-1}. The cumulative gas flux was calculated using Genstat (the 19th edition, VSNI, UK) using the Trapezoidal rule (Meijide et al., 2010). At the end of each incubation, soil was removed from the stainless-steel incubation vessels and sieved before KCl (2M) extraction and analysis of the filtrate for NH_4^+ and NO_3^- using the same methods described above.

References

Bastami, M. saufi, Davey, J., Dave, C., 2018. Methane inhibition from a slurry storage by addition of chemical and natural additives. J. Trop. Agric. and Fd. Sc. 46, 1–16.

Blackwell, M.S.A., Jarvis, S.C., Wilkins, R.J., Beaumont, D.A., Cardenas, L.M., Chadwick, D.R., Collins, A.L., Dungait, J.A.J., Gibb, M.J., Hopkins, A., Lee, M.R.F., Misselbrook, T.H., Murray, P.J., Tallowin, J.R.B., 2018. The Importance of Sustained Grassland and Environmental Research: A Case Study From North Wyke Research Station, UK, 1982–2017, 1st ed, Advances in Agronomy. Elsevier Inc. doi:10.1016/bs.agron.2018.01.004

Cárdenas, L.M., Hawkins, J.M.B., Chadwick, D., Scholefield, D., 2003. Biogenic gas emissions from soils measured using a new automated laboratory incubation system. Soil Biology and Biochemistry 35, 867–870. doi:10.1016/S0038-0717(03)00092-0

Clayden, B. (Benjamin), Hollis, J.M. (John M., Soil Survey of England and Wales., 1984. Criteria for

differentiating soil series. Soil Survey.

Dendooven, L., Anderson, J.M., 1994. Dynamics of reduction enzymes involved in the denitrification process in pasture soil. Soil Biology and Biochemistry 26, 1501–1506. doi:10.1016/0038-0717(94)90091-4

FAO, 2006. Guidelines for soil description (2006): FAO, Rome, 4th edition.

Harrod, T.R., Hogan, D. V, 2008. The Soils of North Wyke and Rowden. Soil Survey of England and Wales.

Kamphake, L.J., Hannah, S.A., Cohen, J.M., 1967. Automated analysis for nitrate by hydrazine reduction. Water Research 1, 205–216. doi:10.1016/0043-1354(67)90011-5

Meijide, A., Cardenas, L.M., Bol, R., Bergstermann, A., Goulding, K., Well, R., Vallejo, A., Scholefield, D., 2010. Dual isotope and isotopomer measurements for the understanding of N2O production and consumption during denitrification in an arable soil. European Journal of Soil Science 61, 364–374. doi:10.1111/j.1365-2389.2010.01233.x

Orr, R.J., Murray, P.J., Eyles, C.J., Blackwell, M.S.A., Cardenas, L.M., Collins, A.L., Dungait, J.A.J., Goulding, K.W.T., Griffith, B.A., Gurr, S.J., Harris, P., Hawkins, J.M.B., Misselbrook, T.H., Rawlings, C., Shepherd, A., Sint, H., Takahashi, T., Tozer, K.N., Whitmore, A.P., Wu, L., Lee, M.R.F., 2016. The North Wyke Farm Platform: effect of temperate grassland farming systems on soil moisture contents, runoff and associated water quality dynamics. European Journal of Soil Science 67, 374–385. doi:10.1111/ejss.12350

Schnürer, J., Clarholm, M., Rosswall, T., 1985. Microbial biomass and activity in an agricultural soil with different organic matter contents. Soil Biology and Biochemistry 17, 611–618. doi:10.1016/0038-0717(85)90036-7

Scholefield, D., Hawkins, J.M.B., Jackson, S.M., 1997. Use of a flowing helium atmosphere incubation, technique to measure the effects of denitrification controls applied to intact cores of a clay soil. Soil Biology and Biochemistry 29, 1337–1344. doi:10.1016/S0038-0717(97)00059-X

Searle, P.L., 1984. The berthelot or indophenol reaction and its use in the analytical chemistry of nitrogen: A review. Analyst 109, 549–568. doi:10.1039/AN9840900549