N cycling and microbial dynamics in pasture soils

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This dissertation is submitted in publication format for the degree of Doctor of Philosophy.
Declaration

This dissertation is the result of my own work and contains nothing which is the outcome of work done in collaboration with others, except where clearly stated.

No part of this dissertation has been submitted for any other degree or qualification.

This thesis project was undertaken in the laboratory of Dr Sergio E. Morales in the Department of Microbiology and Immunology, University of Otago in Dunedin, New Zealand between February 2014 and April 2017. A portion of this research was also performed in the laboratory of Prof. Lars R. Bakken at the Department of Environmental Sciences, Norwegian University of Life Sciences in Ås, Norway between May 2014 and July 2014.
Thesis by publication format

This dissertation has been submitted by publication format. The Otago School of Medical Science and the Department of Microbiology and Immunology provided consultation on how to submit this format. This thesis is presented in accordance with their guidelines.

Chapter two and three are primary research papers that have been accepted for publications. Chapter four has been prepared as a manuscript. Chapter five has been prepared and submitted to a journal. The extended introduction and summary sections were written specifically for this thesis. They serve to place the work in a wider context and bring together the findings as a whole. In accordance with requirements, each chapter has been reformatted for consistency.

I took the leading role in the work presented here. For each study, I designed experiments, performed research, analyzed data and interpreted results, except where clearly stated. I contributed significantly to writing and revising each manuscript as first-author. Each publication also contains contributions by co-authors of varying size and importance, and this is clearly stated for each manuscript. Author contributions are detailed using the initials of each author at the start of each chapter.
Abstract

Pasture soils are a significant source of the greenhouse gas, nitrous oxide (N\textsubscript{2}O) and as such they contribute to global warming. It has been reported that N\textsubscript{2}O is approx. 300 times more potent than carbon dioxide (CO\textsubscript{2}) as a greenhouse gas. Thus, understanding the mechanisms for controlling N\textsubscript{2}O emissions from soil is key to developing new soil management strategies to counter or prevent climate change throughout the world. Despite this, very little is known about the key regulators of production and consumption of N\textsubscript{2}O in pasture soils, especially under urine patch conditions. To address this, we used pasture soils representing both Northern (Ireland) and Southern (New Zealand) Hemispheres in experiments designed to understand both phenotypic and genotypic characteristics associated with N\textsubscript{2}O emissions. We used a combination of gas kinetics, soil physicochemical characterization, metagenomics, 16S amplicon sequencing and quantitative PCR (of denitrifier: nir\textit{S}, nir\textit{K}, nos\textit{ZI} and nos\textit{ZII}; and nitrifier: bacterial and archaeal \textit{amoA} genes) to link physical, chemical and biological parameters associated with emissions. This thesis work was able to show how in nitrate-amended pasture soils the rate of carbon mineralization under oxic and anoxic conditions is positively linked to the rate of denitrification. In addition, the emission ratio of N\textsubscript{2}O is negatively linked to pH. Both pH and N\textsubscript{2}O emission ratio were significantly associated with 16S microbial community composition as well as microbial richness. This result confirms that pH imposes a general selective pressure on the entire community and that this is associated with changes in emission potentials. This supports the general ecological hypothesis that with increased microbial diversity, efficiency of N\textsubscript{2} production increases (i.e. more efficient conversation of N\textsubscript{2}O to N\textsubscript{2}). Worked performed in a simulated urine patch (oxic conditions) suggested other pathway (e.g., nitrifier-denitrification) as a source of N\textsubscript{2}O emissions. No clear trend was observed between emission ratio of N\textsubscript{2}O under urine patch condition and emission ratio under true denitrification conditions (i.e. under anoxic environment). The urine patch accelerated the rate of C mineralization about 10 times, concurrent with a decrease in prokaryotic richness and a shift in community composition. Community response identified two major groups of responders: negatively affected prokaryotes we hypothesized utilized energy from N-linked redox reaction for maintenance and positively responding populations that use this energy for growth. Overall, this study
provides new insights into the N₂O emissions and microbial dynamics for reduction of N₂O in pasture soils.
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Abbreviations

AFP  Air filled porosity
AMO  Ammonium monooxygenase
ANOVA Analysis of variance
AOA  Ammonia-oxidizing archaea
AOB  Ammonia-oxidizing bacteria
ATP  Adenosine Triphosphate
BLAST Basic local alignment search tool
C    Carbon
C:N  Carbon and nitrogen ratio
CaCl₂ Calcium chloride
CEC  Cation exchange capacity
CO₂  Carbon dioxide
Cu   Copper
DI   Deionized water
DNA  Deoxyribonucleic acid
DNRA Dissimilatory nitrate reduction to ammonium
dNTP Deoxynucleotide triphosphates
e⁻   Electron
ECD  Electron capture detector
Fe²⁺ Ferrous
FID  Flame ionization detector
g    Gram
GC   Gas chromatography
GHG  Greenhouse gas
GWP  Global Warming Potential
h    Hour
H⁺   Proton
H₂   Hydrogen
H₂O  Water
ha
ha⁻¹
HAO
He
HM
HURM
\( /N_2O \)
KCl
kg
kPa
LM
M
Mg m⁻³
ml
mm
Mn²⁺
Mo-bis-MGD
mRNA
N₂/N
N₂O
N₂OR
nar
NH₂OH
NH₃
NH₄⁺
nir
NMDS
NO
NO₂⁻
NO₃⁻
NOB
nor
nos
O₂
Hectare
Per hectare
Hydroxylamine oxidoreductase
Helium
High moisture
Hydroxylamine/hydrazine-ubiquinone-redox-module
N₂O production index
Potassium chloride
Kilogram
KiloPascals
Low moisture
Molar
Megagrams per cubic meter
Milliliter
Millimeter
Manganese
Molybdenum-bis-molybdopterin guanine dinucleotide
Messenger RNA
Dinitrogen/Nitrogen
Nitrous oxide
Nitrous oxide reductase
Nitrate reductase
Hydroxylamine
Ammonia
Ammonium
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Non-metric multidimensional scaling
Nitric oxide
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Nitrate
Nitrite-oxidizing bacteria
Nitric oxide reductase
Nitrous oxide reductase
Oxygen
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<tr>
<td>°C</td>
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<tr>
<td>OTUs</td>
<td>Operational Taxonomic Units</td>
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<tr>
<td>PMF</td>
<td>Proton-motive force</td>
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<tr>
<td>ppb</td>
<td>Parts per billion</td>
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<tr>
<td>Ps az</td>
<td>Pseudoazurin</td>
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<td>Q/QH2</td>
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<td>WFPS</td>
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CHAPTER 1

General introduction
1.1. \( \text{N}_2\text{O} \) in the atmosphere

Nitrous oxide (\( \text{N}_2\text{O} \)) is an intermediate product in the natural process of nitrogen (N) cycling and is known as a greenhouse gas (GHG). \( \text{N}_2\text{O} \) is about 298 times more effective at trapping heat than carbon dioxide (\( \text{CO}_2 \)) over a 100-year period and has an atmospheric life of approximately 121 years (IPCC, 2007; Myhre et al., 2013). It is the second most important GHG after \( \text{CO}_2 \), and is known to deplete the stratospheric ozone layer (Ravishankara et al., 2009). The concentration of \( \text{N}_2\text{O} \) in the atmosphere has increased by 20% from 271 ppb to 324 ppb over the last 260 years (Myhre et al., 2013). Soils, sediments, and water bodies all contribute to the production of \( \text{N}_2\text{O} \) as part of microbial and abiotic processes. The major source of \( \text{N}_2\text{O} \) are agricultural soils (Cole et al., 1997; Paustian et al., 2004; Mosier et al., 1998), especially direct \( \text{N}_2\text{O} \) emissions from fertilized soils, animal production (from urine) as well as indirect \( \text{N}_2\text{O} \) emissions from nitrogen (N) used in agriculture (e.g. leaching and runoff, atmospheric deposition) (Mosier, 1998; Syakila and Kroeze, 2011). Combined, emissions from N fertilizer application and animal production (4.3-5.8 Tg \( \text{N}_2\text{O} \)-N yr\(^{-1}\)), and emissions from natural soils (i.e., unmanaged soils; 6-7 Tg \( \text{N}_2\text{O} \)-N yr\(^{-1}\)) represent 56-70% of all global \( \text{N}_2\text{O} \) sources (Syakila and Kroeze, 2011; Butterbach-Bahl et al., 2013). More importantly, grazed pasture soils contribute 41% of global \( \text{N}_2\text{O} \) emissions (direct and indirect) through animal excreta (Oenema et al., 2005).

In New Zealand, pastoral farming is the dominant agricultural sector and is characterized by year-round grazing of clover-based pastures. As a result, N-deposition by grazing animals is the single largest source of direct \( \text{N}_2\text{O} \) emissions in New Zealand contributing over 50% of emissions (de Klein et al., 2003). An additional 30% of emissions were from indirect emission (e.g. leached and volatilized excreta-N) (de Klein et al., 2003). More details about urine patch and how it contributes to \( \text{N}_2\text{O} \) emission in section 1.3.

1.2. The nitrogen (N) cycle

The N cycle involves several redox reactions (i.e. oxidation and reduction ranging from +5 to -3 as illustrated in Figure 1.1) catalyzed by different enzymes within bacteria, archaea and some fungi. This cycle can be broken down into modular reactions which include: ammonification, assimilation, nitrification, denitrification, nitrogen fixation, anammox and dissimilatory nitrate reducing to ammonium (DNRA) pathways.
In pasture ecosystems, N is deposited in soil as urea via urine patches. Urea is an organic compound, which undergoes several N transformation processes: for example, ammonification, nitrification, denitrification. Ammonification is a process in which organic nitrogen is converted to ammonia (NH₃). This process can be performed by many microbes, plants and animals. Ammonia can be exited in the form of ammonium (NH₄⁺) in acidic or neutral environments. NH₄⁺ can be assimilated by many microbes and plants, where they are incorporated into amino acids and other nitrogen-containing biomolecules. In nitrification, NH₃ or NH₄⁺ are oxidized to nitrite ions (NO₂⁻), which is further oxidized to nitrate ions (NO₃⁻) (Figure 1.1). Nitrate ions can be incorporated or assimilated by a wide range of organisms (e.g. bacterial, fungal and algal species) into organic matter via assimilatory NO₃⁻ reduction. Under anaerobic conditions, nitrate ions can act as terminal electron acceptors. This process is known as nitrate respiration, or dissimilatory nitrate reduction. One of the dissimilatory nitrate reduction pathways is called denitrification. In denitrification, NO₃⁻ is first converted into NO₂⁻ then gaseous N (NO, N₂O and N₂). As a result of denitrification, soils lose NO₃⁻ which is one of the important nutrients for farming. However, denitrification can play an important role for removal of NO₃⁻ from wastewater treatment to prevent eutrophication (Knowles, 1982). Other pathways in N cycling includes N fixation, DNRA and anammox. In N fixation, soils gain N from the atmosphere as an inorganic source through N fixation (N₂ to NH₄⁺) using nitrogenase enzyme. In DNRA, NO₃⁻ can be transformed into the NH₄⁺ which is a reverse process of nitrification. In anammox (anaerobic ammonium oxidation), NH₄⁺ and NO₂⁻ are directly converted into N₂. This process has a great interest in wastewater treatment. A detailed description of denitrification and nitrification is given below as both are involved in urine patch kinetics.
Figure 1.1. Microbial transformations within the N cycle. "Org-N" refers to organic nitrogen (e.g. urine/urea). N-transformation pathways and genes denoted as colored arrows and italic-text.

Figure 1.2. Nitrogen transformations within a soil urine patch (modified from Wrange et al., (2001)). For explanation see text.
Figure 1.3. Schematic diagram illustrating the nitrification process by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) at the cellular level. Solid lines represent experimentally verified reactions, dotted lines (with question marks) indicate lack of experimental verification of reactions. Abbreviation: HURM, hydroxylamine/hydrazine-ubiquinone-redox-module; (c)aa3, cytochrome (c)aa3; bc1, cytochrome bc1 (complex II); NirK, Cu-dependent nitrate reductase; c'-β, cytochrome c'-β; c550, cytochrome c552; cM552, cytochrome cM552; c554, cytochrome c554; NXR, nitrite oxidoreductase; P460, cytochrome P460; PMF, proton-motive force; Q/QH2, ubiquinone-ubiquinol pool; sNOR, cNOR, ccNOR, nitric oxide reductase with differing electron acceptor mechanisms. See text for more details. Figure taken from Klotz and Stein (2007).
1.2.1. Nitrification

In general, nitrification is a two-step aerobic oxidative process where NH$_3$/NH$_4^+$ is first oxidized to NO$_2^-$ and subsequently NO$_3^-$ by two different specialist prokaryotic groups, namely the ammonia oxidizing bacteria and/or archaea (AOA & AOB) and the nitrite-oxidizing bacteria (NOB) (van Kessel et al., 2015) (Figure 1.2). The transformation of NH$_4^+$ to NO$_2^-$ produces hydroxylamine (NH$_2$OH) which is one of the first several intermediates during nitrification. This transformation is catalyzed by the enzyme ammonium monooxygenase (AMO). The NH$_2$OH is then oxidized to NO$_2^-$ by hydroxylamine oxidoreductase (HAO), and finally NO$_2^-$ is oxidized to NO$_3^-$ by nitrite oxidoreductase (NRX). Soil NO$_3^-$ can be lost through leaching and/or can be transformed further to gaseous N (NO, N$_2$O, N$_2$) through denitrification. N$_2$O can be produced during nitrification as a result of decomposition of NH$_2$OH or reduction of NO$_2^-$ to N$_2$O and N$_2$ via nitrifier-denitrification by autotrophic ammonia oxidizers (Wrage et al., 2001; Zhu et al., 2013).

1.2.2. Enzymes involved in nitrification

1.2.2.1. Key enzymes of AOB

NH$_3$ is utilized by AOB as the sole source of energy and the reductant requires four specialized proteins: AMO, HAO, cytochromes c554 and cM552 (Whittaker et al., 2000; Arp et al., 2007) (Figure 1.3). AMO is a membrane-bound hetero-trimeric copper enzyme encoded by three gene subunits, amoA (31.4 kDa), amoB (38 kDa) and amoC (31.4 kDa) (Ge et al., 2015). HAO is located in the periplasmic space and composed of multi-c-heme and homotrimer (64 kDa) subunits (Arp et al., 2002). This enzyme is encoded by the hao gene cluster (1710 bp). The AMO initiates NH$_3$ catabolism by oxidizing NH$_3$ to NH$_2$OH. Subsequently, the oxidation of NH$_2$OH to NO$_2^-$ is catalyzed by HAO. As a result of the oxidation process catalyzed by HAO, four electrons are released which then follow a redox cascade via the two tetrahem cytochromes c554 and cM552 to the electron transport chain at the level of ubiquinone (Hooper et al., 1997; Arp et al., 2007). Among the four electrons that are released from the oxidation of NH$_2$OH by HAO, two are moved towards the oxidation of NH$_3$ in the next cycle and the remaining two are utilized for other reductant-requiring cellular processes, for example biosynthesis and ATP generation (Arp et al., 2007). After generation of NO$_2^-$ from the oxidation of NH$_2$OH by HAO, the NO$_2^-$
can be either transformed into NO$_3^-$ through the NXR enzyme or it can be transformed into NO$\rightarrow$ N$_2$O$\rightarrow$ N$_2$ through the process of nitrifier-denitrification.

1.2.2.2. Key enzymes of NOB

NOB gain energy through the one-step oxidation process of NO$_2^-$ to NO$_3^-$ by the key enzyme NXR (Figure 1.3). NXR is a membrane-bound iron-sulfur molybdoprotein, which shuttles two electrons per oxidized NO$_2^-$ into the electron transport chain (Meincke et al., 1992; Lücker et al., 2010). NXR consists of 3 subunits: NxrA ($\alpha$), NxrB ($\beta$) and NxrC ($\gamma$) (Lücker et al., 2010). The subunit NxrA is known as the substrate-binding site and is located in the periplasmic space in Nitrospira (Lücker et al., 2010; Koch et al., 2015), Nitrospina (Lücker et al., 2013), and ‘Candidatus Nitromaritima’(Ngugi et al., 2016). However, in some bacteria (e.g. Nitrobacter, Nitrococcus, and Nitrolancea) NxrA is located in the cytoplasm (Spieck et al., 1996; Starkenburg et al., 2006; Sorokin et al., 2012). The periplasmic NXR contributes proton motive force (PMF) (where the proton is derived from water) as part of the cell’s energy budget whereas in the cytoplasmic NXR the protons do not contribute to creating a PMF (Lücker et al., 2010; Daims et al., 2016). The periplasmic NXRs are phylogenetically affiliated with the type II enzyme of the DMSO reductase family, whereas cytoplasmic NXRs are phylogenetically linked to nitrate reductase (NARs). It is proposed that two types of NXR evolved independently and likely spread by lateral gene transfer into different organisms, representing the large phylogenetic diversity of NOB (Sorokin et al., 2012; Lücker et al., 2010; 2013).

1.2.3. The role of microbes in nitrification

The nitrifiers are chemolithoautotrophic meaning they use chemical energy from nitrification to fix CO$_2$ as their source of carbon (C). Both AOB and AOA are involved in the oxidation of NH$_3$/NH$_4^+$ . AOA are generally found in higher abundance in most soils compared to AOB, but their contribution to nitrification varies (Leininger et al., 2006; Heil et al., 2015). In nitrogen-rich grassland soils, the contribution of AOA is predicted to be small, despite being present in large numbers compared to AOB, suggesting that nitrification is mainly driven by bacteria rather than archaea with the application of ammonia substrate (Di et al., 2009). This means that AOB gain comparative advantage over archaea in fertilized soils due to biochemical adaptation in high nutrient environment. On the other hand, archaea are comparatively better
adapted than bacteria in a low nutrient environment, or in extreme pH, or both (Valentine, 2007; Di et al., 2009).

In addition, a phylogenetically wide range of heterotrophic bacteria and fungi oxidize NH$_3$ using two proposed pathways (De Boer and Kowalchuk, 2001; Heil et al., 2015). In the first pathway, heterotrophic bacteria (e.g. *Paracoccus denitrificans*) use similar enzymes to their autotrophic counterparts (Moir et al., 1996). Some nitrifying bacteria, for example *Thiosphaera pantotropha*, combine their nitrification activity with aerobic denitrification (Kuenen and Robertson, 1994). The second pathway is restricted to fungi and involves N compounds that react with hydroxyl radicals when hydrogen peroxide and superoxide are both present (De Boer and Kowalchuk, 2001). This process can occur during cell lysis and lignin degradation by fungi when oxidases and peroxidases are released into the environment.

1.2.4. Recent discovery in NOB

Recent studies suggest that some strains of NOB (e.g. *Nitrospira*) are complete ammonia oxidizers in a process termed ‘comammox’ (Daims et al., 2016). Organisms able to carry out ‘comammox’ perform complete nitrification (e.g. NH$_4^+$ $\rightarrow$ NO$_2^-$ $\rightarrow$ NO$_3^-$) as they harbor the full genetic complement for both ammonia and nitrite oxidation.

1.2.5. Factors affecting the nitrification process

Nitrification can be influenced by physical, environmental, chemical, and biological factors. The list of factors is shown in Table 1.
Table 1 List of factors that influence the nitrification process *(Sahrawat, 2008)*

<table>
<thead>
<tr>
<th>Physical &amp; environmental factors</th>
<th>Chemical factors</th>
<th>Biological factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate concentrations (e.g. urea, NH₄⁺)</td>
<td>Soil pH</td>
<td>Microbial biomass</td>
</tr>
<tr>
<td>Soil matrix</td>
<td>Nutrient availability</td>
<td>Abundance and diversity of nitrifiers</td>
</tr>
<tr>
<td>Moisture content</td>
<td>C:N ratio</td>
<td>Soil respiration</td>
</tr>
<tr>
<td>Soil temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clay content</td>
<td></td>
<td></td>
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<tr>
<td>O₂ availability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil organic matter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil management practices</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2.6. Denitrification

Denitrification encompasses a series of transformations performed primarily by a wide range of heterotrophic bacteria. This process is also known as a major microbial respiratory process that reduces the anionic form of $N$ ($NO_3^-$ and $NO_2^-$) to gaseous products of NO, N$_2$O and N$_2$ under anoxic conditions. A wide range of diverse microbial genes (mostly bacteria and fungi) are involved in this process ($NO_3^-$ → $NO_2^-$ → NO → N$_2$O → N$_2$) (Figure 1.1). For example, $NO_3^-$ → $NO_2^-$, is the first step of denitrification and is catalyzed by a nitrate reductase encoded by either $narG$ or $napA$ genes. The second step ($NO_2^-$ → NO) is catalyzed by a nitrite reductase which is encoded by one of two different genes ($nirS$ and $nirK$). The third step (NO → N$_2$O) is catalyzed by a nitrite reductase encoded by the $nor$ gene (e.g. $cnorB$, $qnorB$). The final step in the denitrification process (N$_2$O → N$_2$) is catalyzed by the nitrous oxide reductase (N$_2$OR) encoded by the $nosZ$ gene. The N$_2$OR is the only known enzyme capable of reducing N$_2$O to N$_2$ (Jones et al., 2013; Sanford et al., 2012; Hu et al., 2015). Recent studies show that $nosZ$ is represented by two subtypes (clade I and II) each harbored by taxonomically distinct and non-overlapping groups of prokaryotes (Sanford et al., 2012; Jones et al., 2013). The $nosZ$ (clade II) gene is comparatively more diverse compared to $nosZ$ (clade I) gene. The major difference between $nosZ$I and $nosZ$II is secretory pathway (Tat vs. Sec) used to transport proteins across the cytoplasmic membrane. All $nosZ$ (clade I) process the Tat (Twin-arginine translocation) pathway which catalyze the translocation of secretory proteins in their folded state, whereas $nosZ$ (clade II) process the Sec pathway (i.e. general secretory pathway) which catalyze the translocation of secretory proteins in their unfolded state (Sanford et al., 2012; Natale et al., 2008). It should be noted that not all denitrifiers harbor the $nosZ$ genes; for example, $Agrobacterium tumefaciens$ and $Thauera$ (some strains) lack the $nosZ$ gene (Philippot et al., 2011; Bakken et al., 2012). This suggests that the process is modular – and that organisms may able to do part of the process i.e. they can lack any part of the chain but not just $nosZ$.

1.2.7. Enzymes involved in denitrification

Four different reductase enzymes are involved in the complete denitrification process (Figure 1.4). To understand this process at the cellular level we use $Paracoccus denitrificans$ as a model organism for explanation.
Figure 1.4. The schematic diagram illustrating the denitrification process at cellular level in *Paracoccus denitrificans*. Abbreviation: Nap & Nar, nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase. Cyt c₅₅₀, Cytochrome c₅₅₀, Ps az, pseudoazurin. Figure taken from Richardson *et al.* (2009).
1.2.7.1. **Nitrate reductase**

Nitrate to nitrite is catalyzed by nitrite reductase according to the following reaction:

\[
\text{NO}_3^- + 2\text{H}^+ + 2e^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O} \quad [E^{\circ} = +0.43 \text{ V}] \quad ------ (1)
\]

There are two types of nitrate reductase: a periplasmic nitrate reductase, which is known as Nap, and a membrane-bound nitrate reductase, which is known as Nar (Figure 1.4). Nar has 3 subunits: narGHI. (Moura et al., 2004). The catalytic site is encoded by narG. Nar receives electrons from ubiquinol (UQH\textsubscript{2}) at the P-side (positive side) of the membrane. Two protons (2H\textsuperscript{+}) discharge to the periplasm and two electrons (2e\textsuperscript{-}) pass to the cytoplasmic membrane via the cofactor known as Mo-bis-MGD (Molybdenum-bis-molybdopterin guanine dinucleotide). The inward movement of e\textsuperscript{-} is equivalent to the transfer of H\textsuperscript{+} from the cytoplasm to the periplasm which generates proton motive force (PMF) by a redox loop mechanism. The periplasmic nitrate reductase (Nap) is a heterodimer of two subunits (NapA (93 kDa) and NapB (16 kDa)), encoded by the napEDABC gene cluster (Berks et al., 1995). NapA also contains a Mo-bis-MGD cofactor which is similar to the co-factor of the membrane-bound nitrate reductase. The tetra-haem c-type cytochrome (NirC) is an electron-transfer component. The e\textsuperscript{-} is transferred from the UQH\textsubscript{2} to NapA via NapC (Nicholls and Ferguson, 2013).

1.2.7.2. **Nitrite reductase**

Nitrite to nitric oxide is catalyzed by NO\textsubscript{2}\textsuperscript{-} reductase which is located in the periplasm. The reaction is given below:

\[
\text{NO}_2^- + 2\text{H}^+ + 2e^- \rightarrow \text{NO} + \text{H}_2\text{O} \quad [E^{\circ} = +0.35 \text{ V}] \quad ------ (2)
\]

There are two types of NO\textsubscript{2}\textsuperscript{-} reductase: cytochrome cd\textsubscript{1} (both c- and d\textsubscript{1}-type haem centers, cd\textsubscript{1}Nir) and a copper containing NO\textsubscript{2}\textsuperscript{-} reductase (CuNir). The cd\textsubscript{1}Nir and CuNir are respectively encoded by the nirS and nirK genes (Zumft, 1997). Each of the nitrite reducers contains either nirS or nirK genes. (Coyne et al., 1989).
*P. denitrificans* harbors periplasmic NO$_2^-$ reductase (cd$_{1}$Nir), which is a homodimer (approx. 65 kDa) and contains both c- and d$_{1}$-type haem centers. (Ohshima et al., 1993; Zumft, 1997). The c-type acts as an electron transfer center and the d$_{1}$-type acts as a catalytic center. The NO$_2^-$ reductase can get electrons from cyt bc$_1$ via either a haem containing cytochrome C500 (Cyt c$_{500}$) or a copper containing pseudoazurin (Ps az) (Figure 3) (Moir et al., 1993).

Cu-containing CuNir is a homotrimer with two distinct Cu centers (type 1 Cu-center, T1Cu and type 2 Cu-center, T2Cu) in each monomeric unit (Godden et al., 1991; Howes et al., 1994). In general, T1Cu mediate electron transfer, and T2Cu act as active sites where substrate-binding and reduction take place (Howes et al., 1994).

**1.2.7.3. Nitric oxide reductase (NOR)**

NOR is a membrane bound enzyme that catalyzes the conversation of NO to N$_2$O. The reaction is given below:

\[
2\text{NO} + 2\text{H}^+ + 2e^- \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} \quad [E^o^\circ = +1.18 \text{ V}] \quad \text{(3)}
\]

There are 3 types of respiratory NORs (cNOR, qNOR, qCu$_A$NOR) reported from bacteria, but the best characterized NOR (cytochrome-c-dependent, cNOR) is the NorBC enzyme from *P. denitrificans*, which is a two-subunit complex (Field et al., 2008; Richardson et al., 2009). The NorC subunit (17 kDa) contains an N-terminal transmembrane helix that anchors to the periplasmic face of the cytoplasmic membrane. The NorC accepts electrons from two periplasmic electron donors: cytochrome C$_{550}$ and pseudoazurin. The NorB (56 kDa) is a catalytic subunit which consists of 12 transmembrane helices (van der Oost et al., 1994; Field et al., 2008; Richardson et al., 2009).
1.2.7.4. *Nitrous oxide reductase (N₂OR)*

The reduction of N₂O is only possible by the enzyme N₂OR which represents the last step of denitrification (N₂O → N₂). This is the only known biotic sink for N₂O. The reaction requires two protons (H⁺) and two electrons (e⁻). See equation (4) (Zumft *et al*., 2006):

\[
\text{N}_2\text{O} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{N}_2 + \text{H}_2\text{O} \quad [E^{\circ'} (\text{pH } 7.0) = +1.35 \text{ V}; \Delta G^{\circ} = -339.5 \text{ KJ/mol}] \quad \text{(4)}
\]

This reaction shows high positive redox potential at pH 7, \( E^{\circ'} = +1.35 \text{ V} \). N₂OR is a soluble enzyme which is usually located in the bacterial periplasm. The crystal structure of N₂OR is known from several denitrifier species: *Paracoccus denitrificans*, *Pseudomonas nautica*, and *Achromobacter cycloclastes*. All structures of N₂OR look virtually identical (Richardson *et al*., 2009); and they are homodimers and carry multi-copper ions in each monomer. Each monomer consists of two domains: a C-terminal cupredoxin domain (Cu₄A) and an N-terminal seven-bladed beta-propeller domain (Cu₄z) (Haltia *et al*., 2003). The Cu₄z is known as an active site or a catalytic center, and electrons pass from Cu₄A to Cu₄z. The N₂O binds to the active site of Cu₄z (between Cu1 and Cu4) which is suggested by a docking experiment and hence the reduction of N₂O occur by N₂OR (Haltia *et al*., 2003). The N₂OR can be inactivated when it is exposed to O₂. This is apparently due to the trap of cofactor Cu₄z in a redox-inactive form of the state \([\text{Cu}_4\text{S}]^{3+}\) (Rasmussen *et al*., 2002). The activity of N₂OR is also sensitive to the acidic environment (e.g. pH). This was tested in vitro analysis using methylviologen as an electron donor and showed more N₂OR activity at pH>7. A transcriptome study suggests that reduction of N₂O to N₂ is hampered due to posttranscriptional interference with the expression of nosZ (Liu *et al*., 2014). Therefore, the activation and deactivation of N₂OR in bacterial cells play an important role for the production and consumption of N₂O in soils or any other environments.
1.2.8. Factors affecting denitrification and N\textsubscript{2}O emissions

Denitrification can be affected by physiochemical properties (e.g. pH, organic C, mineral N, aeration, and water content), field management practices (e.g. fertilization, liming, irrigation, and tillage) and even genetic potential (e.g. available of genes for denitrification) in soil. These factors can be classified into two groups: proximal and distal controls (Wallenstein et al., 2006). The “proximal controls” on denitrification are defined as environmental conditions and resources that affect immediate changes of denitrification rate and has less direct effects on denitrifier communities in long term. The proximal controls are pH, O\textsubscript{2}, C availability, and temperature. Whereas the “distal controls” on denitrification are defined as those factors that control the diversity and composition of denitrifier communities over the long term (Figure 1.5). The distal controls include both environmental factors and biotic factors.

The availability of N as NO\textsubscript{3}\textsuperscript{-} in soil can be one of the most important factors that regulate denitrification. The concentration of NO\textsubscript{3}\textsuperscript{-} varies and depends on nitrification, N-mineralization, plant N uptake, microbial immobilization and NO\textsubscript{3}\textsuperscript{-} leaching or diffusions (Tiedje et al., 1980; Saggar et al., 2013; Zaman et al., 2007). The ratio of denitrification (N\textsubscript{2}O:N\textsubscript{2}) is also influenced by the availability of nitrate, where the higher concentration of NO\textsubscript{3}\textsuperscript{-} influences the higher N\textsubscript{2}O:N\textsubscript{2} ratio (Senbayram et al., 2012; Firestone et al., 1980).

The rate of denitrification can be influenced by available organic C. Denitrifiers are heterotrophs and they use organic C as an electron donor. With greater availability of organic C, there is an enhancement of denitrification rate under anoxic conditions (Reddy et al., 1982; Burford and Bremner, 1975; McCarty and Bremner, 1992; Senbayram et al., 2012)

Soil pH is considered an important factor that regulates the denitrification process; more importantly it regulates the emission ratio of N\textsubscript{2}O. Denitrification can occur in wide range of pH. Soils with acidic pH have a significant negative relationship with the N\textsubscript{2}O:N\textsubscript{2} ratio; hence, decreasing the pH leads to enhanced emission ratio of N\textsubscript{2}O (Bakken et al., 2012; Senbayram et al., 2012; Liu et al., 2014; Šimek and Cooper, 2002; Qu et al., 2014). In contrast, the alkaline soils show more N\textsubscript{2} as the end product of denitrification which represents a low product ratio of N\textsubscript{2}O.
(Richardson et al., 2009). The probable reason is that under low pH conditions the activity of nitrous oxide reductase (N$_2$OR) enzyme is inhibited due to lack of enzyme assembly in the periplasm (Liu et al., 2014).

Denitrification is an anaerobic process, hence, the availability of oxygen inhibits or represses the process (Knowles, 1982). Oxygen diffusion between soil and atmosphere depends on soil moisture which affects denitrification. In contrast, available oxygen can enhance the production of N$_2$O through nitrification, especially nitrifier-denitrification. It was demonstrated that AOB strain (in batch culture) contributed 11-26% and 43-87% of N$_2$O under 20% and 0.5% O$_2$ respectively as a result of nitrifier-denitrification (Frame and Casciotti, 2010).

There are other factors such as moisture content, temperature, soil type, soil management practices and genetic potentials (e.g. availability of functional genes for denitrification) that can affect the denitrification and N$_2$O emissions. Moisture content regulates the diffusion of oxygen in the soil which affects denitrification. In general, microbial growth is driven by temperature which controls denitrification. Different soil types (e.g. clay, loam and so on) have different components and can play an important role in denitrification.
Figure 1.5. A conceptual schematic diagram of proximal and distal controls on denitrifiers and denitrification. Figure taken from Wallenstein et al. (2006).
1.3. How the urine patch is linked to N$_2$O emissions?

Pastoral agriculture is an important livestock production system where animals graze outdoor pastures. This is a traditional practice for livestock production in many parts of the world including New Zealand. In such a system, the dominant source of N$_2$O is animal excreta, particularly urine, that deposits to the soil during grazing. The rate of N deposition to the pasture soil from a single urination of a dairy cattle can be as high as 700-1000 kg N ha$^{-1}$ (Di and Cameron, 2016). This N deposition from urine is mostly in the form of urea. Urea is hydrolyzed by urease enzyme and produced NH$_3$ (gas) and NH$_4^+$ ions. Most of the NH$_4^+$ (cation) can be retained or absorbed by negatively charged soil cation exchange complex, particularly soil clays and organic matter, despite some NH$_3$ loss through volatilization (Di and Cameron, 2016). The NH$_4^+$ leaching is negligible due to its high cation exchange capacity (CEC). However, NH$_4^+$ can be rapidly oxidized and produce NO$_3^-$ as an end product through nitrification, followed by denitrification where consecutive reductions of NO$_3^-$ to NO$_2^-$ and gaseous products (NO, N$_2$O and N$_2$) is occurring. This process is also called coupled nitrification-denitrification, as the end product of NO$_3^-$ or NO$_2^-$ can be utilized for denitrification (Wrage et al., 2001). The details about nitrification and denitrification processes have been discussed in the earlier sections. The coupled nitrification-denitrification process should not be confused with the term of nitrifier-denitrification. Nitrifier-denitrification is a pathway of nitrification, where oxidation of NH$_4^+/\text{NH}_3$ to NO$_2^-$ is followed by the reaction of NO$_2^-$, N$_2$O and N$_2$ (Wrage et al., 2001) (Figure 1.2). A recent study suggests that N$_2$O can be produced from NH$_2$OH by AOB (e.g. *Nitrosomonas europaea*) without following the nitrifier-denitrification pathway (Caranto et al., 2016).

1.4. Importance of microbial diversity for N$_2$O emissions

Denitrifiers are highly diverse and phylogenetically heterogeneous groups of microorganisms, mostly bacterial species from the phyla Bacteroides, Firmicutes, Actinobacteria, Chloroflexi, Verrucomicrobia, Aquificae and Proteobacteria (Philippot, 2002; Jones et al., 2013). They are also physiologically heterogeneous microorganisms including aerobic & anaerobic taxa, heterotrophs & autotrophs, nitrifiers, N$_2$-fixers, methylotrophs, thiosulphate oxidizers and even extremophiles (Butterbach-Bahl et al., 2013). Denitrifying bacterial communities can be tracked, for
instance, by nirS and nirK encoding NO-reductase that are highly diverse in soil and their abundance can be affected by soil types and soil management practices (Szukics et al., 2010). Another study demonstrated the abundance of nirK gene rapidly increased under wet conditions until the substrate (NO₃⁻) was limited (Azziz et al., 2017). Changes in the community structure were observed in nirK and AOA, indicating dynamic populations, whereas distinct adaptation (i.e. changes in community structure appear after a certain period of time) of the AOB communities, indicating higher stability (Szukics et al., 2010).

Our knowledge about denitrification is mostly related to bacterial denitrification. However, some fungi can produce N₂O from NO₃⁻ and NO₂⁻ under anaerobic conditions. All strain of Fusarium oxysporum (except strain IFO 9967) produce N₂O from NO₂⁻ (Shoun et al., 1992). Some other fungi also exhibited denitrifying activities, for example, Gibberella fujikuroi, Trichoderma hamatum, Cylindrocarpon tonkinense, Fusarium decemcellulare, Fusarium lini, Fusarium solani, Chaetomium sp. and Talaromyces flacus. (Shoun et al., 1992). Still, our knowledge is limited about the overall contribution to N₂O emissions from fungi and their ecological role in pasture soils.

NO-reductase, encoding the nirK gene, has also been identified among extreme halophiles representing an archaeon (e.g. Haloferax denitrificans) (Inatomi and Hochstein, 1996). Archaea are widely distributed and highly abundant in soils (Leininger et al., 2006), although, little is known about archaeal denitrification in soils as they are difficult to culture. Ammonia oxidizing archaea (AOA) have a great importance in the nitrification process. Based on enrichment culture, it was reported that AOA may be a major source of the oceanic N₂O, (Santoro et al., 2011). Another isotopic study reported that N₂O can be produced by AOA (strains from soil) and followed two different pathways (i.e. ammonia oxidation and nitrifier-denitrification) (Jung et al., 2014).

The relationship between the functional genes of denitrification and N₂O emissions and microbial community composition is not well understood. The integration of knowledge about physicochemical, gas kinetics, functional genes, as well as microbial community composition, will help us to understand the role of denitrifier communities and their process for the production and reduction of N₂O in pasture soils.
1.5. Thesis outline

The overall aim of this study is to determine the N cycling process (i.e. denitrification and nitrification) in pasture soils to understand the emission potential of N$_2$O through the analysis of the edaphic factors, gas kinetics, the microbial community structure, and functional gene analysis. To date, significant progress has been made yet the following questions are still poorly addressed:

Denitrification profile of pasture soils:
• Which are the most important drivers of denitrification (and particularly potential N$_2$O emission) in pasture soils?
• Can we predict the denitrification rate based on the rate of C mineralization?

Microbial community profile and its link to pH and N$_2$O emissions:
• What is the role of microbial diversity and richness in terms of soil N$_2$O emissions?
• Are there any potential links between the abundance of denitrification genes with pH as well as N$_2$O emissions?

Urine patch kinetics profile of pasture soils:
• How do urine patches contribute to N$_2$O emissions in pasture soils?
• What is the potential relationship between oxic urine patch kinetics and anoxic nitrate-amended denitrification kinetics?

Microbial community dynamics under urine patches:
• How and which microbes respond to urine addition?
• What are the impacts of N deposition on microbial community dynamics at genome and transcription levels?
• What are the relative contributions of AOA and AOB under urine patches?
• What is the relative contribution of microbes at different taxonomic levels (Phylum to Species)?
• What are the microbial life strategies (growth vs. maintenance)?
This thesis has addressed the above questions completely or partially in the following chapters (Chapter 2 to 5). Each chapter is presented here as a manuscript format.

**CHAPTER 2 | HIGH-RESOLUTION DENITRIFICATION KINETICS IN PASTURE SOILS LINK N₂O EMISSIONS TO PH, AND DENITRIFICATION TO C MINERALIZATION**

Denitrification is a microbial mediated process where soils loose nitrogen as N₂O and/or N₂. The objective of this chapter is to determine the denitrification kinetics profile of 13 pasture soils (New Zealand and Ireland) and how the rate of denitrification is linked to C mineralization. The other objectives were to compare the effect of different soil pH and its relationship to the emission ratio of N₂O and N₂O index.

**CHAPTER 3 | PHYLOGENETIC AND FUNCTIONAL POTENTIAL LINKS pH AND N₂O EMISSIONS IN PASTURE SOILS**

Soil pH regulates the reduction of N₂O to N₂, however, it can affect microbial community composition and the N₂O emission ratio of pasture soils. This chapter is aimed to link phenotypes (Chapter 2) to genotypes (functional potential and community composition) in order to understand the relationship between pH, microbial diversity and N₂O emissions.

**CHAPTER 4 | RUMINANT URINE PATCH REVEALS SIGNIFICANT SOURCES OF N₂O**

This chapter focuses on the simulated urine patch kinetics under oxic conditions. Artificial urine was applied in 13 different pasture soils (same soils that were used in Chapter 2 & 3) under microcosm study. The objective of this chapter is to determine the N transformation process under oxic urine patch conditions and to compare its relationship with denitrification kinetics (Chapter 2 & 3).

**CHAPTER 5 | RESPONSE TO URINE PATCH REVEALS METABOLIC AND ECOLOGICAL STRATEGIES OF SOIL BACTERIA**

The nitrogen cycle represents one of the most well-studied processes in soil, yet taxonomic diversity is mostly unknown or linked to poorly characterized microbial populations. In this study, urea was applied to soil to mimic the ruminant urine
deposition event and its impact on microbial community composition in temporal scale. The hypothesis was that the changes in transcription, or population size, could serve to determine life strategies of microbes utilizing each intermediate (i.e. whether they are used for growth vs. maintenance).
CHAPTER 2

High-resolution denitrification kinetics in pasture soils link N$_2$O emissions to pH, and denitrification to C mineralization

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Cecile de Klein, Karl G. Richards, Gary J. Lanigan, Sergio E. Morales$^*$

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Author Contributions: Authors are listed in order of magnitude of their contribution in each role. Corresponding author is indicated by an asterisk (*). Conceived and designed the experiments: SEM, LRB, MSS, CAMdK, TJC, KGR and GJL. Performed the experiments: MSS and SN. Analyzed the data: MSS and LRB. Contributed reagents/materials/analysis tools: SEM, LRB, CAMdK, TJC, KGR, GJL. Supervisory roles: SEM and LRB. MSS wrote the manuscript. MSS produced all figures and tables. All authors revised the paper.
Abstract

Denitrification in pasture soils is mediated by microbial and physicochemical processes leading to nitrogen loss through the emission of N₂O and N₂. It is known that N₂O reduction to N₂ is impaired by low soil pH yet controversy remains as inconsistent use of soil pH measurement methods by researchers, and differences in analytical methods between studies, undermine direct comparison of results. In addition, the link between denitrification and N₂O emissions in response to carbon (C) mineralization and pH in different pasture soils is still not well described. We hypothesized that potential denitrification rate and aerobic respiration rate would be positively correlated in soils. This relationship was predicted to be more robust when a high resolution analysis is performed as opposed to a single time point comparison. We tested this by characterizing 13 different temperate pasture soils from Northern and Southern hemispheres sites (Ireland and New Zealand) using a fully automated-high-resolution GC detection system that allowed us to detect a wide range of gas emissions simultaneously. We also compared the impact of using different extractants for determining pH on our conclusions. In all pH measurements, soil pH was strongly and negatively associated with both N₂O production index (IN₂O) and N₂O/(N₂O+N₂) product ratio. Furthermore, emission kinetics across all soils revealed that the denitrification rates under anoxic conditions (NO+N₂O+N₂ µmol N/h/vial) were significantly correlated with C mineralization (CO₂ µmol/h/vial) measured both under oxic ($r^2 = 0.62, p=0.0015$) and anoxic ($r^2 = 0.89, p<0.0001$) conditions.
2.1. Introduction

Nitrous oxide (N\textsubscript{2}O) is a potent greenhouse gas contributing 8\% of anthropogenic global warming (Lesschen \textit{et al.}, 2011; IPCC, 2007; Myhre \textit{et al.}, 2013) and responsible for depleting stratospheric ozone (Ravishankara \textit{et al.}, 2009). The N\textsubscript{2}O molecule has a Global Warming Potential (GWP) 298 times higher than carbon dioxide (CO\textsubscript{2}) over a 100-year period and an atmospheric life of approximately 121 years (Myhre \textit{et al.}, 2013). In the atmosphere, N\textsubscript{2}O has increased by 20\% over the last 260 years (1750 to 2011) from 271 ppb to 324 ppb (Myhre \textit{et al.}, 2013). Currently, the major anthropogenic source of N\textsubscript{2}O is agricultural soils (Cole \textit{et al.}, 1997; Paustian \textit{et al.}, 2004). In these N\textsubscript{2}O emitting soils denitrification is thought to be the most important pathway leading to N\textsubscript{2}O loss (Mosier, 1998; Ostrom \textit{et al.}, 2010), although a recent study showed that ammonia oxidation pathways and nitrifier denitrification are significant sources of N\textsubscript{2}O and NO under low oxygen availability (Zhu \textit{et al.}, 2013).

Denitrification is the stepwise process of reducing nitrate (NO\textsubscript{3}\textsuperscript{-}) to N\textsubscript{2}O or N\textsubscript{2}, via nitrite (NO\textsubscript{2}\textsuperscript{-}) and nitric oxide (NO). Four reductase enzymes catalyze the steps: nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N\textsubscript{2}OR) (Regaert \textit{et al.}, 2015; Bakken \textit{et al.}, 2012). The key requirements for biological denitrification, and complete reduction of nitrate to N\textsubscript{2}, can be summarized into two components: 1) the presence of microbes harboring the genetic ability to perform all the steps in denitrification, and 2) suitable environmental conditions for expression of the genetic potential. Changes in these two components can modify N\textsubscript{2}O emissions from soils (Saggar \textit{et al.}, 2013; Morales \textit{et al.}, 2015). For example, some organisms (complete denitrifiers) contain all the genetic information needed to produce the four enzymes, while others (incomplete denitrification) lack a subset of the enzymes and can only catalyze portions of the denitrification process (Regaert \textit{et al.}, 2015; Bakken \textit{et al.}, 2012). Alternatively, changes in the concentration and ratio of electron donors (i.e. available organic carbon compounds), available terminal electron acceptors (e.g. NO\textsubscript{3}\textsuperscript{-}, NO\textsubscript{2}\textsuperscript{-}, NO or N\textsubscript{2}O), and soil redox potential can modulate environmental conditions and thus the efficiency of denitrification in soils (Saggar \textit{et al.}, 2013; Jahangir \textit{et al.}, 2012). The addition of nitrogen fertilizers or manures increases denitrification rates especially when there is an adequate supply of carbon (Lampe \textit{et al.}, 2006; Senbayram \textit{et al.}, 2012). This is due to the fact that, denitrifiers require C to be readily available for reduction of NO\textsubscript{3}•
to occur (Senbayram et al., 2012). The rate of C mineralization in soils is influenced by many factors (e.g. temperature, drying-wetting, tillage, liming, crop residues, fertilizer application, root exudates) and which ultimately have a major impact on the denitrification rate (Saggar et al., 2013).

Known regulators can be difficult to assess in agricultural settings, and even more complicated to manipulate. An important factor that is more amenable for manipulation, and is a strong regulator of soil denitrification at both proximal and distal scales, is pH (Čuhel et al., 2010). Soil pH is a key driver of the microbiological processes affecting N\textsubscript{2}O and N\textsubscript{2} production (Regaert et al., 2015; Saggar et al., 2013), and influences the N\textsubscript{2}O/(N\textsubscript{2}O+N\textsubscript{2}) product ratio and N\textsubscript{2}O production index of soils. Proximal control by pH implices direct changes in N\textsubscript{2}O-reductase activity, while distal control by pH implicates changes in the denitrifier community, which is an important component affecting N\textsubscript{2}O emission rates (Čuhel et al., 2010). The mechanisms producing such effects are not well understood however, recent findings based on gene transcription, protein expression and the kinetics of electron flow at the cellular level have provided promising clues. In the model organism Paracoccus denitrificans, environmental pH hinders the posttranslational assembly of a functional N\textsubscript{2}O-reductase enzyme (Bakken et al., 2012; ISO, 2005; Gawlik et al., 2003). The inactivity of this enzyme results in the accumulation of N\textsubscript{2}O, which in results in soils becoming net N\textsubscript{2}O sources. Since soil pH can be controlled at field scales it represents a potential tool for mitigating N\textsubscript{2}O emissions from soils, but integrating knowledge across studies is made complicated due to variations in methodologies, most commonly the type of extractant used for pH measurements. Several different extractants (e.g. water, CaCl\textsubscript{2} and KCl) are widely used for measuring soil pH (Bergaust et al., 2011; Liu et al., 2014). However, the KCl based pH measurement is less commonly used for agricultural soils because it’s strong nature can alter the original properties of the sample being studied (Liu et al., 2014). This variability limits our capacity to integrate results over studies since the effects these changes can have on measurements are not fully understood.

Here we used a fully automated high-resolution GC detection system for measuring gas emissions under standardized oxic and anoxic conditions in order to assess factors linked to pasture soil N\textsubscript{2}O emission and denitrification potential across soils representing both Northern and Southern hemispheres. Our objectives were: (1) to determine the denitrification kinetics of pasture soils, (2) to determine the effect changing methods (extractant type) for determining soil pH has on observed
relationship with N$_2$O flux, (3) to compare two methods of quantifying N$_2$O emissions from soils (an emission index and ratio), and (4) to investigate the relationship between denitrification and C mineralization in soils.

2.2. Materials and methods

Soil samples were collected (May 2014) from 13 different sites (Figure 2.1) in the Northern and Southern hemispheres: (Ireland- Moorepark, Johnstown, Solohead and New Zealand- Warepa, Otokia, Wingatui, Tokomairiro, Mayfield, Lismore, Templeton, Manawatu, Horotiu, Te Kowhai). Soil properties are presented in Table S2.1. Permission for sampling was not required or in the case of sites located on private land, owner permission was secured for sampling.

At each site multiple (>3) soil cores (25 mm diameter by 100 mm long, and excluding the grass layer) were collected and sieved to 2-4 mm, composited and immediately couriered to the Norwegian University of Life Sciences, Norway for analysis. Soil samples were stored at 4°C in the lab until analyzed (within one week).

2.2.1. Soil pH measurements

Soil pH was measured using three different extraction methods: i) deionized (DI) water, ii) 0.01 M CaCl$_2$ and iii) 2M KCl. All pH measurements were carrying out using a 10 ml soil sample (field moist) measured using a volumetric spoon and transferred to a plastic vial. The respective pH treatment solutions (DI water, 0.01 M CaCl$_2$ or 2M KCl) were added (25 ml) and the vials were sealed and then, mixed thoroughly by hand shaking for 1 minute and left to settle overnight. Immediately prior to measuring the pH, samples were shaken well and allowed to settle for 10 minutes. All pH measurements were done using an Orion 2-star pH Benchtop pH meter (Thermo Scientific) equipped with an Orion 8175BNWP electrode (Thermo Scientific).
Figure 2.1. Geographical location of soil samples. Map showing origin of soil samples used in the study (a) world map, (b) Ireland [Moorespark (MP), Johnstown (JT), Solohead (SH)] and (c) New Zealand [Warepa (WP), Otokia (OT), Wingatui (WT), Tokomairiro (TM), Mayfield (MF), Lismore (LM), Templeton (TP), Manawatu (MM), Horotiu (HR), Te Kowhai (TK)]. The map was generated using open source “R-program (packages ‘maps’ and ‘mapdata’).
2.2.2. Nitrate adjustment

Individual soil samples (100 g dry weight) were placed in 500 ml filter funnels (Millipore) with 4.5 cm diameter (0.2 µm) Millipore membrane filters and subsequently flooded with a 2 mM NH$_4$NO$_3$ solution for 10 minutes. Samples were then drained using a vacuum in order to obtain a homogeneous distribution of NO$_3^-$ in the soils. The moisture content of the soil samples was determined (dried overnight at 105°C) after draining and dry weight equivalents were used for subsequent gas kinetic experiments (Table S2.2). The cation exchange capacity (ECE) is different in all soils and its effect is not tested after addition of NH$_4$NO$_3$.

2.2.3. Gas kinetics under oxic and anoxic conditions

All incubations were performed using slightly modified methods described previously (Raut et al., 2012; Qu et al., 2014; Molstad et al., 2007). In brief, following NO$_3^-$ adjustment, 20 g (dry weight equivalent) of soil was transferred to a 120 ml serum vial and sealed with an airtight butyl-rubber septa and an aluminum crimp cap. Triplicate vials were prepared from each soil sample and incubated at 20 °C using an automated GC system (Molstad et al., 2007). The GC (Agilent GC -7890A) system was equipped with three detectors (an electron capture detector (ECD), a thermal conductivity detector (TCD), a flame ionization detector (FID)) and one Chemiluminescence NOx analyzer (NOx analyzer Model 200A, Advanced Pollution Instrumentation, San Diego, USA). The GC system was integrated with an automated sampling robot (CTC GC PAL). All data presented were from experiments performed over two runs, which included independent standards for each run. Duplicates of four different gas standards were used in this experiment. All standards were prepared using evacuated vials (120 ml with septum) filled with commercially produced standard gases (supplied by AGA). Headspace samples (approx. 1 ml) were taken via needle and measured sequentially every 5 hours. The samples were incubated under oxic conditions for approx. 40 hours and subsequently incubated under anaerobic conditions for the remainder of the incubation (approx. 200 hours total). In order to create anoxic conditions, sampling vials were flushed and evacuated three times with high purity helium (He) gas, and over pressure was released from the vials before GC analysis.
2.2.4. Calculation of C mineralization and denitrification rates

Oxic respiration (i.e. oxic C mineralization) was calculated using the mean production rates of CO₂ (µmol/h per vial) within the first 40 hours when oxygen was present. Denitrification rates and anoxic C mineralization rates were calculated using the mean production rates of NO+N₂O+N₂ (µmol N/h per vial) and CO₂ (µmol/h per vial) respectively, within the first 40 hours following removal of O₂ by replacement of the headspace with helium.

2.2.5. Calculation of N₂O production index and (N₂O/N₂O+N₂) ratio

Characterization of N₂O emissions from each soil were done using two methods: 1) the N₂O production index (Iₙ₂o) as described by Liu et al. (2010) and Qu et al. (2014) and 2) the N₂O product ratio (N₂O/N₂O+N₂) as described by Raut et al. (2012). Calculation of Iₙ₂o was done using a 5 hours interval (i.e. 0 h – 5 h, 5 h – 10 h, 10 h – 15 h, and so on), while the N₂O/(N₂O+N₂) ratio only took into account a single time point (i.e. 0 h, 5 h, 15 h, and so on). All soils were compared based on a 50 h anoxic incubation period for Iₙ₂o. The N₂O/(N₂O+N₂) ratio was calculated using the maximum value during the same 50 h period. Calculation of the N₂O production index (Iₙ₂o) was done using the formula:

\[ I_{\text{N}_2\text{O}} = \int_0^T N_{\text{N}_2\text{O}} (t) \, dt / \left[ \int_0^T N_{\text{N}_2\text{O}} (t) + \int_0^T N_2 (t) \right] \, dt \]

where N₂O (t) is the accumulated flux of N₂O at any time t, N₂ (t) is the accumulated flux of N₂ at any time, and T is the time when a certain amount of NO₃⁻ - N g⁻¹ soil is recovered as (NO₂⁻, NO, N₂O and N₂)-N. Here we considered 50 h as T.

Linear regressions performed on JMP 10 (SAS Institute) were used to identify relationships between variables.

2.4. Results

2.4.1. Gas kinetics

Soil samples incubated under oxic conditions did not produce quantifiable amounts of NO, N₂O or N₂ after 40 h of incubation despite active respiration as determined by consumption of O₂ and production of CO₂ (Figure 2.2). Upon removal of O₂, immediate production of NO, N₂O and N₂ were detected. For all soils, NO and N₂O were converted to N₂, but the kinetics of the conversion varied. Accumulation of NO (mean ± SD) ranged between 100 ± 2.9 and 8390 ± 802 nmol N/vial, corresponding to Templeton and Lismore soils, respectively. While N₂O
accumulation ranged between 2.6 ± 0.5 to 56.5 ± 2.3 µmol N/vial, corresponding to Templeton and Horotiu, respectively.

2.4.2. $I_{N_2O}$ and $N_2O/(N_2O+N_2)$

The $N_2O$ production index ($I_{N_2O}$) and the $N_2O/(N_2O+N_2)$ product ratio were calculated based on the kinetics observed during anoxic incubation (Figure 2.3). Except for Solohead and Otokia, soil samples displayed higher $I_{N_2O}$ (approx. 10%) than $N_2O/(N_2O+N_2)$ with a mean value of 0.77 ± 0.27 and 0.67 ± 0.20 respectively (Fig S2.2). The Solohead soil had both the lowest $N_2O$ production index ($I_{N_2O} = 0.02$) and $N_2O/(N_2O+N_2)$ product ratio (0.23), while the Lismore soil had the highest ($I_{N_2O} = 1$ and $N_2O/(N_2O+N_2) = 0.89$). Values for $I_{N_2O}$ and $N_2O/(N_2O+N_2)$ were from all soils positively correlated ($r^2 = 0.84$ $p<0.001$).

2.4.3. Soil pH and $N_2O$ emissions

Soil pH values were moderately acidic to neutral across all soils (Table S2.1). The pH measurements in the water-based method resulted in a wider range of values (5.57-7.03), while values for the KCl based method resulted in pH values clustered within the acidic range (4.40-6.39). The influence of soil pH on $N_2O$ emissions was examined by comparing pH values obtained, with each of the different pH extraction methods, with the $N_2O/(N_2O+N_2)$ ratio and the $I_{N_2O}$ for all soils (Figure 2.4). Soil pH explained a significant proportion of the variation in relationship to $I_{N_2O}$ regardless of method used to determine pH ($r^2 = 0.85$ in DI H$_2$O; $r^2 = 0.75$ in CaCl$_2$; $r^2 = 0.71$ in KCl; $p<0.05$ all cases). Strong relationships ($p<0.05$) were also observed between pH and the $N_2O/(N_2O+N_2)$ product ratios regardless of soil pH extraction method ($r^2 = 0.82$ in DI H$_2$O; $r^2 = 0.68$ in CaCl$_2$; $r^2 = 0.54$ in KCl). Among the soil samples, one (Solohead) displayed very low $N_2O$ emissions resulting in an outlier (Figure 2.4). To assess its impact, it was removed, and the $I_{N_2O}$ and $N_2O/(N_2O+N_2)$ ratio were recalculated and correlated to pH. Only the DI water-based pH measurement was significantly correlated, but the resulting $r^2$ was lower (In case of $I_{N_2O}$ and pH: $r^2 = 0.62$ $p=0.0025$ in DI H$_2$O; $r^2 = 0.29$ $p=0.07$ in CaCl$_2$; $r^2 = 0.13$ $p=0.24$ in KCl, and in case of $N_2O/(N_2O+N_2)$ product ratios and pH: $r^2 = 0.69$ $p=0.0009$ in DI H$_2$O; $r^2 = 0.43$ $p=0.019$ in CaCl$_2$; $r^2 = 0.17$ $p=0.178$ in KCl).

2.4.4. Links between denitrification and C mineralization

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The rate of soil denitrification under anoxic condition (NO+N\textsubscript{2}O+N\textsubscript{2} µmol N/h/vial) was significantly linked to the rate of C-mineralization (CO\textsubscript{2} µmol/h/vial) under both oxic ($r^2 = 0.62$, $p=0.0015$ and anoxic ($r^2 = 0.89$, $p<0.0001$) conditions (Figure 2.5).
Figure 2.2. Gas kinetics profile of IR and NZ soils under oxic and anoxic conditions. O$_2$, CO$_2$, NO, N$_2$O and N$_2$ emission kinetics during incubation of 13 different temperate soils (3 Ireland (a, b, c) and 10 New Zealand (d to m)) amended with 2 mM nitrate (flooding and draining immediately before incubation). Soil samples (20 g dry weight) were incubated under oxic (first 40 hours) and subsequently anoxic conditions. Dots represent three replicate vials and smooth line is the fitted line for all data.

Figure 2.3. Demonstration of calculation of $\text{IN}_2\text{O}$ and $\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$. Representative curves for a) cumulative N accumulation, b) measured N, and c) N$_2$O production index ($\text{IN}_2\text{O}$) and N$_2$O/(N$_2$O+N$_2$) product ratio over time for one soil (Moorepark). N$_2$O production indices were calculated as $[\text{IN}_2\text{O} = \int_0^T N_2\text{O} (t) \, dt/ \int_0^T N_2\text{O} (t) + \int_0^T N_2 (t) \, dt]$. Curves represent a single flask result. Each vial contained 20 g (dry weight) soil incubated in a 120 ml serum vial under anoxic conditions. Results for all other soils can be found in appendix section Fig S2.1.
Figure 2.4. Relationship between pH and N\textsubscript{2}O emissions. Effect of method (extractant type) for determining soil pH on correlation with (a) N\textsubscript{2}O production index (I\textsubscript{IN\textsubscript{2}O}) and (b) N\textsubscript{2}O/(N\textsubscript{2}O+N\textsubscript{2}) ratio. Calculation of both index and ratio was based on N\textsubscript{2}O emission within the curve (see Fig S1 for each sample) at 50 h under anoxic incubation. Soil pH was measured using three different extractants: i) DI water (■), ii) 0.01 M CaCl\textsubscript{2} (●), and iii) 2M KCl (▲). Dotted lines represent regression lines. Points represent the mean triplicate vials results.

Figure 2.5. Links between denitrification and C mineralization. Relationship between C mineralization rate during both oxic [closed circles] and anoxic phase [open circles] and denitrification (De; i.e. production rates of NO+N\textsubscript{2}O+N\textsubscript{2}). Each point represents mean of triplicates. Linear regression function is shown for both oxic (C\textsubscript{ox}) and anoxic (C\textsubscript{an}) C mineralization.
2.5. Discussion

It is known that soil pH plays a strong role in regulating the loss of N gases (Mørkved et al., 2007). One problem with understanding the pH effect on N\textsubscript{2}O emissions is consolidating the many studies done to date, and their sometimes-conflicting observations (Šimek and Cooper, 2002). Here we tested soil pH using the three most commonly used extractants and found that soil pH measurements vary across all three extractants (approx. 1-2 units within pH range) (Figure 2.4 and Table S2.3). This is likely due to the differences in protons (H\textsuperscript{+}) and hydronium ions (OH\textsuperscript{-}) attracted to exchange sites for each buffer, which causes an electrical potential to develop. Although the different pH extractants yield different soil pH values, the relative ranking of the soils from highest to lowest pH was entirely conserved across all extractants. Thus absolute values of soil pH across studies will be hard to compare but their relative placement within a gradient (higher vs. lower pH) can be used to compare results across independent studies. Evidence in the literature supports the claim for reduced N\textsubscript{2}O reduction and denitrification in low pH systems (Raut et al., 2012; Firestone et al., 1980), leading to the N\textsubscript{2}O production index being strongly correlated with pH (Qu et al., 2014; Liu et al., 2010). The underlying mechanisms involved in the pH control over N\textsubscript{2}O emission have begun to be unraveled in part by the use of model organisms, including Paracoccus denitrificans. Recent work demonstrated that the relative activity of the N\textsubscript{2}O reductase enzyme decreased with lowering of the pH. This decrease in activity was associated with a post-transcriptional effect wherein the assembly of the N\textsubscript{2}OR enzyme was inhibited by low pH (ISO, 2005). However, further work showed that when N\textsubscript{2}OR was expressed at pH 7.0, it remained functional over the entire pH range tested (5.7 to 7.6), suggesting that the role of pH is specific to the folding of the protein upon expression (Gawlik et al., 2003). It is important to understand that although pH in this scenario plays a role as a proximal regulator, it can also play a role as a distal regulator as well by controlling community composition (Rousk et al., 2010) making interpretation complicated.

Independent of the methods used to measure soil pH or the mechanism controlling the pattern, we observed that the I\textsubscript{N\textsubscript{2}O} was higher than the N\textsubscript{2}O/(N\textsubscript{2}O+N\textsubscript{2}) ratio in each soil sample (except for Solohead and Otokia soils). This is likely due to the fact that the I\textsubscript{N\textsubscript{2}O} takes into account a time period (the emission occurring between two given time points), as opposed to single time points as used in the
N₂O/(N₂O+N₂) ratio calculations. As seen in the kinetic profiles (Figure 2.3), the shape of the curve is not always similar and although heights (i.e. maximum values) might be similar, a gentler slope (i.e. slower but more prolonged rates) can lead to an extended period of emissions not accounted for by height alone. The fact that \( I_{N₂O} \) and \( N₂O/(N₂O+N₂) \) were strongly correlated \( (r² = 0.84) \) suggests that both parameters can be used as a measure of the soils’ contrasting propensities to emit N₂O. As the N₂O production index \( (I_{N₂O}) \) is calculated using at least two time points and the area under the curve, it is possibly the best predictor of the propensity of the soils to emit N₂O, as dependent on the ability of the denitrifying community to express N₂O reductase. It cannot be taken as a direct predictor of N₂O emission to the atmosphere under field conditions, primarily because the fraction of denitrification products lost to the atmosphere as N₂O depends on soil moisture content; high soil moisture content retards N₂O diffusion and hence increases the fraction of N₂O reduced to N₂.

Aside from pH, O₂ is also a known ‘master’ regulator of denitrification. Soil samples in this study were incubated in two phases (oxic and anoxic). During the oxic phase, microbial respiration was active as determined by monitoring of the CO₂ produced but there were no emissions of NO, N₂O or N₂. However, upon removal of O₂, emissions of NO, N₂O and N₂ were observed in all soil samples independent of pH. This confirms prior work (Raut et al., 2012; Qu et al., 2014; Liu et al., 2010) indicating that in the hierarchy of regulators of denitrification, O₂ serves as a primary control with pH serving a secondary role, not in controlling the rate of denitrification but the kinetics of the product ratio. Both measurements of emission potential \( (I_{N₂O} \) and \( N₂O/(N₂O+N₂) \)) are strongly related to soil pH \( (r² = 0.53 \) to \( 0.85) \) transient accumulation of N₂O.

In both oxic and anoxic conditions, the C mineralization rates (CO₂ production) for all soils provide an indirect indication of denitrification rates, and serve as a good proxy for predicting N cycling activity (Figure 2.5). Oxic respiration rates (or C mineralization) were 3.2 times higher than the rates of denitrification, likely due to the larger pool of organisms capable of carrying out this general process. When the anoxic C mineralization rate was compared to the rate of denitrification, a strong relationship \( (r² = 0.89) \) was observed, suggesting that denitrification was the dominant pathway for energy generation and responsible for respiration from the selected soils under the experimental conditions. This is expected given the conditions used in this study favor denitrification, and its
intermediates represent the most energetically advantageous alternative electron acceptor. However, we observed that C mineralization rates under anoxic conditions were 10% higher than denitrification rates, which may be due to fermentation process and/or the presence of other alternative electron acceptors in soils (e.g. Fe$^{2+}$, Mn$^{2+}$, SO$_4^{2-}$, etc.). Apart from microbial respiration and fermentation, another probable source of CO$_2$ is from inorganic carbonate (e.g. lime). As denitrification rate is strongly associated with the production rate of CO$_2$ under anoxic conditions, therefore, this may indicate that the sources of CO$_2$ is mainly from C mineralization.

Although measures like C mineralization and denitrification rates, $I$/N$_2$O and N$_2$O/(N$_2$O+N$_2$) allow us to assess the impact of potential regulators, as well as providing easy comparison to prior work, they do not convey all the differences observed. By using a continuous monitoring system, we observed that the gas emission profile (kinetics) (i.e. the production and consumption of the gas intermediates in denitrification) of pasture soils varied greatly across all soils. Some soils (e.g. Lismore, Horotiu, Mayfield and Moorepark) were more prone to producing NO compared to others, but the profiles generated could not be summed based on a single gas. The data generated from these 13 soils suggests that our inability to accurately predict emissions is in part due to the uniqueness of each soil, which is reflected here in their unique gas profiles. Soils such as the Solohead soil would likely generate results that are difficult to interpret based on single time point measurements due to its kinetic profile (extremely fast rates of almost all measured variables). Despite these difficulties, certain conclusions can be made. Soil pH is one of the most important soil factors affecting the denitrification products (i.e. N$_2$O or N$_2$). Here we showed that differences in extractants for measuring pH could account for discrepancies in observations across prior studies. However, a consistent trend of increased N$_2$O emissions with lowering pH was observed independent of pH extractants. Further, two approaches for representing emissions ($I$/N$_2$O than N$_2$O/(N$_2$O+N$_2$)) were examined and shown to be positively correlated, providing alternatives for reporting emissions. Finally, as denitrification rate is closely related to soil C mineralization, therefore C mineralization could be used as an indirect tool for predicting the denitrification rate of NO$_3^-$ amended pasture soils.
CHAPTER 3

Phylogenetic and functional potential links pH and N₂O emissions in pasture soils

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Author Contributions: Authors are listed in order of magnitude of their contribution in each role. Corresponding author is indicated by an asterisk (*). SEM, MSS, LRB, CAMdK, TJC, KGR and GJL designed the experiments. MSS, CAMdK, TJC, KGR and GJL collected samples and processed. MSS, AB, SEM and LRB analyzed the data. SEM and LRB had supervisory roles. MSS produced all figures and tables. MSS and SEM wrote the manuscript. All authors revised the paper.
Abstract

Denitrification is mediated by microbial, and physicochemical, processes leading to nitrogen loss via N₂O and N₂ emissions. Soil pH regulates the reduction of N₂O to N₂, however, it can also affect microbial community composition and functional potential. Here we simultaneously test the link between pH, community composition, and the N₂O emission ratio (N₂O/(NO+N₂O+N₂)) in 13 temperate pasture soils. Physicochemical analysis, gas kinetics, 16S rRNA amplicon sequencing, metagenomic and quantitative PCR (of denitrifier genes: nirS, nirK, nosZI and nosZII) analysis were carried out to characterize each soil. We found strong evidence linking pH to both N₂O emission ratio and community changes. Soil pH was negatively associated with N₂O emission ratio, while being positively associated with both community diversity and total denitrification gene (nir & nos) abundance. Abundance of nosZII was positively linked to pH, and negatively linked to N₂O emissions. Our results confirm that pH imposes a general selective pressure on the entire community and that this results in changes in emission potential. Our data also support the general model that with increased microbial diversity efficiency increases, demonstrated in this study with lowered N₂O emission ratio through more efficient conversion of N₂O to N₂.
3.1. Introduction

The Anthropocene has resulted in a loss of global biodiversity and enhanced greenhouse gas emissions (Vitousek et al., 1997). A major driver of change has been the transformation of land for agriculture purposes, needed to sustain the expanding global populations (Tilman et al., 2002). These changes are expected to drive further reductions in biodiversity and the loss of associated ecosystem services (Tilman et al., 2001). Of the greenhouse gases associated with agriculture, nitrous oxide (N\textsubscript{2}O) is of particular concern due to its global warming potential (> 300 times more powerful as CO\textsubscript{2}) and ozone-depleting capabilities (Robertson, 2000; Ravishankara et al., 2009; Pachauri et al., 2014; Tian et al., 2016).

The mechanisms that control N\textsubscript{2}O production and loss from soils are still being debated, with identified regulators comprising physical, chemical and biological factors (Saggar et al., 2013). Soil pH has been identified as a master regulator of gaseous N emissions, with the propensity of soils to release N\textsubscript{2}O over N\textsubscript{2} tightly linked to this (Samad et al., 2016a). Two mechanisms have been proposed for explaining the role of pH: i) a distal impact on the genetic potential in soils through re-arrangements of the microbial community and ii) a proximal impact driven by modulation of the direct reactions catalyzing the conversion of N\textsubscript{2}O to N\textsubscript{2} by microbial enzymes (Wallenstein et al., 2006). However, emissions of N\textsubscript{2}O are controlled at multiple levels: i) the available genetic potential within the soil microbial community (genotype) (Braker and Conrad, 2011), ii) the activation or de-activation of the potential in response to an environmental signal (transcriptional regulation controlling expression of genotype) (Kern and Simon, 2015; Qu et al., 2016), iii) the translation of transcripts leading to an immature or apoprotein (translational regulation) (Dreusch et al., 1997), iv) maturation of a protein resulting in an active enzyme (post-translational regulation) (Dreusch et al., 1997), v) export of enzymes when activity is not cytoplasmic (e.g. sec / tat dependent secretion as is the case for NosZ) (Bernhard et al., 2000; Heikkilä et al., 2001; Simon et al., 2004), and vi) degradation or turnover rate of enzymes once active (Vogel and Marcotte, 2012). These controls cover both the production of N\textsubscript{2}O and the consumption, or turnover, into N\textsubscript{2} by a different process. As a result emissions are limited by what may be summarized as: i) genetic potential, ii) transcriptional regulation, and iii) enzymatic activity. The outcome is a complex array of regulators and processes that are likely to change across time and space.
Despite the complexity, observations support the role of both distal and proximal regulators (Philippot et al., 2011; Bakken et al., 2012). Distal impacts by pH are proposed to be driven by selecting for community shifts at both functional and phylogenetic levels (Morales et al., 2015) with shifts in available potential (functional gene abundances) resulting in shifts in phenotypes (observed emissions) (Nishizawa et al., 2014; Shiina et al., 2014). Proximal impacts by pH provide a clearer mechanism. Low pH causes a shift in active organisms (Brenzinger et al., 2015), but more importantly pH disrupts the activity of the N₂O reductase by interfering with assembly (Bergaugust et al., 2010; Liu et al., 2010; 2014). Although evidence supports the role of pH in regulating emissions and community structure (Nicol et al., 2008; Lauber et al., 2009; Čuhel et al., 2010; Rousk et al., 2010) studies linking all three remain sparse.

An additional consideration is the role of biodiversity in supporting ecosystem processes like N (nitrogen) cycling. It has been proposed that biodiversity is a universal regulator of ecosystem processes (Tilman, 1999). Although microbial studies that support the role of microbial diversity in controlling productivity (Ptacnik et al., 2008; Schnitzer et al., 2011), N cycling (Griffiths et al., 2000; Wertz et al., 2006; Wittebolle et al., 2009) and even N₂O emissions (Wagg et al., 2014) exist, these rely on single manipulated soils or small sample sizes. However, such studies serve to establish a hypothesis that aligns with ecological theory. That is, with increasing diversity there is increased redundancy and efficiency of ecosystem processes (Loreau et al., 2001; Tilman et al., 2014). This has been observed in some microbial studies (Griffiths et al., 2000; Levine et al., 2011), including those associated with N₂O emissions (Domeignoz-Horta et al., 2015), while others showed no direct effects (Griffiths et al., 2001; Wertz et al., 2006). However, a detailed study linking gaseous emissions (NO, N₂O and N₂), pH and microbial diversity, over soils with varying parent materials and climates, is lacking.

In this study we aimed to link phenotypes (emission potential) to genotypes (functional potential and community composition) across 13 soils with varying pH (5.57 - 7.03) representing both Northern and Southern Hemisphere soils. These soils were selected as they represent the normally observed pH range in agronomic grasslands (recommended pH optima = 6.2-6.5). Using this dataset our goal was to simultaneously explore the relationship between pH, diversity and emissions. We hypothesized that the effect of pH on emissions would be linked to changes in whole communities, and not solely to denitrification functional potential. To test this, we
quantified the abundance of genes involved in denitrification using quantitative PCR and metagenomic analysis, and examined their relationship with the emissions potential ($\text{N}_2\text{O} \text{ ratio} = \text{N}_2\text{O}/(\text{NO+ N}_2\text{O}+\text{N}_2)$). We also determined the microbial community composition and diversity of each soil and identified patterns linked to both changes in pH and emissions.

3.2. Materials and Methods

3.2.1. Sample collection and processing

Soil samples used in this study and their physio-chemical properties have been described previously (Samad et al., 2016a). Soils were selected to represent intensive agricultural grasslands with a representative pH range close to the agronomic optimum of 6.5. Briefly, soil samples were collected from 13 permanent grasslands (managed agricultural) sites in Ireland (Johnstown, Moorepark, Solohead) and New Zealand (Horotiu, Lismore, Manawatu, Mayfield, Otokia, Te Kowhai, Templeton, Tokomairiro, Warepa, Wingatui), representing Northern and Southern hemisphere sites. Soil cores ($n>3$) were collected randomly from each site using a corer (25 mm diameter by 100 mm long), and excluded the grass layer. For each site, replicate cores were sieved to <4 mm, composited and immediately shipped to the Norwegian University of Life Sciences, Norway for analysis. Soil samples for kinetics were stored at 4°C in the lab until analyzed (within one week). Soils for DNA extraction were immediately frozen and stored at -20°C until extracted. Three separate DNA extractions were performed from 0.25 g of soil material from each site (total 39) with the PowerLyzer® PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA) as per manufacturer’s instructions. DNA concentration, purity and contamination with humics were assessed with a Nanodrop Spectrophotometer, ND-1000 (Thermo Scientific). DNA yields ranged between 8-21 ng/µl (median = 13; standard error = 0.6) with no detection of humic acids (median absorbance at 320nm = 0.008; standard error = 0.0010) indicating high quality extractions.

3.2.2. Gas kinetics

Gas kinetics methods were described in detail in Samad et al., 2016 (Samad et al., 2016a). Briefly, soils (100 g dry weight) were provided with nitrate (2 mM NH₄NO₃) by flooding in 500 ml filter funnels (Millipore) with 4.5 cm diameter (0.2 µm)
Millipore filters at least three times for 10 minutes. To obtain a homogeneous distribution of NO$_3^-$ and to remove excess liquid from soils a vacuum was applied. After NO$_3^-$ adjustment, 20 g (dry weight equivalent) of each soil was transferred to a 120 ml serum vial and sealed with an air-tight butyl-rubber septa and an aluminum crimp cap. For each site triplicate vials were prepared and incubated at 20°C using an automated GC system (Molstad et al., 2007). The soils were first incubated for 40 h under oxic conditions and then incubated under anoxic conditions for over 200 h. The emission of NO, N$_2$O and N$_2$ were measured at 5 h intervals under anoxic conditions. The product ratio of N$_2$O (i.e. N$_2$O/(NO+N$_2$O+N$_2$)) was calculated and the maximum value observed during incubation for each soil was used. The maximum value represents the highest potential of each soil to emit N$_2$O. While NO$_3^-$ concentrations are likely to see a small increase due to nitrification of the added NH$_4^+$ (NH$_4$NO$_3$) during oxic incubation, resulting in soil-to-soil differences in available NO$_3^-$ at the beginning of the anoxic incubations, these differences are unlikely to affect the kinetics of denitrification (and the product ratios) since the NO$_3^-$ concentration applied (2 mM) was 2-3 orders of magnitude higher than $K_s$ for NO$_3^-$ reductases (Hassan et al., 2016). Further, wetting of soils did not result in emissions with kinetics only measurable in the presence of exogenously added N.

3.2.3. Quantification of bacterial community and functional gene abundance

Quantitative PCR (qPCR) was performed on all 39 extractions to determine total bacterial abundance and the abundance of four denitrification functional marker genes (nirS, nirK, nosZ (Clade I) & nosZ (Clade II)) in each soil. Reactions were performed in 96-well plates using the ViiA7 real-time PCR system (Applied Biosystems, Carlsbad, CA). Standards for qPCR were generated using a 10-fold serial dilution ($10^8$ to $10^1$) of known copy numbers of pGEM-T easy (Promega, Madison, Wisconsin, USA) cloned template (i.e. specific genes [nirS, nirK, nosZI, & nosZII] were inserted in the cloning vector). All quantifications were performed using 4 technical replicates for each DNA sample loaded into the same plate, with each plate containing replicated standards and no template controls (PCR efficiencies shown in Supplementary Table S3.1. Amplification of nosZ Clade II and nirK targets was not possible with multiple tested polymerase brands even after optimization. As a result, two different master mixes (ABI and Thermo Scientific) were used as specific below. All reactions were performed in 20 µl volumes containing: 1× Master
Mix (ABI for nirS & nosZI or Thermo Scientific for nirK & nosZII), 0.5-1 µM of each primer (0.5 µM for nirS & nosZI and 1µM for nirK & nosZII), 5 ng of template DNA and autoclaved Milli-Q H₂O to a final volume of 20 µl. Primers and qPCR conditions are summarized in Supplementary Table S3.1. A melt curve analysis (95°C for 15 s, 60°C for 1 min then increasing 0.05°C/s (data acquisition) until 95°C) was performed at the end of reactions to test for specificity and to confirm no amplification in the negative control. No inhibition was observed and all samples tested amplified.

3.2.4. Analysis of 16S rRNA gene by amplicon sequencing

16S rRNA gene libraries were created for each DNA extraction using bacterial/archaeal primers 515F/806R targeting the V4 region of the 16S rRNA gene. Library preparation and sequencing were conducted according to the standard protocol (Version 4_13) of the Earth Microbiome Project (Caporaso et al., 2012) and libraries were paired-end sequenced using the Illumina MiSeq platform. Preliminary processing was carried out in Qiime (version 1.9.0) using default parameters (Caporaso et al., 2010). Sequences were clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity using the SILVA version 119 reference library (Quast et al., 2012) and UCLUST (Edgar, 2010). Taxonomic classification was assigned using BLAST analysis against the SILVA database (Altschul et al., 1990). Samples were then rarified and randomly subsampled 10 times (using the Qiime command ‘multiple_rarefactions_even_depth.py’) to equal depths (16,000). Samples below that threshold (1) were removed for a total of 38 samples retained. All 10 OTU tables per sample were subsequently merged and exported for processing in R. All downstream analysis were performed in R (R Development Core Team, 2008) and described in detail in supplemental information. The 16S rRNA amplicon sequences were summited to NCBI, SRA database (SRA accession: SRP080971).

3.2.5. Metagenomic sequence analysis

Six sites (Ireland: Johnstown, Moorepark, Solohead and New Zealand: Horotiu, Lismore, Templeton) representing a range of emission profiles from each country were selected for metagenomic analysis. Libraries for each metagenome were generated using the Illumina Nextera XT library preparation kit. Duplicate
MiSeq 2 X 250 base paired end runs were carried out for each of the 6 samples. Sequences were submitted to and annotated using the MG-RAST server (Meyer et al., 2008). Metagenomic data is available through the MG-RAST server (ID numbers 4644147.3 to 4644142.3). Sequence counts ranged from 2,634,050-4,851,047 before quality control. Sequences were classified taxonomically using the SILVA SSU ribosomal databases and functionally using KEGG with default settings.

3.2.6. *Metagenome quantification of nosZI and nosZII*

To differentiate between Clade I and II variants of the *nosZ* gene, a total of 1463 sequences annotated as being *nosZ* using the KO (KEGG Orthology) database were retrieved from the metagenomic libraries in our study. In order to classify them based on clade and to provide a taxonomic placement a reference database was generated. NosZ amino acid sequences were downloaded from the FunGene database (Fish et al., 2013) and classified as Clade I (*nosZI* [PRK02888; Tat dependent]) or Clade II (*nosZII* [nitrous_nosZ_Gp; Sec dependent]) based on conserved protein domains using CD-Search (Marchler-Bauer and Bryant, 2004). Classification was confirmed by detection of signal peptides using the PRED-TAT algorithm (Bagos et al., 2010). Taxonomy for each reference sequence was retrieved from NCBI using accession numbers associated to reference sequences. Metagenome extracted *nosZ* sequences were annotated by identifying their closest match to the reference database using BLASTX (word_size: 3, E-value: 10). Matches with 60% identity and 40 amino acids coverage (cutoff) were retained and classified based on the best match. A total of 974 sequences of the original 1463 were annotated.

3.2.7. *Statistical analyses*

All statistical analyses were performed in R (R Development Core Team, 2008) using the phyloseq (McMurdie and Holmes, 2013), pvclust (Suzuki and Shimodaira, 2006) and vegan (Oksanen et al., 2013) packages. Detailed descriptions can be found in supplemental methods.
3.3. Results

3.3.1. pH dependent changes in emissions linked to denitrifier community size as well as to total community diversity and composition

The preferential loss of N from soils as N\textsubscript{2}O, or alternatively the efficiency of conversion of N\textsubscript{2}O to N\textsubscript{2}, as determined using the N\textsubscript{2}O ratio ($N\textsubscript{2}O/(NO+N\textsubscript{2}O+N\textsubscript{2})$) was negatively associated with soil pH ($R^2 = 0.83, p<0.001$) (Figure 3.1A). However, when individual gases produced during denitrification were considered, pH was only strongly and inversely associated with emissions of N\textsubscript{2}O ($R^2 = 0.62, p<0.01$), with other gases showing no clear pattern (NO [$R^2 = 0.12, p=0.25$], N\textsubscript{2} [$R^2 = 0.21, p=0.11$]) (Supplementary Fig. S3.1). The N\textsubscript{2}O ratio was negatively, and pH was positively, associated with microbial diversity ($R^2 = 0.57, p<0.01$; $R^2 = 0.49, p<0.01$), as well as to total denitrification gene ($nir$ & $nos$) abundance ($R^2 = 0.57, p<0.01$) (Figure 3.1B-1C and Supplementary Fig. S3.2). Across all soils the Proteobacteria, Actinobacteria and Firmicutes phyla were the dominant phyla, and represented >75% of total microbial populations in pasture soils (Figure 3.1D). Comparison of samples based on 16S rRNA community composition visualised with a non-metric multidimensional scaling (NMDS) plot, using a Bray-Curtis dissimilarity matrix, also displayed a significant link to the N\textsubscript{2}O emission ratio and pH (Figure 3.1E and Supplementary Fig. S3.3-S3.4). A Mantel test, however, supported the correlation between microbial community structure and both the N\textsubscript{2}O ratio ($r = 0.57, p<0.001$) and pH ($r = 0.61, p<0.001$). A pvclust analysis (hierarchical clustering with p-values calculated via multiscale bootstrap resampling, Supplementary Fig. S3.5) demonstrated that while at a 95% confidence level the clusters formed represented replicates for the same site, at lower confidence levels (<95%) soils could be clustered geographically (4 clusters: 1 Ireland; 3 New Zealand: Otago, Canterbury and North Island).

3.3.2. pH and the N\textsubscript{2}O ratio correlate to distinct microbial populations

Operational taxonomic units (OTUs at 97% sequence similarity) significantly associated to changes in emissions, or pH, were identified using Spearman's rank correlation (Figure 3.2). A total of 590 OTUs displaying both a statistically significant result ($p<0.05$) and a strong effect ($r \geq 0.5$ or $r \leq -0.5$), based separately on either variable, were analyzed. The number of detected OTUs was 2.5-fold larger for pH (554 OTUs) than for N\textsubscript{2}O ratio (224 OTUs) (Figure 3.2). Surprisingly, the number of
OTUs either positively or negatively correlated, to either variable, was relatively conserved indicating an almost 1:1 replacement of OTUs along the gradient. For pH, 49.2% of detected OTUs were positively and 50.7% were negatively correlated, whereas for the N2O ratio 47.8% were positively and 52.2% were negatively correlated. As a general trend, taxa showed a strongly conserved antiparallelism in relationship to pH and N2O ratio consistent with prior trends (Figure 3.1). While certain phyla displayed conserved patterns (e.g. Chloroflexi and Bacteroidetes), all phyla had examples of contrasting responses suggesting diverse life strategies. However, certain lineages at lower taxonomic levels did present consistent patterns (e.g. class Ktedonobacteria within the Chloroflexi, Subgroup 1 & 2 of the Acidobacteria, and Frankiales within the Actinobacteria). Lineages with known functional roles associated to N cycling like the Nitrospirae (positive correlation to pH and a negative correlation to N2O ratio) and the Thaumarchaeota (mostly negative correlation to pH and a positive correlation to N2O ratio) showed clear responses. It is also worth noting that candidate phyla (WD272, WS3) as well as other poorly studied phyla (e.g. Armatimonadetes) showed strong correlations with the N2O ratio. For full taxonomic lineages and corresponding response to pH and emissions see Supplementary Table (Samad et al., 2016b).

3.3.3. Linking denitrifying genes with pH and N2O emissions

To determine the effect of varying pH on the genetic potential for denitrification, qPCR analysis was performed for key denitrification genes. Results confirmed a link between pH and the denitrification potential of soils (total [sum] abundance of all measured denitrification genes \([nirS, nirK, nosZ1, nosZII]\)). A positive association with pH (\(R^2 = 0.41, p<0.05\)) was observed, with an inverse response observed based on emissions (negative association with N2O ratio \([R^2 = 0.57, p<0.01]\)) (Figure 3.3). To confirm observations, and to account for potential biases associated with primers and PCR, we determined the total abundance (per 2.63 million reads per sample) of denitrification genes in metagenomes created from 6 soils (Figure 3.3 and Supplementary Fig. S3.6). Trends based on total denitrification gene abundance were conserved between approaches (\(R^2 = 0.66, p<0.05\)), however, discrepancies were observed when clade specific \(nosZ\) gene correlations were performed. For Clade I trends were similar based on either qPCR or metagenome, although these were not statistically significant (\(R^2 = 0.44\)).
However, results for Clade II based on metagenomic data showed a strong and statistically significant link to both pH ($R^2 = 0.69$, $p<0.05$) and N$_2$O ratio ($R^2 = 0.63$, $p=0.059$) that was not consistent with qPCR results. Despite low PCR efficiencies (average 66%), the abundance of nosZ genes belonging to Clade II were consistently higher than Clade I for both methods (~5-fold based on metagenome and 1.02-fold based on qPCR) (Figure 3.3-3.4). Irish soils had significantly higher numbers (1.9-fold, $p<0.05$, Welch’s t-test on metagenome data) of nosZ genes compared to New Zealand. It was also observed that taxonomic richness and diversity for Clade II was approximately 3-fold higher than for Clade I. A total of 11 different phyla (Bacteroidetes, Firmicutes Verrucomicrobia, Gemmatimonadetes, Thermomicrobia, Proteobacteria [Alpha, Beta, Delta and Gamma], Spirochaetes, Aquificae, Euryarchaeota, Crenarchaeota, and Chloroflexi) were identified based on nosZ sequences. The Bacteroidetes dominated those belonging to Clade II (nosZ) while the Alphaproteobacteria dominated within Clade I (Figure 3.4 and Supplementary Fig. S3.7). We also examined the nirS and nirK genes individually, and found a positive association with pH ($R^2 = 0.53$, $p<0.05$) and negative association with N$_2$O ratio ($R^2 = 0.38$, $p<0.05$) for nirS (Supplementary Fig. S3.8). However, no significant associations were observed for the nirK gene.

3.3.4. Linking functional richness with pH and N$_2$O emissions

To account for changes in community metabolic potential outside of those previously explored, trait (function) specific patterns, associated to pH and emissions, were explored by determining the functional richness at two different levels: general N metabolism (all N cycling related genes detected) and total functional potential (total number of different genes detected). No pattern was observed between functional richness (total functional richness as well as functional richness of N-metabolism) and pH or N$_2$O emission ratio in the soil (Supplementary Fig. S3.9).
Figure 3.1. Relationship between soil pH, N₂O emission ratio, community phylogenetic and functional potential. Relationships of N₂O/(NO+N₂O+N₂) with pH (A), Shannon diversity based on 16S OTUs clustered at 97% sequence similarity (B), and total gene abundance (gene abundance per 5 ng soil DNA) for denitrification genes (nirS, nirK, nosZI and nosZII) based on qPCR (C). Changes in community composition at phylum level for Irish (IR) and New Zealand (NZ) soils ranked by country (a-c: IR: Ireland soils, d-m: NZ: New Zealand soils) and decreasing N₂O emission ratio (D). Microbial community dissimilarities of soils with different emission profiles as determined using NMDS (Bray-Curtis) ordination (E).
Figure 3.2. Taxonomic summary of OTUs significantly associated (p<0.05 after BH correction; $r \geq 0.5$ [Red] or $\leq -0.5$ [Green]) to either pH or N$_2$O emissions ratio. The graph represents a cladogram of 590 OTUs. Nodes on the tree (moving outwards from center) correspond to taxonomic level [Domain, Phylum, Class, Order, Family, Genus and OTUs]. Shaded areas of branches delineate defined taxonomic groups. Abbreviations: S, Subgroup-22; H, Holophagae; SG, 7, 10 and 17 denotes Acidobacterial orders (subgroups); Rhodo., Rhodospirillales; Sphing., Sphingomonadales; Xantho., Xanthomonadales; Burk., Burkholderiales; Nit., Nitrosomonadales; Frank., Frankiales; Mic., Micrococcales; Thermo., Thermoleophilia; Acid., Acidimicrobiia; KD4, KD4-96; An., Anaerolineae; Sphingobac., Sphingobacteriia; Cyto., Cytophagia; Flavo., Flavobacteriia; Spa., Spartobacteria; Ver., Verrucomicrobiae; Plancto., Planctomycetacia; Gemma., Gemmatimonadetes; SB, Solirubrobacterales; CO, Comamonadaceae.
Figure 3.3. Relationship between abundance of denitrification genes (based on absolute quantification of metagenome & qPCR abundance of nirS, nirK, nosZI, nosZII), N2O/(NO+N2O+N2) and pH. (A-C) Comparison of gene abundances based on either metagenomic (i.e. gene abundance per 2.63 million reads) or qPCR analysis (gene abundance per 5 ng soil DNA) for 6 soils. (D-F) Response of total denitrification genes, nosZ Clade I and II abundances based on metagenomic analysis for 6 soils against N2O/(NO+N2O+N2) (gray) and pH (black). (G-I) Response of total denitrification genes, nosZ Clade I and II abundances based on qPCR analysis for all 13 soils against N2O/(NO+N2O+N2) (gray) and pH (black).
Figure 3.4. Abundance (genes per 2.63 million reads) and predicted taxonomy of nitrous oxide reductase (nosZ) genes by soil (3 New Zealand [HT, Horotiu; LM, Lismore; TP, Templeton] and 3 Ireland soils [JT, Johnstown; SH, Solohead; MP, Moorepark]). (A), and summarized by Clade (B), based on metagenomics analysis. Clade I: Total abundance (150), Richness (4), Shannon Diversity (0.68), Evenness (0.49). Clade II: Total abundance (824), Richness (14), Shannon Diversity (1.87), Evenness (0.46).
3.4. Discussion

Results support the role of native soil pH in shaping community composition and diversity. Microbial community changes were associated to both geographic changes (country and region) as well as to N$_2$O emissions potential, as has been described previously (Morales et al., 2010; 2015). It is important to note that N$_2$O emissions potential, or ratio, as defined in this study (N$_2$O/(NO+N$_2$O+N$_2$)) refers to the propensity of soils to emit N$_2$O over other denitrification gas intermediates. Here this is accomplished using a controlled environment where all other factors were held constant. While this does not reflect the absolute (total amount) of N lost through the process, it is possibly the best predictor of the propensity of the soils to emit N$_2$O (Saggar et al., 2013; Samad et al., 2016a). However, this potential, and the observed phenotype, can be modulated by fluctuating factors and require observations at the denitrification level through expression profiling (transcriptional/translational level) to identify real time drivers of N$_2$O emissions (Liu et al., 2010; 2014; Brenzinger et al., 2015). Despite these limitations our observations highlight a conserved response to pH in both Northern and Southern Hemisphere soils. This suggests pH is part of a universally conserved mechanism selecting for both emissions and microbial communities. The range of pH observed in our soils (5.57 - 7.03) was sufficient to capture the range at which the N$_2$O reductase and N$_2$O emissions fluctuate in response to pH (Liu et al., 2010; Obia et al., 2015; McMillan et al., 2016; Russenes et al., 2016). Soil pH controls not only the assembly of the N$_2$O reductase (Liu et al., 2010), but also alters general expression patterns (Brenzinger et al., 2015) and selects for shifts in microbial community composition (Rousk et al., 2010) indirectly influencing the abundance and type of functional genes in soils. Thus pH can have confounding effects due to its role in shaping the genotype, expression and eventual phenotype associated with denitrification.

While our findings support prior work, we show that of all the three measured gases only N$_2$O had a significant association with pH when compared to maximum emission levels, with maximum observed N$_2$O emissions decreasing with higher pH (Fig S3.1). This was consistent with a lack of correlation between pH and individual denitrification genes. This is potentially due to the modular nature of denitrification (Zumft, 1997; Philippot, 2002; Philippot et al., 2011) where different steps within the pathway are encoded in distinct operons which do not necessarily depend on nor are associated with each other. Despite no strong correlations between emissions and
denitrification specific genes, we found that of the two clades of nosZ gene one was
dominant. Both qPCR and metagenome results show that Clade II are highly
abundant, despite amplification efficiencies being poor (66%) for Clade II primers.
Further, trends between metagenomic and qPCR data did not match and suggested
that Clade II primers do not provide an accurate view of the abundance within our
soils. Despite an apparent under representation (based on qPCR) for nosZII, the
average Clade II/Clade I abundance ratio was >1 both for PCR-based and
metagenomics analysis and is in line with prior observations of their dominance in
certain soils (Orellana et al., 2014). It also aligns with reports linking the abundance
of Clade II with the emissions potential of soils (Jones et al., 2014). Our results also
support the predicted diversity based on clade, with Clade II being represented in
almost 3-times more phyla (Fig. 4) (Jones et al., 2013). Despite evidence supporting
the taxonomic conservation for the two clades (different nosZ types are found
restricted to certain microbial groups) (Sanford et al., 2012; Jones et al., 2013; 2014)
our data shows that these organisms can be associated with soils displaying
contrasting pH and emissions ratios.

Despite the lack of correlation between specific denitrification genes and pH,
we did observe a trend of decreasing abundance of denitrification genes and overall
diversity (based on 16S analysis) with decreasing pH. The role of diversity in
regulating ecosystem processes has been long debated (Loreau et al., 2001; Tilman
et al., 2014). The significance of microorganisms in this debate has only vaguely
been addressed, relative to their predicted diversity (Locey and Lennon, 2016),
despite their expected importance (Van Der Heijden et al., 2008; Graham et al.,
2014). Available studies suggest that when specific microbial functional groups (i.e.
methanotrophy vs. respiration) are used to test diversity/ecosystem process
relationships, significant trends can be uncovered (Griffiths et al., 2000; Wertz et al.,
2006; Wittebolle et al., 2009; Levine et al., 2011). For N₂O, studies suggest that
diversity plays a role, with decreases in diversity leading to increases in emissions
(Philippot et al., 2013; Wagg et al., 2014). Our results support and expand on those
observations indicating a role for diversity-mediated responses at multiple levels
(from whole community, to specific populations linked to denitrification). Though our
data do not allow a mechanism to be determined, we hypothesize that an increase in
diversity ensures a steady population of microbes that are capable of sustaining a
process (e.g. N₂O reduction) over a range of conditions. This diversity is still under
the proximal control of regulators thus it can be modulated based on spatially and
temporally controlled factors.

Identification of specific organisms responding to either pH or emissions
highlighted co-varying trends. For example, while many organisms associated to
changes in pH were identified as being associated to changes in emissions, not all
organisms were. This implies that while certain organisms are selected by pH, they
may not play a role in controlling emissions. Alternatively, some organisms that do
play a role, might not be selected for by pH alone. While such correlations allow for
development of new hypotheses they serve only as a first step in identifying the
mechanisms controlling emissions and the role individual organisms may play. Our
study also does not address the role or contributions other pathways (like
nitrification) might play in regulating N₂O emissions.
CHAPTER 4

Ruminant urine patch reveals significant sources of $\text{N}_2\text{O}$

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Author Contributions: Authors are listed in order of magnitude of their contribution in each role. Corresponding author is indicated by an asterisk (*). SEM, LRB, and MSS designed the experiments. MSS, SN, CAMdK, TJC, KGR and GJL collected samples and processed. MSS and LRB analyzed the data. SEM and LRB had supervisory roles. MSS wrote the manuscript. MSS produced all figures and tables.
Abstract

Nitrous oxide (N₂O) emissions in soils predominantly arise from denitrification under anoxic conditions. However, the emission of N₂O under oxic conditions is poorly understood or sometimes underestimated. Here we used high-resolution automated gas-chromatography to track the urine patch kinetics profile (NO, N₂O and N₂) of 13 different pasture soils from Northern (New Zealand) and Southern hemispheres (Ireland) under oxic conditions. We observed significant production of NO and N₂O compared to controls (no urine) which may indicate other process apart from denitrification. Urine addition elevated the C-mineralization (i.e. production of CO₂) by approx. 10-fold. The production rate of N (i.e. NO+N₂O+N₂ µmol/h) from the urine patch was significantly associated (R²= 0.9, p<0.001) with the maximum emission of N₂O ratio (i.e. N₂O/(NO+N₂O+N₂)). This means higher denitrification rates enhance the emission ratio of N₂O. No significant relationship was observed between pH and the emission ratio of N₂O under this simulated urine patch, although such relation was obvious under true denitrification process (i.e. anoxic conditions). Multiple correlation analysis is showed that NO₂⁻ concentration has significantly correlated to both pH and the emission ratio of N₂O. In addition, oxic respiration (O₂ rate) was negatively correlated with gaseous N rate but positively correlated with ammonia oxidizing bacteria (AOB).
4.1. Introduction

Nitrous oxide (N\textsubscript{2}O) is a potent greenhouse gas about 298 times more effective in trapping heat than carbon dioxide (CO\textsubscript{2}) (IPCC, 2007; Myhre et al., 2013), while also contributing to the depletion of the stratospheric ozone layer (Ravishankara et al., 2009). The concentration of N\textsubscript{2}O in the atmosphere has increased by 20 % from 271 ppb to 324 ppb over the last 260 years (Myhre et al., 2013). The major source of this N\textsubscript{2}O is agricultural soils (Cole et al., 1997; Paustian et al., 2004; Mosier et al., 1998), with ruminant urine representing a significant source of nitrogen (N) linked to global N\textsubscript{2}O emissions (Oenema et al., 2005; van Groenigen et al., 2008).

Grazed pasture soils receive N as organic urea in the form of ruminant excreta, with urine being the dominant source. A single ruminant urination event contributes a fertilization equivalent of approx. 1000 kg N (per ha) to the soil (Di and Cameron, 2002). The transformation of urine to N\textsubscript{2}O or dinitrogen (N\textsubscript{2}) is regulated through both biotic (e.g. nitrification, nitrifier-denitrification and denitrification) and abiotic processes (e.g. chemodinitrification) (Van Cleemput and Samater, 1996; Saggar et al., 2013). Urine is first hydrolyzed into ammonia (NH\textsubscript{3}) or ammonium ions (NH\textsubscript{4}\textsuperscript{+}), and depending on site conditions (i.e. pH, oxic status) can proceed down different pathways, with coupled nitrification-denitrification expected to be the dominant pathway (Wrage et al., 2001). Nitrification is a stepwise aerobic process where biological NH\textsubscript{3} or NH\textsubscript{4}\textsuperscript{+} is first converted into nitrite (NO\textsubscript{2}\textsuperscript{-}) by nitrifying or ammonia-oxidizing bacteria, and then into nitrate (NO\textsubscript{3}\textsuperscript{-}) by nitrite-oxidizing bacteria. Further conversions are carried out via a denitrification cascade. Denitrification is an anaerobic stepwise process catalyzed by predominantly heterotrophic bacteria where NO\textsubscript{3}\textsuperscript{-} is converted first into nitric oxide (NO), then into N\textsubscript{2}O, and finally into N\textsubscript{2}. While this process is divided amongst the different group of prokaryotes, the alternative process of nitrifier-denitrification combines both stages within the same organism (Wrage et al., 2001). In this combined pathway, AOB emit N\textsubscript{2}O along with other gases under oxic conditions without following the anoxic denitrification pathway (Zhu et al., 2013; Stein and Yung, 2003). In this pathway (by AOB), the oxidation of NH\textsubscript{3} to NO\textsubscript{2}\textsuperscript{-} occurs first, followed by the reduction of NO\textsubscript{2}\textsuperscript{-} to NO, N\textsubscript{2}O, and N\textsubscript{2}. In addition, NO\textsubscript{3}\textsuperscript{-} is not produced under nitrifier-denitrification process, which is a linking compound between two pathways (nitrification-denitrification). However, recent findings suggest that N\textsubscript{2}O production could completely bypass denitrification with
N₂O production under anaerobic conditions occurring immediately through hydroxylamine oxidoreductase (Caranto et al., 2016).

The complex array of potential transformations leading to N₂O suggests that soil conditions can alter the contributions of each pathway (Zhu et al., 2013), but it also implies that chemical transformations within each pathway can transiently alter the conditions overriding intrinsic regulators. This is especially true for pH, considered a master regulator of N cycling and N₂O emissions (Čuhel et al., 2010). Under true (anaerobic) denitrification in soils, the emission ratio of N₂O and pH are negatively associated with each other (Šimek and Cooper, 2002; Bakken et al., 2012; Raut et al., 2012; Qu et al., 2014; Samad et al., 2016a). The current hypothesis is that low pH hinders the posttranslational assembly of a functional N₂O-reductase enzyme (Bakken et al., 2012; Bergaust et al., 2010; Liu et al., 2014). However, most studies have utilized nitrate or nitrite as an N source. Within urine patches the dominant N source is urea, and its hydrolysis and sequential transformation can result in large pH fluctuations (Sherlock and Goh, 1985; Clough et al., 2017; Samad et al., 2017). An initial ‘liming’ effect is consistently recorded and suggests that regulation by intrinsic soil pH can be decoupled allowing other regulators to become more important within urine patches.

Here, we used a fully automated high-resolution gas chromatography (GC) system for measuring gas kinetics immediately after applying artificial urine in 13 different soils representing Northern (Ireland) and Southern Hemispheres (New Zealand) soils. Our objectives were: (1) to determine the urine patch kinetics of soils, (2) to investigate the relationship between pH and the emission ratio of N₂O (i.e. N₂O/(NO+N₂O+N₂)) under a urine patch, (3) to determine if regulators identified under denitrification conditions still exert a role within urine patches, and finally (4) to determine which variables are linked to production of gaseous N rate (i.e. NO+N₂O+N₂ μmol/h) as well as emission ratio of N₂O.

4.2. Materials and methods

4.2.1. Study sites, and sample collection

Soil samples were collected from 13 different sites in both Northern (Ireland [Moorepark, Johnstown, Solohead]) and Southern (New Zealand [Warepa, Otokia,
Wingatui, Tokomairiro, Mayfield, Lismore, Templeton, Manawatu, Horotiu, Te Kowhai] hemispheres as described previously (Samad et al., 2016a).

4.2.2. pH measurements

Soil pH was measured using deionised (DI) water as in (Samad et al., 2016a). All pH measurements were done using an Orion 2-star pH Benchtop pH meter (Thermo Scientific) equipped with an Orion 8175BNWP electrode (Thermo Scientific).

4.2.3. Nitrate adjustment

Nitrate levels in soils (150 g dry weight) were adjusted by placing samples in 500 ml filter funnels with 4.5 cm diameter (0.2 µm) Millipore membrane filters and subsequently flooding them with a 2 mM NH$_4$NO$_3$ solution for 10 minutes. Samples were immediately drained by applying a vacuum. The moisture content of drained samples was determined and dry weight equivalents were used for gas kinetic experiments.

4.2.4. Artificial urine preparation

Artificial urine was prepared by following the protocol of Kool et al., (2006) and pH adjusted if needed (pH = 7). There are some reasons why artificial urine was used instead of ruminant urine. N concentration is fixed in the artificial urine and easy to reproduce for multiple experiments at any time. Nitrogen concentration and volume was adjusted to simulate a urine patch in the field (final dose was equivalent to 1000 kg N/ha, or 13.3mg N/vial delivered in a 1.29 ml dose for a final concentration of 0.66 mg N/g of soil (dry weight)).

4.2.5. Gas kinetics of urine cascade

All samples were processed using a slightly modified version of the method described previously (Samad et al., 2016a). For each sample, 20 g (dry weight equivalent) of nitrate adjusted soil was placed inside a 120 ml serum vial and compressed to obtain 70% water filled porosity (WFPS) and 30% air filled porosity (AFP) which mimicked natural soil conditions. Vials were sealed with an air-tight butyl-rubber septa and an aluminium crimp cap, followed by three rounds of flushing and evacuating using 20% pure O$_2$ (80:20 He:O$_2$ mix). Urine treated vials received 1.29 ml (13.3mg N/vial) of artificial urine delivered via a syringe needle one minute
before the first GC reading of all samples. For each soil and treatment (urine and without urine), triplicate vials were prepared and incubated at 20°C using an automated GC system (Molstad et al., 2007). All data presented were from experiments performed in two runs, with each run containing independent standards (duplicates of four different gas standards). All standards were prepared using evacuated vials (120 ml with septum) filled with commercially produced standard gases (supplied by AGA). Headspace samples (approx. 1 ml) were measured every 5 hours (O₂, CO₂, NO, N₂O, N₂) using an autosampler. To prevent anaerobiosis additional pure O₂ (final conc. ~20%) was injected (1-4 times depending on O₂ consumption rate) to the vials throughout the incubations. Incubations lasted approx. 180 hours. The emission ratio of N₂O (i.e. N₂O/(NO+N₂O+N₂)) was determined at every time points and the maximum observed value was used for downstream analysis.

4.2.6. Nitrite (NO₂⁻) measurements

To allow destructive sampling and monitoring of NO₂⁻ levels, 7 additional vials per soil (12 ml serum vials, each containing 2 g of soils (dry weight) under the same treatment) were used and kept offline (not processed for gas kinetics). These vials were only incubated for 25 hours, with sampling occurring at different time intervals. NO₂⁻ concentrations were determined by distilled water extractions (4 ml per vial and shaking for 1 minute). A 1ml aliquot of extract was transferred to a microcentrifuge tube and centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed and NO₂⁻ concentration was measured immediately using a Sievers Nitric Oxide Analyzer (NOA 280i; GE Instruments, USA).

4.2.7. Calculation of gaseous N (NO+N₂O+N₂) emissions and C mineralization rates

Gaseous N emissions and C mineralization rates were calculated using the mean production rate of NO+N₂O+N₂ (µmol/h per vial) and CO₂ (µmol/h per vial) respectively, within the first 40 h.

4.2.8. Quantification of ammonia oxidizers

The ammonia oxidizers (archaeal [AOA] & bacterial [AOB] ammonia monooxygenase gene; amoA), and total prokaryotes (16S rRNA genes) were
quantified by quantitative (qPCR). All reactions were performed in 384-well plates using the QuantStudio 6 real-time PCR (Applied Biosystem, CA, USA). Absolute quantification was preformed using a 10-fold dilution series (\(10^8\) to \(10^1\)) of known copy numbers of plasmid templates, generated from pGEM-T easy (Promega, Madison, Wisconsin, USA). Each target was run in separate plates (384-well) and included cloned standards and no template controls. All targets (AOA, AOB and 16S rRNA gene) were run in quadruplicates to determine abundance. The relative abundance of each target was then calculated as a percent ratio (e.g. gene abundance of each target/total prokaryotes (16S rRNA)).

All reactions were performed in 10 µl volumes containing: 1x Master Mix (Fast SYBR Green Master Mix, ABI), 0.2-0.6 µM of each primer [0.2 µM for AOA (Tourna et al., 2008), 0.6 µM for AOB (Avrahami et al., 2003), 0.5 µM for 16S rRNA (Hartman et al., 2009)], 2 µl of target DNA (5 ng total) and autoclaved Mili-Q H₂O to a final volume of 10 µl. qPCR details are summarized in Table S4.1.

4.2.9 Statistical analyses

Analyses were performed in JMP (SAS Institute, Cary, NC, USA) and R (R Development Core Team, 2008). Statistical significance was determined by means of independent t-test for comparison of treatments. Linear regressions were used to identify the relationship between two variables. Multiple correlation tests (Spearman correlation) were performed across variables.
4.3. Results

4.3.1. N kinetics under urine patch

Soil samples were incubated under oxic conditions for 180 hours (Figure 4.1 and Supplementary Figure S4.1). A significant (p<0.001) increase in all measured gases was observed in response to urine addition (Supplementary Figure S4.2). Kinetic profiles demonstrate active respiration, with the extent of activity varying across soils as reflected in both oxygen consumption and mineralization rates (Figure 4.1). The rate of CO₂ production as a result of C-mineralization was increased by 10-fold under urine patch conditions compared to control (without urine treatment). The maximum concentrations of NO and N₂O were 24.2 nmol N/vial (mean 16.8±6.9 nmol N/vial) and 19 µmol N/vial (mean 6.7±4.7 µmol N/vial) respectively after urine addition. NO₂⁻ was produced in all soils upon urine addition. The maximum concentration of NO₂⁻ was 20.6 nmol/vial (mean 3.5±2.6 nmol/vial [20 g soil]) (Figure 4.2).

4.3.2. Regulators of N₂O emissions under true denitrification (nitrate + anoxic) vs. urine patch (nitrate + urea + oxic) conditions

It was observed that maximum observed N₂O ratios under denitrifying vs. urine patch conditions did not correlate (R²=0.1, Figure 4.3). Further, while pH was a strong driver of N₂O ratio under denitrifying conditions (R²=0.83, p<0.001), no relationship was found under urine patches even when soil properties that could affect conditions at the aggregate level (i.e. drainage class) were accounted for. Instead, we found that the N₂O ratio within urine patches increased in a linear manner as the rate of gaseous N (NO+N₂O+N₂) increased (Figure 4.4).

4.3.4. Multiple correlation analysis across variables from urine patch, soil properties and ammonium oxidizers (AOA and AOB)

A Spearman correlation test was performed to investigate the relationship across variables including urine patch kinetics, soil chemistry and relative abundance of ammonia oxidizers (AOA & AOB) (Figure 4.5). It was observed that NO₂⁻ conc. (max.) under urine patch conditions positively correlated with soil pH (r = 0.61,
p<0.05), the gaseous N rate (r = 0.66, p<0.05) as well as the emission ratio of N₂O (r = 0.74, p<0.01). C mineralization rate was positively correlated with moisture content (r = 0.58, p<0.05), but negatively correlated with pH (r = -0.77, p<0.01). Oxic respiration rate was positively correlated with AOB (%) (r = 0.63, p<0.05), but negatively correlated with gaseous N rate. Positive correlation was observed between AOB (%) and AOA (%) (r = 0.56, p<0.05).
Figure 4.1. Gas kinetics (O₂, CO₂, NO, N₂O, N₂) of urine cascade events in 13 different soil samples (10 New Zealand and 3 Ireland soils) under oxic incubation. Artificial urine (dose was 1000kgN/ha or 13.3 mgN/vial (= 1.29 ml per vial)) simulating the urination event of a cow was injected into each vial just before the first GC measurement. The GC measurements were performed continuously at 5-hour intervals for 180 hours. The top row of the figure represents O₂ conc. of each soil (per vial). To keep the vial under aerobic conditions additional O₂ was added to each vial (2-4 times). Figure represents mean (dot points), SE, and a smooth line which is a fit line of replicates (n = 3).
Figure 4.2. Nitrite (NO$_2$) concentration was measured at different time intervals for 25 hours after treatment with urine.

Figure 4.3. Relationship between pH and N$_2$O emission under both oxic (urine patch) and anoxic (true denitrification) conditions. (A) Relationship between pH and N$_2$O emission ratio under anoxic condition. (B) Relationship between pH and N$_2$O emission ratio under oxic condition (with urine treatment). (C) Relationship between N$_2$O emission ratio under oxic conditions (urine patch) and N$_2$O emission ratio under anoxic conditions (without urine patch).
Figure 4.4. Relationship between N rate (i.e. production rate of NO+N$_2$O+N$_2$) under urine patch conditions and emission ratio of N$_2$O (i.e. N$_2$O/(NO+N$_2$O+N$_2$)) under true denitrification conditions.
Figure 4.5. Heatmap shows spearman correlations across variables—under urine patch kinetics, soil chemistry and relative abundance of ammonium oxidizing bacteria and archaea (AOB, AOA). Significant values are shown (after BH-corrected) as asterisk (*p<0.05; **p<0.001).
4.4. Discussion

Very little is known about gas kinetics profile of pasture soils, especially N\textsubscript{2}O emissions under ruminant urine patches. Here we showed the significant (p<0.001) production of NO and N\textsubscript{2}O under oxic urine patch conditions in 13 different pasture soils, representing from Northern and Southern Hemispheres. We also observed the production of NO\textsubscript{2}\textsuperscript{-} in almost all soils. Our results probably indicate that apart from denitrification other pathway is dominant under urine patch conditions. This is a rapid and alternative pathway for N\textsubscript{2}O emissions under oxic or low oxygen conditions in soils. No isotopic measurements were done in our experiment and that is why we cannot able to specify the pathway. The process has been shown in previous \textsuperscript{15}N study where significant production of NO and N\textsubscript{2}O from urea and ammonium-sulfate amended soils were driven by nitrifier-denitrification under low oxygen availability (Zhu et al., 2013).

Soil pH is known to be an important edaphic factor in the regulation of the emission ratio of N\textsubscript{2}O (i.e. N\textsubscript{2}O/(NO+N\textsubscript{2}O+N\textsubscript{2})) under true denitrification conditions (Qu et al., 2014; Raut et al., 2012). The pH interferes with the function of the nitrous oxide reductase enzyme resulting in more N\textsubscript{2}O emissions compared to slightly alkaline or neutral pH (Liu et al., 2010; 2014). In our previous studies, we showed that there was a significant linear relationship between pH and emission ratio of N\textsubscript{2}O (Samad et al., 2016a; 2016b) where 13 different pasture soils were used. By using the same types of pasture soils in this study, we wanted to investigate further whether the same trend could be observed or not, under urine patch conditions. We observed that emission ratio of N\textsubscript{2}O is not significantly associated with pH. The reason could be that the urine addition in soils disrupts or elevates the soil pH due to urea hydrolysis (Sherlock and Goh, 1985; Cabrera et al., 1991). Furthermore, it is completely a different processes (anoxic vs. oxic) and different microorganisms are involved. In general, this process is regulated by ammonia oxidizers; whereas, in denitrification, heterotrophic bacteria are predominantly responsible. These could possibly explain why the emission ratio of N\textsubscript{2}O from this process is differed from denitrification and is not linked to pH.

We have observed that the rate of C-mineralization was higher compared to control (without urine treatment) under urine patch conditions. This suggests that
oxic C-mineralization process may support the transformation of NO and N₂O. This trend was observed previously in denitrification under anoxic conditions, where C-mineralization enhanced the denitrification (Reddy et al., 1982; Zimmerman and Benner, 1994; Samad et al., 2016a).

There was a positive linear relationship between the gaseous N rate (i.e. NO+N₂O+N₂ µmol/h) and emission ratio of N₂O (i.e. N₂O/(NO+N₂O+N₂)). This means, higher N rate can contribute the higher emission ratio of N₂O. Furthermore, the gaseous N rate can be used as an alternative predictor for modeling the emission ratio of N₂O.

Furthermore, we did not observe any strong correlation between ammonia oxidizers (AOA and AOB) with gaseous N rate and emission ratio of N₂O. The AOA and AOB abundance analysis were done based on field soils, and compared here with urine patch kinetics. As a result, this could affect our ability to see the genotypic relation with urine patch kinetics. However, we could generate a hypothesis, for example, the relative abundance of AOB in soil is linked to the oxic respiration (O₂ µmol/h) under urine patch conditions. This gives us an indication where the abundance of AOB can be regulated by the availability of oxygen. Importantly, the higher the rate of oxic respiration could support the growth of AOB.

We observed that NO₂⁻ concentrations, which play an important role in urine patch to understand the emission ratio of N₂O and gaseous N rate as observed positive correlation. This was also demonstrated in the previous study where NO₂⁻ intensity was strongly correlated with N₂O emissions (Maharjan and Venterea, 2013).

In summary, we observed the gas kinetics of NO, N₂O and N₂ in urine treated soils in oxic conditions suggest other process apart from denitrification (i.e. nitrifier-denitrification, co-denitrification). As this process was different from denitrification (anoxic), therefore no relationship was observed between soil pH and emission ratio of N₂O under urine patches.
CHAPTER 5

Response to nitrogen addition reveals metabolic and ecological strategies of soil bacteria

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Abstract

The nitrogen (N) cycle represents one of the most well studied systems yet the taxonomic diversity of the organisms that contribute to it is mostly unknown, or linked to poorly characterized microbial groups. While progress has allowed functional groups to be refined, they still rely on *a priori* knowledge of enzymes involved, and the assumption of functional conservation, with little connection to the role the transformation plays for specific organisms. Here, we use soil microcosms to test the impact of N deposition on prokaryotic communities. By combining chemical, genomic and transcriptomic analysis we are able to identify and link changes in community structure to specific organisms catalyzing given chemical reactions. Urea deposition led to a decrease in prokaryotic richness, and a shift in community composition. This was driven by replacement of stable native populations, which utilize energy from N-linked redox reactions for physiological maintenance, with fast responding populations that use this energy for growth. This model can be used to predict response to N disturbances and allows us to identify putative life strategies of different functional, and taxonomic, groups thus providing insights into how they persist in ecosystems by niche differentiation.
5.1. Introduction

Modern microbiology techniques have given us unprecedented access to the microbial world (Spiro, 2012; Rinke et al., 2013), yet soil microbial communities remain poorly understood (Delmont et al., 2015). While many studies have focused on the diversity or abundance of key populations (Taylor et al., 2012; Gubry-Rangin et al., 2015; Wei et al., 2015), fewer have looked at the transcriptional profiles over time (Nicol et al., 2008; Morales and Holben, 2013), and even less have done so for multiple groups at the same time (Liu et al., 2010; Brenzinger et al., 2015). This is particularly true of organisms involved in nitrogen (N) cycling in soils. The complexity of the underlying processes combined with the diversity of microbes contributing to each process provides a large challenge to identifying mechanisms active at any given time (Butterbach-Bahl et al., 2013). Currently we lack enough information to understand basic ecological concepts linked to N cycling in situ such as: i) substrate competition at both inter and intra species level, ii) full diversity of both present and active N cycling populations, iii) and the life strategies of these populations which in turn control their responses (both as observed growth or transcriptional changes).

The initial discovery of ammonia oxidizing archaea (AOA) and recognition as important players in the N cycle (Leininger et al., 2006; Hatzenpichler, 2012; Stahl and la Torre, 2012) highlighted the unexpected gaps in knowledge. Later studies have suggested different life strategies for AOA when compared to ammonia oxidizing bacteria (AOB) (Sterngren et al., 2015), but this may be complicated by variance across strains (Bayer et al., 2015). One major unknown is whether observations made in studies, or organisms, from one ecosystem translate to others.

It is well established that individual intermediates in the N cycle can be used for specific reasons (i.e. ammonia oxidation provides electrons, while denitrification intermediates accept reducing equivalents), but the purpose of the reactions for any organism is another major unknown. That is, while some organisms carry out these processes for electrogenic purposes that can result in growth, others do it in order to maintain redox homeostasis (e.g. to dissipate excess reductants) (Green and Paget, 2004). Unfortunately examples where an organism harbors multiple versions of the same enzyme for completely different purposes (respiration vs. redox balance) exist (Hartsock and Shapleigh, 2011), and are likely to limit generalizations.

Despite this, studies focusing on population changes in response to manipulations have consistently recorded conserved patterns (e.g. growth of AOB
but not AOA (Jia and Conrad, 2009; Di et al., 2009; Pratscher et al., 2011)) suggesting that responses by specific populations in a given location or ecosystem are predictable. However, the debate continues on whether niche specialization and differentiation can be determined based solely on correlations, without analyzing the wider array of processes that contribute or influence any given N transformation (Prosser and Nicol, 2012). This is relevant in ecosystems such as agricultural grassland where an understanding of N cycling is crucial for management of both productivity and greenhouse gases (Herrero et al., 2016), of which nitrous oxide (N$_2$O) is a key player (Reay et al., 2012).

In grazed pastures (i.e. agricultural grasslands) N deposition through ruminant urine drives the emissions of N$_2$O (Saggar et al., 2013). In this system a full cascade of transformations begins with urea and can result in accumulation of any intermediate depending on conditions, but with a final end product of N$_2$ or N$_2$O. While the chemical transformations have been explored (Hamonts et al., 2013; Baral et al., 2014; de Klein et al., 2014a; 2014b), mechanistic understanding of the populations catalyzing the reactions, and the purpose they serve for the organisms is less clear. In this study, we aimed to identify active N-transformation pathways as well as changes in microbial populations/taxa abundance and transcriptional activity for organisms involved in N loss (through gases) in response to urea (simulated ruminant urine deposition event) and varying moisture content. Observed chemical transformations were linked to changes in genotype (functional potential through DNA; a proxy for population changes), expression of genotype (RNA profiles), and total community composition (specific taxonomically defined populations based on the 16S ribosomal rRNA gene). We hypothesized that sequential transformation of nitrogenous intermediates would be coupled to changes in expression of functional genes catalyzing production and consumption of intermediates. Alternatively, transformations not linked to population, or expression changes, would be driven by other (abiotic) pathways. We also hypothesized that changes in transcription, or population size, could serve to determine life strategies of microbes utilizing each intermediate (whether they are used for growth vs. physiological maintenance). To test this we mimicked a ruminant urine-N deposition event using repacked soil cores (soil bulk density= 1.1 Mg m$^{-3}$) on tension tables monitored for 63 days. Soils were treated with urea under two different moisture contents: high (near saturation; -1.0 kPa) and low (field capacity; -10 kPa) moisture. Simultaneous measurements of soil chemistry, gas kinetics, microbial community composition (by 16S rRNA gene
amplicon sequencing) and functional gene abundance (for nitrification and denitrification) at DNA (gene) and RNA (transcript) levels were performed to determine the active populations and pathways.

5.2. Materials and methods

5.2.1. Sample collection and experimental design

A detailed methodology can be found in (Clough et al., 2017). In brief, soil was collected from a permanently grazed agricultural grassland (dairy pasture) in March (early spring) at the Teagasc Moorepark Research Center, County Cork, Ireland (8°15'W, 52°9'N). The soil is classified as a Typical Brown earth from the Clashmore Series (Gardiner and Radford, 1980). Soil was sampled after the turf was removed and a spade was used to randomly sample the A-horizon (5-20 cm depth, excluding grass layer). To avoid fresh N loading, fields had not been grazed for over a month. Field moist samples were immediately shipped to Lincoln University, New Zealand and kept at 4°C until processed. Prior to use, soil was sieved (≤ 2 mm) to remove any stones, plant roots or earthworms and packed into stainless steel rings (7.3 cm internal diameter, 7.4 cm deep) to a depth of 4.1 cm at *in situ* soil bulk density (1.1 Mg m⁻³ with a gravimetric water content (θ₉) of 0.24 g water g⁻¹ soil). The resulting cores had a total porosity of 0.58 cm³ pores cm⁻³ soil and were arranged in a factorial experiment replicated four times. Soil cores were maintained at two moisture contents: high (near saturated; -1.0 kPa) and low (field capacity; -10 kPa) moisture using tension tables (Romano et al., 2002). These moisture contents, -1 and -10 kPa respectively, corresponded to 53% and 30% volumetric water content, or 91% and 52% water-filled pore space (WFPS). Nitrogen was applied as a urea solution at 2141 kg urea/ha dry soil (equivalent to a single urination event at the higher rate expected under bovine urine deposition of 1000 kg N ha⁻¹). Four treatments in total were carried out (replicated four times each for a total of 112 cores analyzed) representing two levels of urea and two levels of moisture: urea + high moisture (HM +N; Urea _-1.0kPa), urea + low moisture (LM +N; Urea _-10kPa), no urea + high moisture (HM –N; No Urea _-1.0kPa) and no urea + low moisture (LM –N; No Urea _-10kPa). All cores where held at 20°C for a period of 63 days.

5.2.2. Soil pH, and inorganic-N measurements
Soil pH was monitored throughout the experiment using a flat surface pH electrode (Broadley James Corp., Irvine, California). Inorganic N concentrations (NH$_4^+$, NO$_2^-$, NO$_3^-$) were determined by destructively sampling batches of soil cores (16 soil cores, 4 treatments x 4 replicates) on days 0, 3, 7, 14, 21, 35 and 63. Each core was homogenized and a subsample was extracted (10 g dry soil: 100 ml 2M KCl shaken for 1 hour), filtered (Whatman 42) and analyzed using flow injection analysis (Blakemore *et al*., 1987). N$_2$O flux was determined by placing a soil core into a 1-L stainless steel tin fitted with a gas-tight lid and rubber septa. The headspace was sampled after 15 and 30 minutes and analyzed using an automated gas chromatograph (8610; SRI Instruments, Torrance, CA), linked to an autosampler (Gilson 222XL; Gilson, Middleton, WI) as previously described (Clough *et al*., 2006).

5.2.3. Nucleic acids extraction

Samples for RNA and DNA extraction were collected simultaneously with samples for inorganic N analysis, but only samples at 0, 7, 14, 21, 35, 63 days were processed for nucleic acids. Each biological replicate was extracted and analyzed separately. For each extraction 2 g (wet weight) of soil were processed using the PowerSoil Total RNA Isolation and DNA Elution Accessory Kits (MoBio, Carlsbad, CA) as per manufacturer’s instructions, with slight modifications. Bead beating was done in a Geno/Grinder 2010 (SPEX SamplePrep, LLC, Metuchen, NJ) using two rounds of beating (1750 strokes/min) for 15 s with a 1 min pause in between. The total elution volume for RNA and DNA was 60 µl and 100 µl respectively. RNA was treated with DNase I (RNase-Free) (New England Biolabs, USA) as per the manufacturer’s protocol. RNA quality was assessed by denaturing gel electrophoresis. RNA and DNA concentration, purity and humic acid contamination were determined using a Nanodrop Spectrophotometer, ND-1000 (Thermo Scientific). All extractions were stored at -80 °C until downstream analyses.

5.2.4. Reverse transcription (RT)

Triplicate cDNA conversions (technical replicates) were performed for each RNA extraction using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) according to manufacturer’s protocol. Each 20 µl reaction contained: 13 µl of RNA (208 ng Total RNA), 1 µl of random hexamers (100 pmol), 1µl of dNTP mix (0.5 mM final conc.) and 5 µl of master mix (4 µl of 5X RT buffer and 1 µl Maxima H
Minus reverse transcriptase). All technical replicates for a sample were combined and stored at -80°C until further analysis. All further analyses were performed on the same cDNA pool for each sample.

5.2.5. 16S rRNA gene amplicon sequencing

16S rRNA gene amplicon sequencing was performed using primers 515F/806R (V4 region of the 16S gene) and the Earth Microbiome Project conditions (Version 4_13) (Caporaso et al., 2012). All samples were run simultaneously on a single Illumina MiSeq run. Sequences were first processed in Qiime (version 1.9.1) using default parameters (Caporaso et al., 2010). Sequences were clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity using the SILVA (version 119) reference library (Quast et al., 2012) and UCLUST (Edgar, 2010) following the open-reference Operational Taxonomic Unit (OTU) picking protocol. Taxonomic identification was done using BLAST against the SILVA database (max-e value = 0.001) (Altschul et al., 1990). Subsampling and rarefactions (10 times) were performed to equal read depths of 7,400 per sample, and samples below that threshold were removed. After rarefaction, all 10 OTU tables were merged and exported for further processing in R (R Development Core Team, 2008).

5.2.6. Quantification of gene and transcript abundance

Quantitative PCR (qPCR) was performed in 384-well plates using the ViiA7 real-time PCR system (Applied Biosystems, Carlsbad, CA). Absolute quantification was done using a 10-fold serial dilution (10^8 to 10^1) of known copy numbers of pGEM-T easy (Promega, Madison, Wisconsin, USA) cloned templates as standards. For all targets qPCR runs included cloned standards, no template control and no reverse transcription controls (RNA) run in triplicate. No inhibition or positive amplification on negative controls was observed for any target. All DNA and cDNA samples were run in quadruplicates to determine abundance of: prokaryotes (16S rRNA gene), ammonia oxidizers (archaeal [AOA] & bacterial [AOB] ammonia monooxygenase gene; amoA), denitrifiers (cytochrome cd1-type nitrite reductase gene; nirS, and Clade I nitrous oxide reductase gene; nosZI) and nitrogen fixers (nitrogenase gene; nifH).
All reactions were performed in 10 µl volumes containing: 1× Master Mix (Fast SYBR Green Master Mix, ABI), 0.2-0.6 µM of each primer [0.2 µM for AOA (Tourna et al., 2008), 0.6 µM for AOB (Rotthauwe et al., 1997; Avrahami et al., 2003) 0.5 µM for 16S rRNA (Hartman et al., 2009); nirS (Throbäck et al., 2004; Yergeau et al., 2007), nosZI (Henry et al., 2006) & nifH (Rösch and Bothe, 2005)], 2 µl of template [DNA (1 ng total) or cDNA (80× diluted RT reaction, i.e. total 0.13 ng RNA)] and autoclaved Milli-Q H2O to a final volume of 10 µl. Primers and qPCR conditions are summarized in Table S5.1. A melt curve analysis (95°C for 15 s, 60°C for 1 min then increasing 0.05°C/s (data acquisition) until 95°C) was performed to test for specificity and to confirm no amplification in the negative controls.

5.2.7. Statistical analyses

All statistical analyses were performed in R (R Development Core Team, 2008) using the phyloseq (McMurdie and Holmes, 2013), pvclust (Suzuki and Shimodaira, 2006), vegan (Oksanen et al., 2013) and mpmcorrelogram packages. Detailed descriptions can be found in supplemental methods.

5.2.8. Growth rate estimation and prediction of rRNA operon (rrn) copy numbers

rrn copy numbers for identified OTUs were predicted using the ribosomal RNA operon copy number database (rrnDB) (Stoddard et al., 2015). For each OTU, information from the closest strain available was selected. In instances where a closely related organism was not available, the mean copy number for the closest taxonomic group (i.e. genus, class, etc.) was used. Copy numbers where then compared to the maximum observed abundance and the maximum observed fold change (calculated based on lowest observed abundance for the same organism in a preceding time point for OTUs showing growth or succeeding time points for those decreasing in abundance). An estimated growth rate was calculated for OTUs showing increases in population size in response to N using the following formula:

\[ N_t = N_0 \cdot e^{rt} \]

where: \( N_t \): The amount at time t; \( N_0 \): The amount at time 0; \( r \): exponential growth rate; \( t \): Time passed
5.2.9. Fit model for *rrn* copy numbers

Both non-linear (Michaelis-Menten) and linear regressions were used to fit *rrn* copy numbers and population changes (i.e. maximum abundance and fold-change), and growth rate (per day). The fit model was performed in R using “drc” and “ggplot2” packages.

5.3. Results

5.3.1. Soil pH and N transformation dynamics in response to urea

Soil pH increased from acidic (pH = 5.5 ± 0.1, i.e. mean ± SD) to alkaline reaching a maximum (pH = 8.7 ± 0.2) at day 3 in urea treated soils. Return to baseline pH was modulated by soil moisture with high moisture (HM; -1.0kPa) soil reaching baseline at day 35 and low moisture soils (LM; -10kPa) doing so at day 53 (Figure 5.1). This shift in pH was linked to a successive N transformation process initiated with urea hydrolysis and leading to nitrification and denitrification: urea → $\text{NH}_4^+$ → $\text{NO}_2^-$ → $\text{NO}_3^-$ → $\text{N}_2\text{O}$ → $\text{N}_2$ (Figure 5.1). Sequential peak activity was observed for each transformation with the response modified by moisture. Maximum production (mean µg N g$^{-1}$ soil) for each transformation was observed at day 3, 21 and 35 respectively for $\text{NH}_4^+$ (HM+N = 1758; LM+N= 1730), $\text{NO}_2^-$ (HM+N = 79.2; LM+N= 39.7) and $\text{NO}_3^-$ (HM+N = 429.2; LM+N= 335). Two distinct production peaks were observed for $\text{N}_2\text{O}$, with a short pulse (0 to 5 days) reaching a maximum at day 2 for HM soils (11602.8 µg m$^{-2}$ h$^{-1}$) and day 3 for LM soils (46.8 µg m$^{-2}$ h$^{-1}$) (Figure 5.1 and Supplementary Fig. S5.1). A second, longer duration (10 to ~50 days), $\text{N}_2\text{O}$ pulse reached a maximum at day 28 for HM soils (6405.1 µg m$^{-2}$ h$^{-1}$) and day 30 for LM soils (448.9 µg m$^{-2}$ h$^{-1}$). The large $\text{N}_2\text{O}$ spike (first peak) between days 0 to 5 in the HM+N treatment was about 11.6% of the total $\text{N}_2\text{O}$ cumulative flux over 63 days, whereas in the LM+N treatment the 0 to 5 day periods accounted for 22.3% of the total $\text{N}_2\text{O}$ cumulative flux over 63 days.

5.3.2. Population and transcription dynamics for nitrogen related functional groups

Significant changes (ANOVA, p<0.05) in relative activity (mRNA abundance/16S rRNA gene abundance) were observed promptly between day 0 & 3 for all functional groups (except AOA and N-fixers in HM soil) in response to urea (Figure 5.1). However, maximum relative transcription did not match maximum
production peaks for corresponding substrates, or products, for each functional group. Nitrifiers (ammonia oxidizers) displayed niche differentiation, with time, length and strength of response differing between bacterial (AOB) and archaeal ammonia oxidizers (AOA). Relative activity of AOA increased (4.6-fold for LM and 1.6-fold for HM) under urea treatments at day 3 only, with a subsequent decrease (-19.3-fold for LM and -7-fold for HM) resulting in lower expression than in untreated soils (Figure 5.1). AOB relative activity also increased but was sustained for a much longer period (3-63 days), with maximum activity (>11-fold change) seen at 21 and 35 days for HM+N and LM+N respectively (Figure 5.1). Denitrifiers (both nitrite and nitrous oxide reducers) showed similar responses as AOA, with peak activity at day 3 and a rapid return to baseline, in the case of nitrite reducers decreasing to levels below those observed in non-urea treated soils (Figure 5.1). To account for endogenous sources of N, N\textsubscript{2} fixers were monitored through the activity of the nitrogenase gene (\textit{nifH}). No significant changes were observed except for day 3 (LM +N only), with a subsequent decrease in activity below background. This decrease below background was observed for all N treated samples.

Changes in the relative contribution to total community composition were calculated by normalizing functional gene abundance to total 16S rRNA gene abundance per sample for each functional group (Figure 5.1). The maximum observed relative abundance of each functional group differed for each group (HM|LM, respectively): AOB, 19|12%; AOA, 8|13%; \textit{nirS}, 6.3|2.9%; \textit{nosZI}, 3.3|3.4%; \textit{nifH}, 4.7|4.32%. Further, large population changes over time were mostly limited to AOB. Generally, AOB comprised <1% of the total community, but in response to urea increased up to 29-fold to make up 19% (day 21 for HM) and 20-fold to make up 12% (day 35 for LM) of the community in urea treated soils. In contrast, AOA were found at consistently high levels (median=4.2%) in untreated soils, but numbers decreased >7-fold in response to urea (~1.3% at least 63 day). Similarly, other functional groups (\textit{nosZI}, \textit{nifH}) decreased or remained stable (\textit{nirS}) in response to urea. Similar patterns for both activity and population changes were observed when absolute values were analyzed (Supplementary Fig. S5.2).

5.3.3. \textit{N} deposition induces both a genotypic and a transcriptional response at the community level that is modified by soil moisture content
Urea deposition imposed a general negative selective pressure leading to decreases in OTU level prokaryotic diversity (Shannon, -1.2-fold change), richness (-1.5-fold change) and evenness (-1.1-fold change) at DNA level (Figure 5.2a, Supplementary Fig. S5.3). The same pattern was observed when active microbes (based on RNA) were analyzed with decreases in OTU level prokaryotic diversity (Shannon, -1.3-fold change), richness (-1.9-fold change) and evenness (-1.2-fold change). Moisture was found to have a smaller, but significant, effect compared to urea, with LM samples consistently resulting in lower diversity and richness when compared to their HM pairs. Richness and diversity losses were not recovered even after 63 days. In contrast, samples where no urea was applied remained stable (i.e. constant diversity and richness).

Urea deposition significantly altered community structure (Adonis test: $F=18.04, p<0.001$ for 16S rDNA and $F=26.27, p<0.001$ for 16S rRNA) as shown in a non-metric multidimensional scaling (NMDS) plot using a Bray-Curtis dissimilarity matrix (Figure 5.2b and Supplementary Fig. S5.4). At both DNA and RNA level community changes along the first axis corresponded with changes in response to urea treatment, with the second axis accounting for changes in moisture. A pvclust analysis (hierarchical clustering with p-values calculated via multiscale bootstrap resampling, Supplementary Fig. S5.5) confirmed two major clusters [100% AU (Approximately Unbiased) and 100% BP (Bootstrap Probability)] formed by urea treated (HM+N and LM+N samples, excluding day 0), vs. untreated soils (HM-N, LM-N, field samples, and HM+N & LM+N at Day 0). Temporal variance within each cluster was confirmed using a Mantel correlogram analysis (Figure 5.2c). Urea treated samples had significant changes in community composition immediately upon treatment (Day 0 to 7), with no return to baseline conditions by the end of the experiment. In contrast, untreated samples did not change significantly over time (Supplemental Fig. S5.6)

Changes in community structure were associated with shifts in major taxonomic lineages (Figure 5.3). In general, phylum level changes in abundance and transcription where correlated to each other (Supplementary Table S5.2 and Fig. S5.7, S5.8). Urea deposition induced temporal changes in phylum level abundance with observed maximum fold changes per group (HM & LM at DNA level) being: Acidobacteria, -4.6 & -3.7; Actinobacteria, 2.4 & 5.3; Bacteroidetes, 4.6 & 2.2; Candidate Division WS3, -10.5 & -7; Chloroflexi, -2.9 & -2.6; Firmicutes, 10.8 & 16.2;
Normalized transcriptional activity (reads of 16S rRNA/reads of 16S rDNA) identified the Firmicutes and members within classes of the Proteobacteria as the most transcriptionally active. While abundant phyla tended to have high levels of normalized transcription, less abundant organisms like the Thaumarchaeota, were observed to have high normalized transcriptional activity especially under background conditions (Supplementary Fig. S5.7). In contrast, groups traditionally considered slow growers (e.g. Nitrospirae and Gemmatimonadetes) had low normalized transcription. It was also noted that while normalized transcription levels remained stable without urea, N deposition induced changes. These changes in normalized activity did not always match trends observed at individual DNA or RNA level (e.g. Firmicutes).

5.3.4. Shifts in N and moisture status trigger OTU response linked to divergent life strategies

Since Figure 5.3 only represents a taxonomic summary of all OTUs (irrespective of their response to treatments), it does not provide a clear indication of who is changing and why. To account for this, urea responsive OTUs were identified independently in RNA and DNA profiles (under each treatment) through a SIMPER analysis. OTUs accounting for 50% of the variance were analyzed (Figure 5.4). Response patterns for detected OTUs were conserved between RNA and DNA profiles. However, while some OTUs responded similarly to urea under varying moisture conditions, marked differences were observed with no detectable pattern based on taxonomy.

OTUs within the Proteobacteria identified in the SIMPER analysis did not display a conserved response to urea, however when lower taxonomic levels were examined patterns emerged. A consistent positive response was seen for OTUs within the class Betaproteobacteria and the family Hyphomicrobiaceae, amongst others. Positive responses to urea were also observed at the phylum level for the Firmicutes, Bacteroidetes, Actinobacteria, Gemmatimonadetes and Planctomycetes, although the level of response varied across lower taxonomic levels. In contrast, with only some exceptions, OTUs within the phyla Acidobacteria, Verrucomicrobia,
Nitrospirae, Candidate Division WS3 (also referred to as candidate phylum Latescibacteria) and the Thaumarchaeota all were negatively impacted by urea deposition.

To account for response patterns over time, we focused on OTUs that accounted for 30% of the variance in the SIMPER analysis (36 total), with individual OTU contributions ranging from 5 to 0.1 percent at the DNA level and 5 to 0.06 percent at the RNA level. Temporal patterns were conserved between DNA and RNA profiles (Supplementary Fig. S5.9, S5.10), despite differences in absolute abundance. Once again, moisture acted as a modulator of response with the extent of impact dependent on the OTU (Figure 5.5 and 5.6). While most functional groups responded immediately (at both DNA and RNA level), positively affected OTU responses were observed along all time points creating a succession of positively selected organisms. In contrast, negatively affected OTUs all responded within the first 2 time points indicating an immediate negative selective pressure (Figure 5.6).

Large variances in absolute changes were observed, even within similar organisms (e.g. Pedobacter), with fold changes ranging from -10.5 to 410 across both positively and negatively affected OTUs. Despite this, OTU response was noted to correspond to taxonomy, with both the effect (positive or negative) and the extent of response (fold change or total abundance) in line with predicted ecological growth strategies (r vs. k) predicted for different taxa. To test this, we predicted rRNA operon copy numbers (rrn) for all 36 OTUs and compared them to the observed maximum abundance, max fold change in population or observed growth rate per day. We consistently observed a non-linear response with an asymptote reached at higher copy numbers (Figure 5.7). These trends were consistent independent of which moisture conditions were present at the time of response. To account for preferential response due to moisture, we selected the highest response for each organism and saw no clear difference in patterns. To account for potential biases due to uneven representation, OTUs were grouped into low (1-2 copies of rrn) or high (>2) copy number organisms (Supplementary Table S5.3). While significant changes (p < 0.05, Supplementary Fig. S5.11) were observed in most instances, exceptions were noted (e.g. growth rate under HM).
Figure 5.1. Chemical transformations and biological (functional group) response in soils treated with urea (+/- 1000 µg N/g dry soil) under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]). Error bars are the standard error of the mean (n ≥ 3, except gene abundance data of day 7 [n=1; LM soil] and day 21 [n=1; LM soil]) for replicate mesocosms. Gene and transcript abundance were measured by qPCR targeting: nitrifiers (AOA, ammonia oxidizing archaea; AOB, ammonia oxidizing bacteria), denitrifiers (nirS, cytochrome cd$_{1}$-containing nitrite reductase; nosZI, nitrous oxide reductase) and nitrogen fixers (nifH, nitrogenase reductase). All qPCR results are normalized to 16S rRNA copy numbers and presented as percent of the nucleic acid pool.
Figure 5.2. Total microbial community response (based on 16S rRNA gene amplicon profiling and clustering of sequences at OTU level (97% sequence similarity)) to urea (+/-1000 µg N/g dry soil) under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]) at both DNA and RNA level. Error bars are the standard error of the mean (n = 3, except day 7 [n=1; LM soil] and day 21 [n=1; LM soil]) for replicate mesocosms. (a) Changes in microbial diversity (Shannon) index over time in response to treatment. (b) Non-metric multidimensional scaling (NMDS) ordination plots based on Bray-Curtis distances showing relationships among samples based on OTU level changes in community composition. (c) Mantel correlogram showing autocorrelation on community composition by performing sequential Mantel tests between the Bray-Curtis dissimilarities and the grouping of samples using a time period index (index 1 represents 0-7 days; 2 represents 7-14; 3 represents 14-21; 4 represents 21-35; 5 represents 35-63). Filled circles represent significant correlation (p < 0.05) in community composition at specific time periods, with open circles indicating no significant correlation.
Figure 5.3. Phylum and class level (for Proteobacteria only) changes in abundance (DNA) representing relative contribution >1% of all detected phyla (based on OTUs clustered at 97% sequence similarity). A total of 7,400 sequences were examined per sample. Error bars are the standard error of the mean (n = 3, except day 7 [n=1; LM soil] and day 21 [n=1; LM soil]) for replicate mesocosms. Treatments = +/- N [+/-1000 µg N/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]). Abbreviations: c: Class; p: Phylum. See supplemental Fig. S8 for relative abundance
Figure 5.4. **Taxonomic summary of OTUs responsive to urea treatment identified through similarity percentages (SIMPER) analysis (representing top 50% cumulative sum).** The 4 outer rings represent fold changes in response to urea under high and low moisture content (MH & LM respectively) at either DNA or RNA level, with blank gaps indicating OTUs not identified in SIMPER analysis under the specified ring condition. Nodes on the tree (moving outwards from center) correspond to taxonomic level [Domain, Phylum, Class, Order, Family, Genus and Species/OTUs]. Nodes are colored based on dominant response (>50% conserved fold change response across OTUs within a node) with black notes indicating equal representation of positive and negatively responding OTUs. Shaded areas of branches delineate defined taxonomic groups.
Figure 5.5. Population (16S rDNA) changes (abundance based on 7400 reads per samples) for OTUs identified as positively responsive to urea treatment based on similarity percentages (SIMPER) analysis (representing top 30% cumulative sum). Treatments = +/- N [+/−1000 μg N/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]).
Figure 5.6. Population (16S rDNA) changes (abundance based on 7,400 reads per samples) for OTUs identified as negatively responsive to urea treatment based on similarity percentages (SIMPER) analysis (representing top 30% cumulative sum). Treatments = +/- N [+/-1000 µg N/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]).
Figure 5.7. Relationship between predicted ribosomal RNA operon (rrn) copy numbers and growth rate (per day), maximum observed population change, or fold change in response to N treatment under both high moisture (HM) content, low moisture (LM) content and best growth either in HM or in LM (based on maximum observed growth). Copy number was estimated using rrn database (Stoddard et al., 2015). Copy number values were obtained by finding the closest match (lowest taxonomic level possible) to each OTU and retrieving the mean rRNA copy number for that group.
5.4. Discussion

Functional profiling (identification and quantification of specific functional genes/transcripts) is normally utilized to link chemical transformations to specific microbial populations capable of catalyzing reactions. However, functional groups are comprised of taxonomically diverse species of microbes with different lifestyle strategies that are unlikely to share a conserved response to an ecosystem disturbance (Ho et al., 2012). While functional profiling allows us to measure the net response of a functional group, and could serve as a proxy for determining the importance of the group in a sample, it does not identify how specific organisms benefit from a catalyzed transformation. Here we used a controlled microcosm experiment to measure the response of soil communities to a disturbance in the form of changes in moisture and nitrogen (urea) deposition. Functional analysis (qPCR) demonstrated a biological response to urea, but differing responses to moisture depending on group (Figure 5.1). Responses are potentially linked to different life strategies amongst these groups. Ammonia oxidizers displayed contrasting population and expression profiles, suggesting niche differentiation driven by time and/or substrate concentration. AOA responded early, and declined as new N was made available while AOB responded later with population swings spanning from near detection limit to most dominant group. These observations match prior reports showing AOA prefer low N concentrations, while AOB respond vigorously to N deposition (Di et al., 2010; Sterngren et al., 2015). This has been interpreted as evidence for differing lifestyles for AOB and AOA, with AOA preferring nutrient poor conditions and AOB dominating in rich ones (Sterngren et al., 2015). However, prior assertions that AOB are solely important for driving nitrification might be overstated given that transcriptional activity for both groups is comparable if compared at peak time (Di et al., 2009). This contrasting use of energy between functionally redundant organisms might explain the low correlations between processes and the abundance of their respective functional populations (Rocca et al., 2015). When we examine the response of other functional groups benefiting from influxes of N, like denitrifiers, we see no significant change in population sizes suggesting that either energy is being utilized for physiological maintenance or otherwise for redox balance/homeostasis (Hartsock and Shapleigh, 2011; Li et al., 2012; Dietrich et al., 2013). The distinction here being that we use the term physiological maintenance as it refers to the state of energetics in a cell where the energy consumed is used for functions other than the
production of new cell material (i.e. growth) (van Bodegom, 2007; Lipson, 2015). Alternatively, redox balance reactions are used to maintain viable metabolic processes by controlling the redox state of all the cellular components (Green and Paget, 2004). In contrast, organism adapted to low N concentrations, like N fixers, decline in response to exogenous N demonstrating real time selective pressure in a complex ecosystem. These responses also highlight the temporal nature of these relationships and how by following niche differentiation high number of functionally redundant organisms can be maintained (Stempfhuber et al., 2015). However, the use of very high concentrations of urea (leading to rapid hydrolysis to ammonium followed by substantial nitrification) has major consequences for soil pH, physicochemical parameters, and potentially other factors (e.g. osmolarity). Without accounting for those it is unclear what the direct mechanism causing an increase or decrease in the relative abundance of a specific population is.

Despite this, our observations highlight how lifestyle preferences for organisms may be reflected in their dominance in the ecosystem. Prior work suggests that AOA dominate in soils with low N inputs, but AOB numbers are higher at times of high N loading or in ecosystems with consistent N deposition (Gong et al., 2013; Sterngren et al., 2015; Venterea et al., 2015; Li et al., 2016). This would suggest that a dynamic ecosystem with varying nutrient levels would select for a higher diversity of organisms that maintain ecosystem processes stable over time and space (Wang and Loreau, 2014). Indeed, our data supports this with alpha diversity (calculated based on 16S amplicon analysis at both DNA and RNA) decreasing in response to urea. This is inconsistent with plant responses to nutrient deposition in which multiple resources need to be added to elicit a response (Harpole et al., 2016), although contrasting results have been observed (Suding et al., 2005; Bai et al., 2010; Song et al., 2011; 2012). For microbes, high site to site variance is reported (De Schrijver et al., 2011; Leff et al., 2015), but similar negative responses are suggested and could be linked to increased competition in the absence of natural ecosystem variability. However, links between microbial and plant response suggest interplay between the response of macro and microbiota (Zeng et al., 2016). While previous work suggests an important role for moisture in controlling community composition (Waldrop and Firestone, 2006), we only observed a modifier role in our experiment.

Although broad observations align with ecological theory, precise identification of responsive organisms is rarely carried out. Here we note that while at phylum level
clear responses (+/- fold change) are observed, variance is seen at the OTU level suggesting intra-taxonomic (i.e. same phylum but different species or OTUs) diversity. We hypothesized this reflects the life history strategies of the different organisms. Attempts to link specific transformations to organisms failed, potentially due to the succession of functionally redundant organisms that respond at different time with non-overlapping optima. That is, while functional gene abundance provides the population size of organisms capable of carrying out a process, the group may be composed of many OTUs with divergent life strategies or metabolic potentials that affect when they can respond. This makes functional gene measurement an average of all OTU subpopulations carrying that gene. However, community response allows us to identify OTUs responsive to N deposition, which when analyzed independently, provides insights into metabolic preferences (i.e. aerobic vs. anaerobic, nitrifier vs. denitrifier) based on time and response to treatments. Taxonomic groups regularly recognized as native to, or abundant in, oligotrophic conditions declined in the presence of urea. Most of these groups are still poorly understood, and included the Acidobacteria, Verrucomicrobia, Nitrospirae, Candidate Division WS3 (also referred to as candidate phylum Latescibacteria) and the Thaumarchaeota. These organisms are predicted to be slow growers with the Thaumarchaeal response confirming the AOA patterns observed at the functional level. In contrast, positively responding organisms are those generally associated with groups considered eutrophic or capable of fast response. This discrepancy based on life history strategies has been proposed and applied to microbes previously, and suggests that an organisms’ ability to grow, utilize carbon, generate proteins and efficiently transform resources to biomass, amongst others, is related to its rRNA operon copy number (Klappenbach et al., 2000; Stevenson and Schmidt, 2004; Dethlefsen and Schmidt, 2007; Roller et al., 2016). When applied to communities, it is associated with microbial successions in which decreases in copy numbers are associated with later stages of succession including in soils (Nemer gut et al., 2015). For example, two OTUs matching the Verrucomicrobial OTU DA101 where found to be negatively affected by urea, and at least one was found to be highly abundant under background conditions. DA101 seems to be a common soil (and grassland) organism identified throughout the world (Felske and Akkermans, 1998; O’Farrell and Janssen, 1999; Brewer et al., 2016). Based on growth (Sangwan et al., 2005) and genome reconstructions (Brewer et al., 2016), these organisms are predicted to be slow but efficient growers (k strategists). In contrast, most of the positively affected organisms seemed to posses higher rrn
copy numbers and included members of the Proteobacteria and Bacteroidetes in line with prior predictions (Fierer et al., 2007). Statistical analysis supported this interpretation with low copy numbers (1-2) significantly associated to a negative response to N deposition, while high copy numbers (>2) were linked to increased capacity for growth, growth rate and maximum abundance. However, we found a non-linear relationship between increased rrm copy numbers and growth capacity, best fitted by models reaching an asymptote. These are first order models that suggest that while a benefit exists where increased copy numbers lead to increased growth rate, after a certain threshold other variables might limit any benefit. Alternatively, a decrease in growth rate might be observed with increasing copy numbers once a tradeoff threshold is passed (Lipson, 2015). However, when rrm copy numbers are log2 transformed, a significant linear fit was observed as seen in prior studies (Roller et al., 2016). In our study these predictions are made complicated due to the observed intra-taxonomic variance that can arise from the lack of accurate knowledge of copy numbers for many organisms, or from metabolic plasticity at higher taxonomic levels. In addition, our analysis focused on N responsive organisms only, and with only 38 identified it indicates that most organisms were neither positively nor negatively affected. This could explain why certain organisms (e.g. Actinobacteria) expected to be k strategist, based on their ability to produce secondary metabolites (Abdelmohsen et al., 2015) and compete with other organisms (Barka et al., 2015), showed a positive response to N deposition. Alternatively, the low number of responsive organisms could indicate that our false discovery rate corrections were too restrictive.

These findings help us get closer to understanding not just the metabolic potential of organisms in soils, but the role specific pathways play for an organism. It also allows us to understand the repercussion of disturbances and management of soils on below ground biodiversity. The knowledge gained through these type of observations, and integration of life history strategies into microbial ecology, will get us one step closer to microbiome management as part of soil care.
CHAPTER 6

Conclusions and future perspectives
6.1. Summary

Pastoral farming in New Zealand contributes large amounts of nitrogen to soils, largely via livestock urine. Through a combination of soil nitrogen cycling processes, this nitrogen is often transformed to the gaseous product \( \text{N}_2\text{O} \), a potent greenhouse gas and ozone depleter. Our understanding of the interconnecting pathways leading to the production and consumption of this \( \text{N}_2\text{O} \) are not well understood, especially under urine patch conditions. This PhD thesis contributes new insights into the N cycling process (i.e. denitrification and nitrification) in pasture soils to understand the emission potential of \( \text{N}_2\text{O} \) through the analysis of the edaphic factors, gas kinetics, the microbial community structure, and functional gene analysis.

6.1.1. Understanding of denitrification kinetics, C mineralization, pH, and their link to microbial diversity and richness in pasture soils

Soil pH is one of the most important edaphic factors regulating the emission ratio of \( \text{N}_2\text{O} \) or \( \text{N}_2\text{O} \) index (chapter 2) under anoxic conditions. We observed that low pH or acidic soils have more propensity to emit \( \text{N}_2\text{O} \) compared to slightly alkaline or neutral soil pH, and vice versa. Both soil pH and emission ratio of \( \text{N}_2\text{O} \) had a strong link to microbial community composition, diversity (Shannon) and richness. Our study suggests that pH imposes a general selective pressure on the entire microbial community and that resulted in changes in emission potential. Diversity (Shannon) and richness were higher in slightly alkaline or neutral soil pH where low emission ratio of \( \text{N}_2\text{O} \) was observed. The opposite pattern was observed in low soil pH where the emission ratio of \( \text{N}_2\text{O} \) was comparatively higher. Our results imply that higher microbial richness and diversity, and soil pH management (i.e. keeping the soil pH at neutral level) strategies in soils can overcome the higher emission ratio of \( \text{N}_2\text{O} \) through the rapid transformation of \( \text{N}_2\text{O} \) to \( \text{N}_2 \).

We observed a positive linear relationship between the rate of denitrification and the rate of C mineralization (under oxic and anoxic conditions). This may have implications in a soil management perspective as we can measure the rate of denitrification indirectly in nitrate-amended soils by estimating the rate of C mineralization.

6.1.2. Abundance and diversity of \( \text{nosZI} \) and \( \text{nosZII} \), and how these link to emission ratio of \( \text{N}_2\text{O} \)

Nitrous oxide reductase (\( \text{N}_2\text{OR} \)) is purported to be the only sink of terrestrial \( \text{N}_2\text{O} \). This enzyme is encoded by \( \text{nosZ} \). Recent findings have shown that there are
two types of \textit{nosZ} (clade I and clade II), with most \textit{nosZI} belonging to the Proteobacteria (alpha) phylum, and most \textit{nosZII} to the Bacteroidetes phylum. Recently, it has been shown that \textit{nosZ} (clade II) are highly abundant in the natural environment (Sanford \textit{et al.}, 2012; Orellana \textit{et al.}, 2014; Jones \textit{et al.}, 2013). Our results confirmed that irrespective of soil type, \textit{nosZII} is highly abundant compared to \textit{nosZI} in pasture soils (chapter 3). In addition, higher abundance of the \textit{nosZII} gene was observed in slightly alkaline or neutral pH soils compared to acidic soils. Furthermore, a lower emission ratio of N$_2$O was observed from the highly abundant of the \textit{nosZII} gene compared to lower abundance of \textit{nosZII} in pasture soil. Our results suggest that both \textit{nosZI} and \textit{nosZII} are a phylogenetically diverse group of prokaryotes, with their abundance and activity in soils ultimately regulating the production and consumption of N$_2$O.

6.1.3. \textbf{Does pH link to the emission ratio of N$_2$O under oxic urine patches?}

Our denitrification study (NO$_3^-$-amended pasture soils) showed a linear relationship between pH and emission ratio of N$_2$O under anoxic conditions (chapter 2). However, this trend was not observed under oxic urine patch conditions (chapter 4). The probable reason could be that urine patches disturb the soil pH as well as microbial community composition as seen in chapter 5.

6.1.4. \textbf{Response of microbial community, diversity, and richness under urine patches}

Urine patch contributed significant emission of N$_2$O (Chapter 4 & 5). However, it is still not known how microbial community composition changes under urine patch conditions, or which microbes are involved in N transformation process at DNA and transcription levels. We observed that soil urea treatment altered soil microbial community composition and reduced microbial diversity (Shannon) and richness. We suggested a model (chapter 5) that fast growing microbial populations utilized energy from the N-linked redox reactions for growth while others used it for physiological maintenance.

6.1.5. \textbf{Abundance and activity of AOB and AOA under urine patches}

Despite the higher abundance of AOA in pasture soils, asynchronous microbial activity was observed between AOA and AOB populations. AOA activity was observed during early N transformation process but then declined over time. On the other hand, the AOB population was initially very small (<1%) in pasture soils, but bloomed after just a few days (>10%). The immense growth of AOB was supported by energy utilization derived from the N transformation process. Hence,
understanding the growth and activity of AOA and AOB in pasture soils indicate the genetic potential for N transformation process which can be applied in soil management perspectives.

6.2. Future perspectives

Our studies linked N cycling processes and outcomes with edaphic factors and prokaryotic microbial communities in pasture soils but did not investigate the fungal communities and their role in N\textsubscript{2}O emissions. Although, some progress has been made recently to target the fungal nir\textsubscript{K} gene (Long et al., 2015; Wei et al., 2015b), and show their symbiotic roles (Bender et al., 2014), the overall contributions of fungal populations along with the prokaryotic communities, and their interactions in the N cycling process are still poorly understood.

We have identified pasture soil microbes which respond to urine treatments (i.e. N transformation process). However, their ecology and physiology are still not well known as they are mostly uncultured and their complete genomes are not yet sequenced. The isolation, culturing and genome sequencing of these nitrifying and denitrifying microbes from the soil could open up new doors for a deeper understanding of the N cycling process.

Here, we quantified only a handful of N cycling genes through qPCR, giving a useful but narrow picture of the relationship between N cycling gene abundance and soil N transformations. In the future, metagenomic and metatranscriptomic quantification of all N cycling genes could give a more complete picture of N cycling gene abundances, expression and their relationship to N transformations in pasture urine.

Our studies were conducted under laboratory conditions. This resulted in exclusion of some biological (e.g. grasses, plants, earthworms) and environmental factors (e.g. diurnal temperature variation, rainfall) from our experiments. Therefore, field level studies are important to corroborate our findings.
Supplementary Information

Supplementary materials for chapter 2

Supplementary Figures
Supplementary Fig. S2.1. Emission profile of N\textsubscript{2}O production index (I\textsubscript{N\textsubscript{2}O}) and N\textsubscript{2}O/(N\textsubscript{2}O+N\textsubscript{2}) product ratio over time. N\textsubscript{2}O (μmol N/vial) and N\textsubscript{2} (μmol N/vial) emissions from the anoxic incubation of soils over time (1a-13a), and N\textsubscript{2}O production index (I\textsubscript{N\textsubscript{2}O}) and N\textsubscript{2}O/(N\textsubscript{2}O+N\textsubscript{2}) product ratio of denitrification over time (1b-13b).

Soils were collected from Ireland (1: Moorepark, 2: Johnstown, 3: Solohead) and New Zealand (4: Warepa, 5: Otokia, 6: Wingatui, 7: Tokomairiro, 8: Mayfield, 9: Lismore, 10: Templeton, 11: Manawatu, 12: Horotiu and 13: Te Kowhai). Values represent the mean and standard error of triplicate flask results.
Supplementary Fig. S2.2. Maximum $N_2O$ production index ($I_{N_2O}$) and $N_2O/(N_2O+N_2)$ product ratio values observed in all soils. Values represent the mean and standard error of triplicate flask results.
**Supplementary Tables**

**Supplementary Table S2.1.** Physical and chemical characteristics of soil samples.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Country</th>
<th>Latitude/Longitude</th>
<th>Region</th>
<th>Soil type</th>
<th>Soil classification</th>
<th>Lowland/hill country</th>
<th>Drainage class</th>
<th>Management/farming practice</th>
</tr>
</thead>
<tbody>
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<td>NZ</td>
<td>37°46'22&quot;S, 175°21'13&quot;E</td>
<td>Waikato</td>
<td>Silt loam</td>
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<td>Lowland</td>
<td>Freely</td>
<td>Intensively grazed flatland dairy pasture</td>
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<td>Gleysol</td>
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<td>Intensively grazed flatland pasture</td>
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<td>NZ</td>
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<td>Fine sandy loam</td>
<td>Pallic Orthic Brown</td>
<td>Hill country</td>
<td>Freely</td>
<td>Intensively grazed flatland pasture</td>
</tr>
<tr>
<td>Mayfield</td>
<td>NZ</td>
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<td>Canterbury</td>
<td>Silt loam</td>
<td>Typic Argillie Pallic Soils</td>
<td>Lowland</td>
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<td>Moderately intensively grazed flatland sheep pasture</td>
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<td>Freely</td>
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</tr>
<tr>
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<td>NZ</td>
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<td>Fragile Perch-gley Pallic soil</td>
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<td>Weathered Fluvial Recent soil</td>
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<td>Moderately intensively grazed flatland sheep pasture</td>
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<table>
<thead>
<tr>
<th>Soil</th>
<th>Total C%</th>
<th>Total N%</th>
<th>C:N ratio</th>
<th>Soil Density (g/cm³)</th>
<th>Soil Porosity (%)</th>
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<td>69.0</td>
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### Table S2.3. Soil moisture content measurements (before and after adjustment of NH₄NO₃)

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Country</th>
<th>% Moisture (before flooding)</th>
<th>% Moisture (after flooding)</th>
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<td>Warepa</td>
<td>NZ</td>
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### Table S2.2. Soil pH measurements. Soil pH values as determined using three different extraction methods (DI water, 0.01 M CaCl₂ and 2M KCl).

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<th>Soil</th>
<th>pH (H₂O)</th>
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<td>Solohead</td>
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<td>6.39</td>
</tr>
</tbody>
</table>
Supplementary materials for chapter 3

Phylogenetic and functional potential links pH and N₂O emissions in pasture soils
Supplementary Methods

Analysis of microbial community composition
The rarified OTU table (biom file) was imported into R using the Phyloseq package (McMurdie and Holmes, 2013). To account for the multiple rarifications (10 total) abundances were normalized by dividing by 10 and rounding values to whole integers using the `transform_sample_counts()` command. Taxa (OTUs) with less than 1 count were removed using the `prune_taxa()` command. Alpha diversity (Shannon and richness) were calculated using the `estimate_richness()` command.

The NMDS plot was created using a Bray-Curtis distance matrix through Phyloseq. A Mantel test was performed to test the relationship between pH, as well as N₂O emission ratio, and microbial community composition using the Vegan package (Oksanen et al., 2013). To determine grouping of samples a cluster analysis was performed in using the Pvlust package (method = Ward; distance matrix = Bray-Curtis; bootstrap value, n=1000) (Suzuki and Shimodaira, 2006). Clusters were marked boxes (red) at 95% confidence interval.

Identifying OTUs correlated to change in pH and N₂O/(NO+N₂O+N₂)
In R, the Phyloseq file was transformed into a matrix. The variables (pH and N₂O/(NO+N₂O+N₂)) were converted into new data frames (df). A Spearmans correlation test (`cor.test(variable$pH,x, method = "spearman")`) was performed and results were further processed by adjusting p-value using a false discovery rate adjustment based on the Benjamini & Hochberg method (`p.adjust(p.vals,method = "BH")`). Results were subsetted to include only data with an adjusted p-value of < 0.05 & Rho >= 0.5 | Rho <= -0.5. OTU names were then used to subset the full Phyloseq file based on the significantly correlated OTUs using the `subset_taxa()` command. The significant OTUs and their full taxonomic classification were exported from R as a text file before visualization using Graphlan (Asnicar et al., 2015).

Normalization of metagenome sequences
Normalization was done based on equal number of sequence reads per sample (i.e. 2.63 million reads per sample).

**Functional richness**

Total functional richness (i.e. number of different functional genes) and functional richness at specific category (N-metabolism, level 3) were calculated from metagenome in MG-RAST (ID numbers 4644147.3 to 4644142.3) using KO annotation method with default MG-RAST settings (i.e. maximum e-value cutoff: 1e-5; minimum % identity cutoff: 60%; minimum alignment length cutoff: 15 aa). The functional richness data were exported from the MG-RAST and normalized based on equal number of sequence reads per sample (2.63 million reads per sample).
Supplementary Figures

Supplementary Fig. S3.1. Relationship between soil pH and maximum emission of NO, N₂O and N₂ under anoxic incubation for all 13 soils.

Supplementary Fig. S3.2. Shannon diversity based on microbial OTUs across all sites for both Irish (IR) and New Zealand (NZ) soils. Color gradient denotes influence of pH ($R^2 = 0.49$, $p<0.01$).
Supplementary Fig. S3.3. Microbial community dissimilarities of soils (Irish and New Zealand) with different pH as determined using NMDS (Bray-Curtis) ordination.

Supplementary Fig. S3.4. Stress plot for Figure 3.1E
Supplementary Fig. S3.5  Pclust tree using Bray-Curtis distance of 16S rRNA microbial community composition and including p values for each node [AU (approximately unbiased) BP (bootstrap probability)]. Red boxes mark clusters with 95% confidence. Clusters at lower confidence are labeled by region.
Supplementary Fig. S3.6. Relationships between total denitrification genes (genes per 2.63 million reads) & N₂O emission ratio (N₂O/NO+N₂O+N₂), and total denitrification genes (genes per 2.63 million reads) & pH (A). The black circles represent the relationship between denitrification genes & pH, and the gray circles represent the relationship between denitrification genes & N₂O emissions ratio. The bottom stack bar plot shows the relative abundance of denitrification genes according to pH gradient (high to low) (B). The abundance of denitrification genes was calculated from metagenome analysis (annotation source: KO) by detection of the following genes: nosZ, norC, norB, nirK+S, napB, napA, narJ, narI, narH, narG.
Supplementary Fig. S3.7. Maximum likelihood phylogeny of short-length nosZ amino-acid sequences (129 aa) obtained from metagenomes. A multiple sequence alignment was performed with CLUSTALW on MEGA 6. After alignment, sequences were trimmed outside of conserved (90-100 %) regions (at C terminal LGPLHT--- and at N terminal ---EPH) containing approx. 129 aa sequences. The phylogenetic tree was constructed preliminary with MEGA 6 using the maximum likelihood approach and JTT matrix-based model, and finally visualized with iTOL.
Supplementary Fig. S3.8. Relationship between abundance of nir genes (based on absolute quantification of metagenome & qPCR of nirS & nirK), N₂O/(NO+N₂O+N₂) and pH. (A-B) Comparison of gene abundances based on either metagenomic (i.e. gene abundance per 2.63 million reads) or qPCR analysis (gene abundance per 5 ng soil DNA) for 6 soils. (C-D) Response of nirS and nirK abundances based on metagenomic analysis for 6 soils against N₂O/(NO+N₂O+N₂) (gray) and pH (black). (E-F) Response of nirS and nirK abundances based on qPCR analysis for all 13 soils against N₂O/(NO+N₂O+N₂) (gray) and pH (black).
Supplementary Fig. S3.9. Relationship between functional richness (A: at N-metabolism level and B: total functional richness), N\textsubscript{2}O emission ratio (gray) and pH (black). The x-axis denotes richness i.e. number of different genes per 2.63 million sequence reads. The functional richness was calculated from metagenome analysis (annotation source: KO).
### Supplementary Table S3.1. The primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target group</th>
<th>Function</th>
<th>Amplicon (bp)</th>
<th>Sequences (5´-3´)</th>
<th>Polymerase</th>
<th>Cycling conditions &amp; data acquisition</th>
<th>Efficiency (%) &amp; R²</th>
<th>References</th>
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<tbody>
<tr>
<td>UniF</td>
<td>16S rRNA</td>
<td>Ribosomal RNA</td>
<td>180</td>
<td>ACTCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGGC</td>
<td>Fast SYBR Green Master Mix</td>
<td>95°C for 10 minutes, followed by 40 cycles of 10 s at 95°C, 20 s at 65°C, then followed by 20 s at 72°C for fluorescent acquisition</td>
<td>106 &amp; 0.99</td>
<td>(Hartman et al., 2009)</td>
</tr>
<tr>
<td>UniR</td>
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<tr>
<td>cd3AF</td>
<td>nirS</td>
<td>NO₃⁻ reduction (cytochrome cd₉-containing)</td>
<td>425</td>
<td>GTSAACGTSAAGGARACSGG GASTTCGGRTGSCTTTGA</td>
<td>Fast SYBR Green Master Mix</td>
<td>95°C for 10 minutes, followed by 40 cycles of 10 s at 95°C, 20 s at 58.5°C, 20 s at 72°C then followed by 20 s at 77°C for fluorescent acquisition</td>
<td>94.67 &amp; 0.99</td>
<td>(Throbäck et al., 2004; Yergeau et al., 2007)</td>
</tr>
<tr>
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<td>F1aCu</td>
<td>nirK</td>
<td>NO₂⁻ reduction (Cu containing)</td>
<td>474</td>
<td>ATCATGGCTCTGGCCGC GCCTCGATCAGRTTGTGGTT</td>
<td>Luminaris HiGreen Low ROX qPCR Master Mix</td>
<td>95°C for 10 minutes, followed by 6 cycles of 15 s at 95°C, 30 s at 60-55°C (1°C per cycle), 30 s 72°C, and then followed by 44 cycles of 15 s at 95°C, 30 s at 54°C, 30 s at 72°C and 30 s for fluorescent data acquisition (82°C).</td>
<td>66.12 &amp; 0.99</td>
<td>(Jones et al., 2013)</td>
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<tr>
<td>nosZ2F</td>
<td>nosZ I</td>
<td>N₂O reduction (Tat dependent)</td>
<td>267</td>
<td>CGCRACGGCAAAAGGTSMSGTC AKRGCAGGTTGTTTGGGCAGA</td>
<td>Fast SYBR Green Master Mix</td>
<td>95°C for 10 minutes, followed by 40 cycles of 10 s at 95°C, 20 s at 58.5°C, 20 s at 72°C then followed by 20 s at 75°C for fluorescent acquisition</td>
<td>99.3 &amp; 0.99</td>
<td>(Henry et al., 2006)</td>
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<tr>
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<td>nosZ-II-F</td>
<td>nosZ II</td>
<td>N₂O reduction (Sec dependent)</td>
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<td>CTGGGICGIYTCAYAC GCIGARCARAATCBGTRC</td>
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<td>66.12 &amp; 0.99</td>
<td>(Jones et al., 2013)</td>
</tr>
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<td>nosZ-II-R</td>
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</table>
Supplementary materials for Chapter 4

Ruminant urine patch reveals significant sources of N₂O.
Supplementary Figures

Supplementary Fig. S4.1 Gas kinetics (O₂, CO₂, NO, N₂O, N₂) of 13 different soil samples (10 New Zealand and 3 Ireland soils) under oxic incubation (without urine treatment).
**Supplementary Fig. S4.2.** Emissions (CO₂, NO and N₂O) comparison between with urine and without urine.
Supplementary Table S4.1 Primer pairs used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target group</th>
<th>Function</th>
<th>Amplicon size (bp)</th>
<th>Sequence (5’-3’)</th>
<th>Polymerase used</th>
<th>Cycling &amp; data acquisition</th>
<th>Efficiency (%) &amp; R²</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>UniF</td>
<td>16S rRNA gene</td>
<td>Ribosomal RNA</td>
<td>180</td>
<td>ACTCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGGC</td>
<td>Fast SYBR Green Master Mix</td>
<td>95°C for 10 minutes, followed by 40 cycles of 15 s at 95°C, 20 s at 65°C, then followed by 20 s at 72°C for fluorescent acquisition</td>
<td>99.3 &amp; 0.99</td>
<td>(Hartman et al., 2009)</td>
</tr>
<tr>
<td>UniR</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Crenamo A23F</td>
<td>Archaeal amoA</td>
<td>Ammonia oxidation</td>
<td>628</td>
<td>ATGGTCTGGCTWAGACGGCATACTCCATCTGTATGTC</td>
<td>Fast SYBR Green Master Mix</td>
<td>95°C for 10 minutes, followed by 6 cycles of 15 s at 95°C, 30 s at 60-55°C (-1°C per cycle), 30 s 72°C, and then followed by 36 cycles of 15 s at 95°C, 30 s at 54°C, 30 s at 72°C and 30 s for fluorescent data acquisition (75°C).</td>
<td>86.1 &amp; 0.98</td>
<td>(Tourna et al., 2008)</td>
</tr>
<tr>
<td>Crenamo A616R</td>
<td></td>
<td></td>
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<tr>
<td>amoA1F</td>
<td>Bacterial amoA</td>
<td>Ammonia oxidation</td>
<td>491</td>
<td>GGGGGTTTCTACTGGTGGTCCCCTCGGGAAAGCCTTC</td>
<td>Fast SYBR Green Master Mix</td>
<td>95°C for 10 minutes, followed by 45 cycles of 15 s at 95°C, 30 s at 57°C, 40 s at 72°C then followed by 20 s at 82°C for fluorescent acquisition</td>
<td>84.6 &amp; 0.99</td>
<td>(Avrahami et al., 2003)</td>
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<td>amoAR1</td>
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</table>

Note: The efficiency and R² were calculated from the standard curve (10-fold dilution series) of each target (gene) from a single run in a 384-well plate.
Supplementary materials for chapter 5

Response to nitrogen addition reveals metabolic and ecological strategies of soil bacteria
Supplementary Methods

Analysis of microbial community composition

The rarified biom file was exported from Qiime and then processed in R using the phyloseq package (McMurdie and Holmes, 2013). To account for the multiple rarifications (10 total) abundances were first normalized by dividing by 10 followed by rounding values to whole integers using the `transform_sample_counts()` command. Taxa (OTUs) with less than 1 count were deleted using the `prune_taxa()` command. Alpha diversity (Shannon and richness) were calculated using the `estimate_richness()` command.

The NMDS plot was created using a Bray-Curtis distance matrix through “phyloseq” and “vegan” (Oksanen et al., 2013) packages. Significant treatment effects by urea where determined using an Adonis test. To determine samples forming statistically significant groups, a cluster analysis was performed using the pvclust package (method = Ward; distance matrix = Bray-Curtis; bootstrap value, n = 1000) (Suzuki and Shimodaira, 2006). Significant groups (representing 95% confidence) were marked with boxes (red). To understand the temporal variation within microbial community in each treatment (4 treatments: HM+N [high moisture soil with urea]; LM+N [low moisture soil with urea]; HM-N [high moisture soil with no urea] and LM-N [high moisture soil with no urea]), a Mantel correlogram analysis was performed using “vegan” and “mpmcorrelogram” packages. Control samples with no N added were stable and only sampled 4 times (+N treatment was sampled 7 times).

Identifying OTUs affected by N treatment through SIMPER analysis

Both DNA and RNA data (16S sequencing reads) were subset into two groups based on moisture treatment (i.e. high moisture [HM] and low moisture [LM]). For each moisture treatment, OTUs identified within urea treated (+N) and untreated (-N) samples were compared after samples from day 0 (immediately after N application where removed). OTUs responsible for dissimilarities between N treatments for each moisture content were identified using similarity percentage analysis (SIMPER) (Clarke, 1993).
$qPCR$ inhibition test

Low conc. of DNA and cDNA samples were used for qPCR templates to avoid PCR inhibition. This was tested on some DNA and cDNA samples by making a dilution series (low vs. high concentration of DNA or cDNA) along with qPCR standard curve.
## Supplementary Table S5.1 Primer pairs used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target group</th>
<th>Function</th>
<th>Amplicon size (bp)</th>
<th>Sequence (5’-3’)</th>
<th>Polymerase used</th>
<th>Cycling &amp; data acquisition</th>
<th>Efficiency (%) &amp; R²</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>UniF</td>
<td>16S rRNA gene</td>
<td>Ribosomal RNA</td>
<td>180</td>
<td>ACTCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGGC</td>
<td>Fast SYBR Green Master Mix</td>
<td>95°C for 10 minutes, followed by 40 cycles of 15 s at 95°C, 20 s at 65°C, then followed by 20 s at 72°C for fluorescent acquisition</td>
<td>99.3 &amp; 0.99</td>
<td>(Hartman et al., 2009)</td>
</tr>
<tr>
<td>UniR</td>
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</tr>
<tr>
<td>CrenamoA 23F</td>
<td>Archaeal amoA gene</td>
<td>Ammonia oxidation</td>
<td>628</td>
<td>ATGGTCCTGCGTAWAGCG GCCATCCATCTGTATGTCCA</td>
<td>Fast SYBR Green Master Mix</td>
<td>95°C for 10 minutes, followed by 6 cycles of 15 s at 95°C, 30 s at 60-55°C (-1°C per cycle), 30 s 72°C, and then followed by 36 cycles of 15 s at 95°C, 30 s at 54°C, 30 s at 72°C and 30 s for fluorescent data acquisition (75°C).</td>
<td>86.1 &amp; 0.98</td>
<td>(Tourna et al., 2008)</td>
</tr>
<tr>
<td>CrenamoA 616R</td>
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<tr>
<td>amoA1F</td>
<td>Bacterial amoA gene</td>
<td>Ammonia oxidation</td>
<td>491</td>
<td>GGGGGTTTCTACTGTGTGT CCCCTCGGAAAGCCTTC TTC</td>
<td>Fast SYBR Green Master Mix</td>
<td>95°C for 10 minutes, followed by 45 cycles of 15 s at 95°C, 30 s at 57°C, 40 s at 72°C then followed by 20 s at 82°C for fluorescent acquisition</td>
<td>80.6 &amp; 0.99</td>
<td>(Rotthauwe et al., 1997; Avrahami et al., 2003)</td>
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<tr>
<td>cd3AF</td>
<td></td>
<td>NO₃ reduction (cytochrome cdₑ-containing)</td>
<td>425</td>
<td>GTSAACGTSAGGARACSGG GASTTCCGRTGGTCGCTGA</td>
<td>Fast SYBR Green Master Mix</td>
<td>95°C for 10 minutes, followed by 40 cycles of 10 s at 95°C, 20 s at 58.5°C, 20 s at 72°C then followed by 20 s at 77°C for fluorescent acquisition</td>
<td>94.2 &amp; 0.99</td>
<td>(Throbäck et al., 2004; Yergeau et al., 2007)</td>
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<tr>
<td>nosZ2F</td>
<td>nosZ I N₂O reduction (Tat dependent)</td>
<td>267</td>
<td>CGCRACGGCAASAAGGTSMSGG T CAKRGCAKSGCRTGGCAGAA</td>
<td>Fast SYBR Green Master Mix</td>
<td>95°C for 10 minutes, followed by 40 cycles of 10 s at 95°C, 20 s at 58.5°C, 20 s at 72°C then followed by 20 s at 75°C for fluorescent acquisition</td>
<td>99.3 &amp; 0.99</td>
<td>(Henry et al., 2006)</td>
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<tr>
<td>nifHF</td>
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<td>AAAGGYYGWTACGGYAARTCCA CCAC TGSGCYTTTGCYTCRRCGGATBGG CAT</td>
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<td>94.6 &amp; 0.99</td>
<td>(Rösch and Bothe, 2005)</td>
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</table>

Note: The efficiency and R² were calculated from the standard curve (10-fold dilution series) of each target (gene) from a single run in a 384-well plate.
Supplementary Table S5.2 Pairwise correlation between observed phylum (or class) abundance at DNA and RNA level for urea (+N) treated soils. Correlation analysis was done between DNA (16S rDNA) and RNA (16S rRNA) samples based on mean absolute abundance (per 7,400 sequence reads) at each time point (day 0, 7, 14, 21, 35, 63). Only Proteobacteria shown at class level.

<table>
<thead>
<tr>
<th>Phylum or class</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
<th>Correlation (r)</th>
<th>p-value</th>
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<tbody>
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<td>Acidobacteria</td>
<td>0.71</td>
<td>0.98</td>
<td>0.91</td>
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<td>Bacteroidetes</td>
<td>0.30</td>
<td>0.92</td>
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<td>CD WS3</td>
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<td>Chloroflexi</td>
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<td>0.96</td>
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<td>Thaumarchaeota</td>
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<td>0.93</td>
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<td>Verrucomicrobia</td>
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Note: Lower 95% and Upper 95% represent confidence limit. Statistically significant correlations (p<0.05) are in bold.
**Supplementary Table S5.3:** Two sample t-test for mean comparison between low copy number *rrn* (rRNA operon) samples (1-2) and high copy number of *rrn* samples (>2). The significant correlation (p<0.05) are showed as bold.

<table>
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<tr>
<th>t-test (Welch)</th>
<th>Group1 mean (&gt;2 <em>rrrn</em>)</th>
<th>Group2 mean (1-2 <em>rrrn</em>)</th>
<th>t value</th>
<th>df</th>
<th>p</th>
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</thead>
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<tr>
<td>HM (growth rate)</td>
<td>0.44</td>
<td>0.40</td>
<td>0.58</td>
<td>23.92</td>
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<tr>
<td>HM (Max. abundance)</td>
<td>176.62</td>
<td>7.88</td>
<td>3.20</td>
<td>30.66</td>
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<tr>
<td>HM (Fold change)</td>
<td>166.77</td>
<td>54.01</td>
<td>2.71</td>
<td>22.42</td>
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<td>LM (growth rate)</td>
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<td>0.36</td>
<td>0.97</td>
<td>20.38</td>
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<tr>
<td>LM (Max. abundance)</td>
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<td>21.62</td>
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<tr>
<td>LM (Fold change)</td>
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<td>3.17</td>
<td>14.42</td>
<td><strong>0.006</strong></td>
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<tr>
<td>Best (HM/LM) (growth rate)</td>
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<td>Best (HM/LM) (Max. abundance)</td>
<td>282.68</td>
<td>28.67</td>
<td>3.74</td>
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<td><strong>0.0007</strong></td>
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<tr>
<td>Best (HM/LM) (Fold change)</td>
<td>267.87</td>
<td>78.51</td>
<td>3.52</td>
<td>22.45</td>
<td><strong>0.002</strong></td>
</tr>
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</table>
**Supplementary Fig. S5.1.** \( \text{N}_2\text{O} \) response in soils treated with urea (+/- 1000 µg N/g dry soil) under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]). Error bars are the standard error of the mean (n ≥ 3) for replicate mesocosms.
Supplementary Fig. S5.2. Functional group response (absolute quantification) in soils treated with urea (+/-1000 µg N/g dry soil) under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]). Gene and transcript abundance were measured from DNA template (1 ng of DNA) and cDNA template (1 ng RNA). Error bars are the standard error of the mean (n = 3, except day 7 [n=1; LM soil] and day 21 [n=1; LM soil]) for replicate mesocosms. Absolute gene and transcript abundance were measured by qPCR targeting: 16S (total prokaryotic community), nitrifiers (AOA, ammonia oxidizing archaea; AOB, ammonia oxidizing bacteria), denitrifiers (nirS, cytochrome cd1-containing nitrite reductase; nosZI, nitrous oxide reductase) and nitrogen fixers (nifH, nitrogenase reductase).
Supplementary Fig. S5.3. Changes in microbial a) Richness and b) Evenness (Pielou’s) over time in response to treatment.
**Supplementary Fig. S5.4.** Stress plots for Fig. 5.2.b.
Supplementary Fig. S5.5. Pvclust tree displaying sample clustering based on Bray-Curtis distances calculated from 16S rRNA gene community composition and indicating significant clusters based on p values ([AU (approximately unbiased) BP (bootstrap probability)]) for each node. Red boxes mark clusters with 95% confidence. Bootstrap replication (n=1000). Two clusters: with urea (light red box) and no urea + day 0 N treated samples (light green box).
Supplementary Fig. S5.6. Mantel correlogram showing autocorrelation on community composition by performing sequential Mantel tests between the Bray-Curtis dissimilarities and the grouping of samples using a time period index (index 1 represents 0-7 days; 2 represents 7-21; 3 represents 21-63). Opened circles represent no significant correlations (p > 0.05) in community composition at specific time periods.
Supplementary Fig. S5.7 Changes in abundance (DNA), activity (RNA) and RNA/DNA ratio for phyla, or classes, representing top 11 phyla (based on OTUs clustered at 97% sequence similarity). A total of 7,400 sequences were examined per sample. Error bars are the standard error of the mean (n = 3, except day 7 [n=1; LM soil] and day 21 [n=1; LM soil]) for replicate mesocosms. Treatments =
+/- N [+/- 1000 µg N/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]). Abbreviations: Firmi., Firmicutes; Verru., Verrucomicrobia; Bact., Bacteroidetes; Acido., Acidobacteria; Actino., Actinobacteria; Planct., Planctomycetes; Gemma., Gemmatimonadetes; Thaum., Thaumarchaeota; Chloro., Chloroflexi, Nitro., Nitrospirae
Supplementary Fig. S5.8 Phylum level changes (relative abundance) in genome (16S rDNA) and transcript (16S rRNA) levels representing relative contribution >1% of all detected phyla (based on OTUs clustered at 97% sequence similarity). A total of 7,400 sequences were examined per sample. Treatments = +/- N [+-1000 µg N/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]).
Supplementary Fig. S5.9 Transcriptional (16S rRNA) and population (16S rDNA) changes (abundance based on 7400 reads per samples) for OTUs identified as positively responsive to urea treatment based on similarity percentage (SIMPER) analysis (representing top 30% cumulative sum). Treatments = +/- N [+/- 1000 µg N/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]).
OTU29: c_Acidobacteria, o_Subgroup 6
OTU30: c_Alphaproteobacteria, f_Xanthobacteraceae
OTU31: c_Alphaproteobacteria, o_Rhodospirillales, f_I-10
OTU32: c_Deltaproteobacteria, o_GR-WP33-30
OTU33: p_Thaumarchaeota, c_Soil Crenarchaeotic Group(SCG)
OTU34: p_Verrucomicrobia, f_DA101 soil group
OTU35: p_Verrucomicrobia, f_DA101 soil group
OTU36: p_Verrucomicrobia, f_FukuN18 freshwater group

**Supplementary Fig. S5.10** Transcriptional (16S rRNA) and population (16S rDNA) changes (abundance based on 7400 reads per samples) for OTUs identified as negatively responsive to urea treatment based on similarity percentage (SIMPER) analysis (representing top 30% cumulative sum). Treatments = +/- N [+/- 1000 µg N (urea)/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]).
Supplementary Fig. S5.11 Relationship between predicted ribosomal RNA operon (rrn) copy numbers and observed growth rate (per day), maximum observed population change, or fold change in population abundance for OTUs responsive to N treatment under both high moisture (HM) content. Copy number was estimated using rrn database (Stoddard et al., 2015). Predicted rrn copy numbers represent the mean rRNA copy number for the closest taxonomic match (at the lowest taxonomic level possible) for each OTU. The rrn copy numbers were log2 transformed before linear regression analysis. Significant “p” value is marked with an asterisk (*p<0.05; **p<0.01; ***p<0.001)
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