THE INFLUENCE OF SOIL PARAMETERS AND DENITRIFIERS ON N_2O EMISSIONS IN NEW ZEALAND DAIRY-GRAZED PASTURE SOILS

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ABSTRACT

Denitrification is a primary source of nitrous oxide (N_2O) production found globally in temperate grasslands and in New Zealand pasture soils. The various reductase enzymes coded by specific denitrifier genes, and influenced by soil and environmental factors, regulate the N_2O production and reduction during the denitrification process. An understanding of the soil and environmental factors that have the potential to enhance the activity of denitrifiers reducing N_2O to dinitrogen (N_2) contributes to the development of novel and effective N_2O mitigation technologies.

This thesis attempts to address a critical gap in our understanding of the role of bacterial denitrifier communities and their abundance in denitrification under various field-moist and incubation conditions in New Zealand dairy-grazed pasture soils. This thesis consists of a literature review (Chapter 2) that describes the biochemical and molecular aspect of denitrification, and identifies key factors that affect the process and the denitrifier community structure and its abundance. It addresses the advantages and limitations of available techniques to measure denitrification, the denitrifier community structure and abundance, and also any available mitigation options to reduce denitrification losses. The review concludes by identifying gaps in our knowledge of the denitrifier communities, and their abundance and activities in New Zealand soils.

The acetylene (C_2H_2) inhibition technique (AIT) was standardised (Chapter 3) for the measurements of denitrification enzyme activity (DEA) and denitrification rate (DR) used during the research. Similarly, terminal restriction fragment length polymorphism (T-RFLP) and quantitative polymerase chain reaction (qPCR) were standardised to assess denitrifier community structure (numbers of *nir*S+*nir*K and *nos*Z gene T-RFs) and abundance (*nir*S+*nir*K and *nos*Z gene copy numbers) in soils.

Soil samples were obtained from 10 sites with known management histories, representing dairy farms across the North and South Islands of New Zealand, to determine the soil factors contributing to spatial variability in DEA, DR and denitrifier community structure and abundance within and across dairy-grazed pastures (Chapter 4). Despite the spatial variability at each site, the DEA results show large differences in potential denitrification in these soils. The outcome of this study painted an overall picture of the distribution of denitrification enzyme

activities in the various New Zealand dairy-grazed pasture soils and the biochemical characteristics and presence of denitrifiers related to denitrification in contrasting soils.

The bacterial denitrifier genes richness correlated significantly ($P < 0.05$) to Olsen P, microbial biomass C (MBC), and NH_4^+-N contents of soil, whereas these gene abundances correlated to MBC and NO_3^--N contents in soils, thus confirming inherent soil characteristics that influence denitrifier populations in soils. *Nir*S and *nir*K gene copy numbers correlated positively with N_2O emissions (from nitrification and denitrification). There was no clear relationship between *nos*Z gene copy numbers and denitrification rate in the field-moist soils, which could be due to less anaerobic condition for denitrifiers to carry denitrification.

To determine the effect of increasing soil water content (SWC) on changes in denitrification, 5 soils contrasting in DEA were incubated at field capacity (FC) and saturation SWC (Chapter 5). The measured DRs were higher in soils incubated at saturation, ranging from 21.5 to 73.9 μg N_2O-N kg^{-1}soil hr^{-1}, than in soils incubated at FC, in which DR varied from 0.8 to 50.4 μg N_2O-N kg^{-1}soil hr^{-1}. Although the direction of change in denitrification with the increase in SWC was similar among the soils, the magnitude of increase was variable among the five soil types. This variability was mainly driven by their inherent biochemical (NO_3^--N, TC, TN, MBC, Olsen P, DEA,) and molecular characteristics (denitrifier richness and denitrifier abundance).

A subsequent incubation experiment was conducted to investigate the effect of water only, cattle urine, and cattle urine with added nitrification inhibitor dicyandiamide (DCD) on denitrification and numbers of denitrifier gene T-RFs and their copy numbers in three pasture soils with contrasting DEA. The results of this incubation are described in Chapters 6 and 7. The results described in Chapter 6 explore the effect of saturation (only water addition) on DRs, denitrifier gene richness, and denitrifier gene copy numbers in three soils. At saturation soil water content in the incubated soils, the increases or decreases in DRs with incubation time were variable in three soils and depended on their TC, TN, Olsen P, MBC, and denitrifier gene richness. It is inferred from the results that, with increasing SWC, a denitrifier community of a constant size was maintained in the incubated soils.

The results in Chapter 7 describe the effect of urine with and without DCD on denitrification, denitrifier richness, and denitrifier gene abundance. There were changes in soil pH, NO_3^--N, NH_4^+-N, soluble C, and microbial biomass C in soils with urine application. The

numbers of denitrifier gene T-RFs and copy numbers either remained unchanged or were lower under urine + DCD treatments than urine only and related to N_2O productions (from both nitrification and denitrification) during the incubation.

There was an overall increase in DR with application of urine and urine + DCD to soils. Comparatively higher N_2O-NA (N_2O productions from non- acetylene jars) were observed in urine-only treatments than in urine + DCD. The cumulative N_2O-NA with the addition of urine and urine + DCD were variable among the three soils. During 4 weeks incubation the N_2O-NA ranged from 20.9 to 26.7 mg N_2O-N kg^{-1} soil in urine treatments and from 19.6 to 21.7 mg N_2O-N kg^{-1} soil in urine + DCD treatments in three soils. The proportion of total N denitrified during the entire incubation ranged from 6.3 to 22.4 % in urine treatments and 3.6 to 5.6 % in urine + DCD treatments in three soils.

Denitrifier gene richness and gene copy numbers during incubation helped identify overall changes in the denitrifier community with the application of treatments and link these changes to N_2O-NA across various soils. Overall, in the urine-applied samples the N_2O-NA (positively) was significantly correlated with their DEA, MBC, Olsen P, and numbers of denitrifier gene richness and denitrifier gene copy numbers.

The information obtained in this research helped enhance the understanding of the variability in denitrification and the denitrifier community in the New Zealand dairy pasture soils. The molecular measurements of the soils helped identify differences in N_2O productions in soils at similar incubation conditions. Soils with low denitrifier richness and, more importantly, lower abundance of complete denitrifiers had limited capacity to denitrify available inorganic N. Measurements of DEA, denitrifier richness, and denitrifier abundance in soils, along with their N_2O productions (with and without acetylene), indicated the inherent potential of soils to carry denitrification. Under urine application, soil with the most abundant denitrifier population and the highest DEA responded to added treatment quickly and produced more N_2O-NA and N_2O-A than the other two soils did. On the other hand, soils with lower DEA and less abundant denitrifier community showed lower N_2O production (with and without acetylene) even under anaerobic condition than did other soils.

DEDICATION

This work is dedicated to my parents, my husband, and my parents-in-law

for their consistent encouragement and support

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AN	Avogadro's Number
ANOVA	Analysis of variance
AOA	Ammonia oxidising archaea
AOB	Ammonia oxidising bacteria
bp	Base pairs
BSA	Bovine serum albumin
C	Carbon
Cd	Cadmium
C_2H_2	Acetylene
cnorB/cnorBp	Cytochrome nitric oxide reductase enzyme
CO_2	Carbon-dioxide
Cp	Crossing Point
CT	Control
Cu	Copper
DCD	Dicyandiamide
DEA	Denitrification enzyme activity
DNA	Deoxyribonucleic acid
DNRA	Direct nitrate reduction to ammonia
dNTPs	Deoxinucleotide triphosphates
DR	Denitrification rate
ds	double stranded
ECD	Electron capture detector
EDTA	Ethylenediaminetetraacetic acid
Eq	Equation
F	Forward
FAM	6 carboxyfluorescein
Fe	Iron
GC	Gas Chromatograph
GHG	Greenhouse gas
H^+	Hydrogen ions
H'	Shannon Diversity Index
HR	Horotiu silt loam
hrs	hours
HSD	Honestly significant difference
IGEPAL	octylphenoxypolyethoxyethanol
IPCC	Intergovernmental panel for climate change
J	Pielou's Coefficient of Evenness
K_2SO_4	Potassium Sulphate
KCl	Potassium chloride
LM	Listmore stony silt loam
MF	Mayfield silt loam
Mf.E	Ministry of Environment
$MgCl_2$	Magnesium chloride

Mn	Manganese
Mo	Molybdenum
MPN	Most Probable Number
mRNA	Messenger ribonucleic acid
MS	Microsoft
MW	Manawatu fine sandy loam
MWEI	Manawatu fine sandy loam (Effluent irrigated)
N	Nitrogen
N_2	Dinitrogen
N_2O	Nitrous oxide
N_2OR	Nitrous oxide reductase
NAR	Nitrate reductase
NEB	New England Biolabs
NH_3	Ammonia
NH_4^+	Ammonium
NIR	Nitrite reductase
Nir	Nitrite reductase gene
*Nir*K	Copper containing nitrite reductase gene
*Nir*S	Cytochrome-cd1 nitrite reductase gene
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOR	Nitric oxide reductase
*Nos*Z	Nitrous oxide reductase gene
NZ	New Zealand
O_2	Oxygen
OH	Otorohanga silt loam
OH^-	Hydroxyl ion
OTUs	Operational taxonomic units
P	Phosphorus
PCA	Principal components analysis
PCR	Polymerase chain reaction
PL	Paparua silt loam (Lincoln)
PS	Paparua silt loam (Springston)
Q_{10}	Temperature coefficient
qPCR	Quantitative polymerase chain reaction
R	Reverse
RNA	Ribonucleic acid
s	second
S	Sulphur
SWC	Soil water content
TAE	Tris-ascetic acid EDTA
Taq	*Thermus aquaticus*
TeK	Te Kowhai silt loam
TET	Tetrachlorofluorescein
TM	Tokomaru silt loam

T-RFLP	Terminal restriction fragment length polymorphism
T-RFs	Terminal restriction fragments
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propandiol Hydrochloric acid
U	Cattle urine only
UI	Cattle urine +DCD
UV	Ultra violet

Chapter 1

Introduction

1.1 BACKGROUND

In New Zealand nearly 80 % of agricultural land is under grazed grasslands (MfE., 2007). These pastures are considered a major source of N_2O emission, contributing 46–52 % of the global anthropogenic N_2O flux, nearly 2.8 Tg N_2O-N $year^{-1}$ (IPCC, 2007). Nitrous oxide (N_2O) is a long-lived, potent greenhouse gas (GHG) with a warming potential 298 times greater than that of CO_2, and in 2012, contributed 14.3 % of the total greenhouse gas (GHG) emissions in New Zealand, of which 97.1 % was from agriculture (MfE, 2014). Between 1990 and 2012 New Zealand's total GHG emissions increased by 25.4 %, with a 32 % increase in N_2O emissions (MfE, 2014). This increase is directly linked to the intensification in dairying and increased use of mineral N fertilizers on farms.

Major sources for atmospheric N_2O are biotic (nitrification, denitrification, nitrate ammonification, nitrifier denitrification, and co-denitrification) and chemical decomposition of nitrite (chemodenitrification) and hydroxylamide occurring in soil, sediment, and sub-surface ecosystems (Bouwman *et al.*, 2002; Bremner & Blackmer, 1979; Bremner, 1997; Hénault *et al.*, 2012; McMahon & Dennehy, 1998). When the supply of oxygen (O_2) is limited in soil, denitrification becomes the major N_2O, producing mechanism in many soils (Šimek *et al.*, 2002). Microbial denitrification is carried out by facultative anaerobes. In the absence of O_2, the denitrifiers reduce nitrogen oxyanions like nitrate (NO_3^-) and nitrite (NO_2^-) to N_2O or dinitrogen (N_2) (Hallin & Pell, 1994).

Denitrification reaction rate and the relative production of N_2, N_2O, and NO depend on complex interactions between soil properties, climatic factors, agricultural practices, and soil microorganisms. Important factors include the availability of mineral N (NH_4^+ and NO_3^-), organic C (Burford & Bremner, 1975; Payne, 1981; Reddy *et al.*, 1982; Robertson & Tiedje, 1984), temperature and pH (Šimek *et al.*, 2002), together with conditions that lower the oxygen

diffusion in the soil (Firestone *et al.*, 1979; Tiedje, 1988), such as changes in soil moisture (de Klein & Van Logtestijn, 1994a, b; Ledgard *et al.*, 1999; Luo *et al.*, 2000; Saggar *et al.*, 2004a; 2009). In temperate grassland soils under a wide variety of management conditions, denitrification is the chief source of N_2O production (de Klein & Van Logtestijn, 1994b; Saggar *et al.*, 2004c; 2007; Wrage *et al.*, 2001; Zhong *et al.*, 2014). In temperate grasslands the annual loss of N globally through denitrification has been estimated to be 5.6 Tg N (Saggar *et al.*, 2013).

Despite a broad understanding of the multiple soil and environmental factors that regulate the rates of denitrification in pastoral soils, we lack both reliable quantitative denitrification rates for grazed pasture soils and knowledge of how these rates vary spatially and temporally across these grasslands. Understanding the biotic processes (microorganisms and enzymes involved in denitrification) and soil and environmental factors that have the potential to enhance the reduction of N_2O during denitrification is one of the steps to the development of new and effective N_2O mitigation technologies. Mitigation approaches should then focus on ways to decrease total denitrification and recognise the potential of increasing total denitrification, thus enhancing the reduction of N_2O to N_2 during denitrification, and lowering the proportion of N_2O in the total denitrification product.

Most of the recent research in New Zealand has focused on reducing N_2O emissions in grazed pastures by applying nitrification and/or urease inhibitors to retain N in NH_4^+ form and/or reduce the hydrolysis of urea-N (Di & Cameron, 2006; Singh *et al.*, 2008; Zaman *et al.*, 2008a; Zaman & Blennerhassett, 2010). However, we lack a comprehensive, quantitative understanding of denitrification rates and controlling factors across agro-systems.

The purpose of this thesis is to develop an understanding of the soil biochemical factors, denitrifier community structure, and denitrifier abundance that affect denitrification that might lead to N_2O emissions in New Zealand dairy-grazed pastures.

1.2 THESIS OBJECTIVES

The objectives of this study were to:

- identify the key soil factors affecting denitrification rate, and denitrifier community structure and abundance in a range of pasture soils
- understand how the changes in soil water content (SWC) between field-moist soils and saturation change the denitrification products, denitrifier richness and denitrifier abundance in pasture soils with contrasting denitrification enzyme activities
- determine the effect of cattle urine with and without DCD on denitrification rate, denitrifier richness, and denitrifier abundance in selected pasture soils.

1.3 THESIS STRUCTURE

This thesis comprises 8 chapters (Figure 1.1).

Chapter 1 gives a brief introduction of processes leading to N_2O emission globally and in New Zealand dairy pasture. This chapter also includes the overall objective of this PhD.

Chapter 2 provides a detailed overview of the available information regarding denitrification, microbiology of denitrification, soil and environmental factors that affect the process, methods of measurement of denitrification, denitrifier community structure and abundance. This chapter also summarises mitigation techniques to reduce N_2O losses through denitrification.

Chapter 3 outlines the methodology developed during the study to determine denitrification enzyme activity (DEA), denitrification rates (DR) using acetylene inhibition technique, measuring denitrifier gene richness using Terminal Restriction Fragment Length Polymorphism (T-RFLP), and denitrifier gene abundance using quantitative Polymerase Chain Reaction (qPCR).

Chapter 4 reports variability in the physical, chemical characteristics, and DEA and DRs in field-moist samples collected across 10 sites in New Zealand. Variability in the numbers of denitrifier gene (*nir*S, *nir*K and *nos*Z) T-RFs and their gene copy numbers have also been described. The results of this experiment suggested that soils with similar physicochemical characteristics represented similar DEA and DRs. Also the inherent chemical characteristics of soils influenced their denitrifier community structure and abundance.

Chapter 5 presents the result of laboratory incubation conducted on the five selected New Zealand dairy-grazed pasture soils with contrasting DEA at field capacity (FC) and saturation. The measurements of soil NO_3^-, NH_4^+, N_2O production rate (from acetylene and non-acetylene jars), at original field-moist, field capacity and saturation SWC have been compared. The soils incubated at FC and saturation had higher DRs as compared to field-moist soils. However, changes in DRs varied among the five incubated soils and depended on their inherent physicochemical characteristics, denitrifier gene richness and abundance.

Chapter 6 provides the result of laboratory experiment conducted on the three selected New Zealand dairy-grazed pasture soils with contrasting DEA incubated at saturation for 28 days. The measurements of soil pH, NO_3^-, NH_4^+, N_2O production rate (from acetylene and non-acetylene jars), numbers of denitrifier gene T-RFs and gene copy numbers at original field-moist, and saturation have been presented. The increase in N_2O production rate (from acetylene jars) at saturation with increasing incubation duration was variable among the three incubated soils and depended on changes in their chemical characteristics, numbers of denitrifier gene T-RFs and gene copy numbers during 28 days incubation.

Chapter 7 describes the result of a laboratory experiment conducted on the three selected New Zealand dairy-grazed pasture soils with contrasting DEA incubated at saturation with application of cattle urine with and without DCD for 28 days. The measurements of soil pH, NO_3^-, NH_4^+, N_2O production rate (from acetylene and non-acetylene jars), numbers of denitrifier gene T-RFs, and gene copy numbers during incubation have been presented. The addition of urine and urine + DCD resulted in higher N_2O production (from acetylene and non-acetylene jars), and also have higher numbers of *nir*S+*nir*K and *nos*Z gene T-RFs and gene copy numbers compared with samples applied only with water.

Chapter 8 is the overall summary of the experiments conducted during the study and the conclusions derived from the obtained results. This chapter also discusses future research needs to facilitate better understanding of denitrification and development of mitigation techniques to reduce N_2O emission through denitrification.

Chapter 1 Introduction

Introduces the research topic and summarises the overall objective of conducting this research.

Chapter 2 Review of Literature

Provides an overview of the available literature on denitrification and identifies the gaps in the required information, thus justifying the need for the current study.

Chapter 3 Materials and Methods

Protocols of denitrification measurement, and chemical and molecular analyses used in this study.

Chapter 4 Characterization of NZ dairy-pasture soils

Measurements and description of chemical & physical characteristics of soils, their denitrification and denitrifier community structure under field-moist condition.

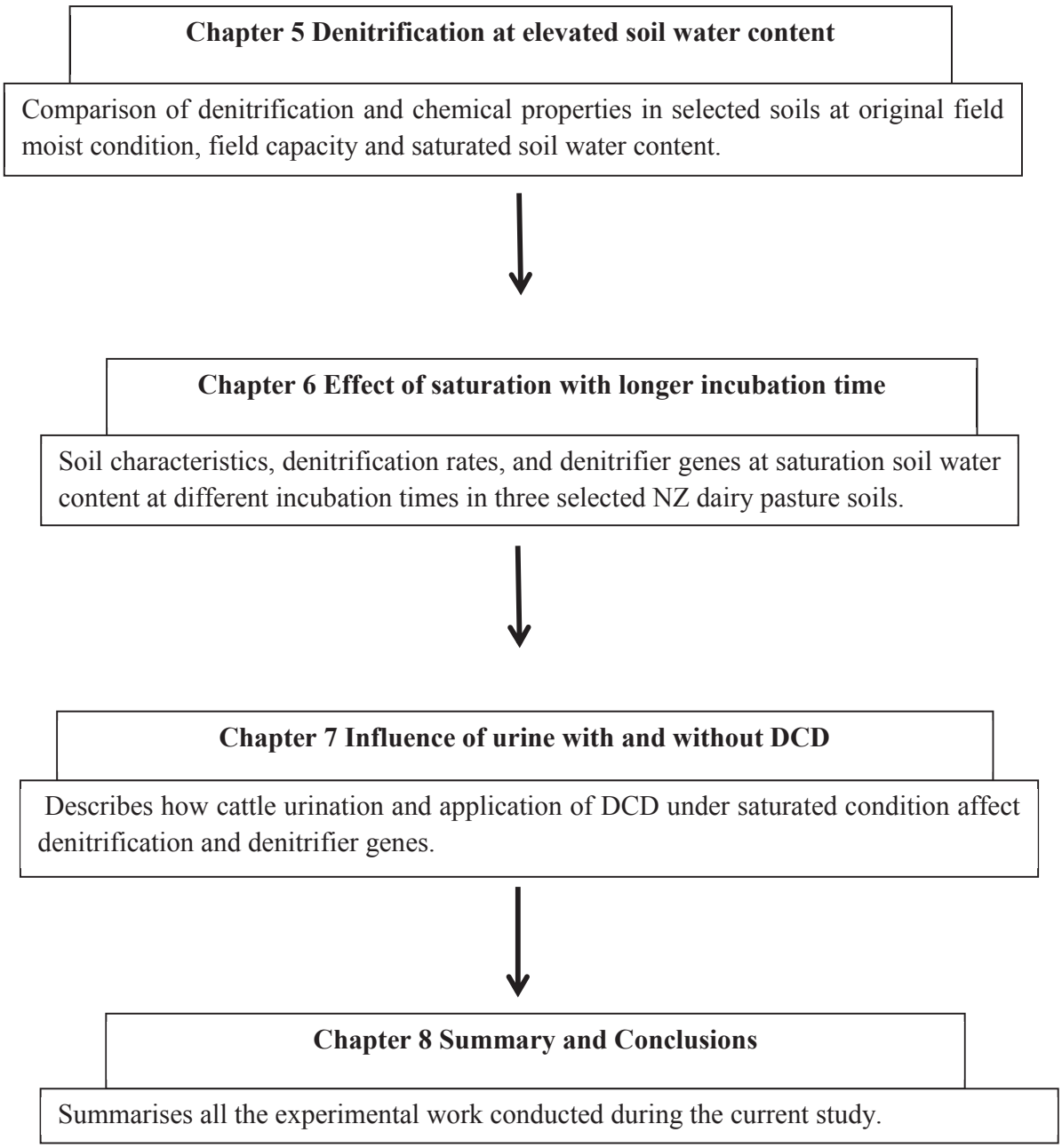


Figure 1.1 Thesis structure.

Chapter 2

Review of literature

2.1 INTRODUCTION

Denitrification is a microbial process that occurs in a wide range of habitats including sediments, marine, freshwater, ground water, and soils under a wide range of land uses (Wallenstein *et al.*, 2006). The presence of high moisture maintains the anaerobic condition and availability of substrates in the form of nitrate (NO_3^-), and carbon (C) ensures denitrification in these ecosystems (Seitzinger *et al.*, 2006). The complete process of denitrification takes place by a stepwise reduction of N oxides with the addition of either one or two electrons per N atom at each step. In total, 10 electrons are consumed in converting 2 NO_3^- ions to dinitrogen (N_2). The sequential denitrification N transformations are presented as follows (Felgate *et al.*, 2012):

$$2NO_3^- + 4e^- + 4H^+ \rightarrow 2NO_2^- + 2H_2O \quad \text{(Eq 2.1)}$$

$$2NO_2^- + 2e^- + 4H^+ \rightarrow 2NO + 2H_2O \quad \text{(Eq 2.2)}$$

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad \text{(Eq 2.3)}$$

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \quad \text{(Eq 2.4)}$$

Complete denitrification involves the stepwise conversion of NO_3^-) to N_2 gas (Eqs 2.1 – 2.4) and leads to the removal of excess reactive N from soil without harmful nitrous oxide (N_2O) emissions (Butterbach-Bahl *et al.*, 2013). Denitrification (Eqs 2.1 – 2.3) serves as the major process of harmful N_2O emission to the atmosphere globally (Kroeze *et al.*, 1999; Mosier *et al.*, 1998). Denitrification can occur through both biological and abiotic processes. Biological denitrification is carried out by diverse group of prokaryotic and eukaryotic organisms representing more than 60 genera of bacteria, archaea, fungi, protozoa, and other eukaryotes (Baggs, 2011). Most of these denitrifiers are facultative anaerobes and use NO_3^- as the final electron acceptor in the absence of O_2. Most commonly found denitrifiers are heterotrophs, using organic carbon compounds as the energy source. However, there are also autotrophic (chemolithotrophic) denitrifiers that use inorganic carbon compounds as a source of carbon and

reduce inorganic compounds, such as Mn_2^+, Fe_2^+, S and H_2, as electron sources. Various genera of prokaryotic denitrifiers, including *Pseudomonas*, *Bacillus*, *Alcaligenes*, and *Flavobacterium, Achromobacter*, *Agrobacterium*, *Chromobacterium*, and *Hyphomicrobium*, are responsible for denitrification in soil (Cheneby *et al.*, 2000; Tiedje, 1988; Zumft, 1992).

Abiotic denitrification contributes only a small portion of total N_2O emissions. Among abiotic processes of N_2O emission chemo-denitrification produces the highest amount of N_2O, when soil nitrite (NO_2^-) reacts with the phenolic compounds present in soil (Bremner, 1997).

The rates of denitrification are affected by the interaction of various soil physical, chemical, and biological conditions (Koponen *et al.*, 2006; Saggar *et al.*, 2013; Skiba & Smith, 2000; Skiba & Ball, 2002). Numerous studies have investigated the factors that control denitrification in an attempt to understand the process better (Firestone *et al.*, 1979; Thomas *et al.*, 1994; Weier *et al.*, 1993a). The most important regulators are soil texture, soil water content, pH, and the presence of denitrifier community (Čuhel *et al.*, 2010; Miller *et al.*, 2009a; Mørkved *et al.*, 2007; Pihlatie *et al.*, 2004; Skiba *et al.*, 1998; Smith *et al.*, 1998; Stevens *et al.*, 1998). Urine deposited by grazing animals is the chief source of N_2O production on a grazed pasture (Oenema *et al.*, 2005). In New Zealand > 80 % of N_2O emission from soils is generated from urine deposited by grazing animals (MfE., 2012), which far exceeds the N_2O produced by the application of synthetic fertilizers or N fixed by leguminous plants.

An important step towards minimising N_2O emissions through denitrification is to understand the soil and environmental conditions affecting the N transformations on farm. Therefore, this review is focussed on the overview of denitrification process, addressing the various factors affecting denitrification, listing the available methods of measuring denitrification, and examining both the possible mitigation techniques currently in use and thhe future aspects of some of the mitigation technologies to reduce denitrification and, more importantly, N_2O emission during the process.

Some parts of this review chapter have been adapted from a recently published review (Saggar *et al.*, 2013) to which I significantly contributed as one of the co-authors.

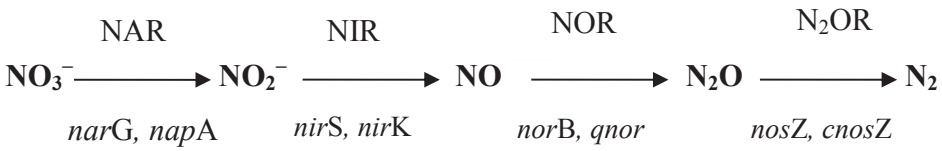


Figure 2.1 Schematic representation of complete denitrification: showing reductase enzymes in regular upper case and genes encoding respecting enzymes in italics.

2.2 BIOLOGICAL DENITRIFICATION

Biological denitrification is a microbially mediated process of the enzymatic reduction of N oxides to N_2. The ability to denitrify is distributed among phylogenetically diverse and unrelated group of organisms. When bacteria are dominant microorganisms in a biological system a large proportion of denitrification is carried out by denitrifying bacteria. Bacterial denitrification is a community process mediated by a set of four reductase enzymes and some of the denitrifiers are unable to produce complete set of enzymes needed for complete denitrification (Zumft, 1997).

The four reductase enzymes involved in the complete denitrification process are NO_3^- reductase (NAR) that converts NO_3^- into NO_2^-, the conversion of NO_2^- into NO is mediated by NO_2^- reductase (NIR), nitric oxide (NO) reductase (NOR) changes NO into N_2O, and N_2O reductase (N_2OR) facilitates the conversion of N_2O to N_2 (Rich & Myrold, 2004; Zumft, 1997) (Figure 2.1). These enzymes and other associated proteins are known as the denitrification proteome, the translation of which is initiated under anaerobic conditions and repressed under aerobic conditions.

There are two subtypes of each bacterial denitrifying enzyme. The two forms of NAR are membrane bound (Nar) and Periplasmic (Nap); denitrifiers may possess either of these two enzymes or may possess both. The two forms of NIR include homotrimeric Copper NIR (NirK) and homodimeric cytochrome Cadmium (Cd) NIR (NirS), of which denitrifiers can possess only one of these two enzymes at a time. Cytochrome C or Pseudoazurin (cNor) and Quinol pool (qNor) comprise two forms of NOR. It was previously believed that denitrifiers possessed only

one form of the N_2OR enzyme; however, recent report suggests the presence of both periplasmic N_2OR and cytochrome cN_2OR (Pauleta *et al.*, 2013).

The denitrifier reductase enzymes are encoded by genes responsible for their presence and activation. The catalytic subunit of membrane bound and periplasmic NAP are coded by *nar*G and *nap*A genes respectively (Bru *et al.*, 2007). The cd_1-Nir and the cu-Nir are coded by *nir*S and *nir*K genes respectively (Liu *et al.*, 2010). So far *nos*Z is the only gene known to encode N_2OR enzyme (Burger & Matiasek, 2009). The best characterised *nir*S genes clusters are those from *Pseudomonas aeruginosa* (*nir*SMCFDLGHJEN), *Pseudomonas denitrificans* (*nir*XISECFDLGHJN) and *Pseudomonas strutzeri* (*nir*STBMCFDLGH). The *nos* is a homodimer of a 65-KDa Copper-containing subunit in which each monomer is made up of the CuA and CuZ domains. The *nos* gene clusters often comprise the *nos*RZDFYLX genes. The *nos*Z gene encodes the monomers of N_2OR. The *nos*DFYL genes encode proteins that are apparently required for Cu assemblage into N_2OR, although their specific role still remains unknown. The NosRX proteins regulates transcription, activation, and Cu assemblages of N_2OR (Zumft & Kroneck, 2006).

Denitrifying bacteria are abundant in agricultural fields counts have shown millions of bacteria in per gram soil in fields. While denitrifying bacteria are abundant in soils, these are not the dominant bacteria generally found in soils. The active denitrifying species are generally limited to the genera *Pseudomonas*, *Bacillus* and *Paracoccus*, although *Thiobacillus denitrificans* and an occasional *Chromobacterium*, *Corynebacterium*, *Hyphomicrobium* or *Serratia* species can also catalyse denitrification. Denitrifiers are among the most diverse group of bacteria and they are studied because of their importance in nitrogen cycling (Cavigelli & Robertson, 2000; Tiedje, 1988; Zumft, 1992).

Using the complete genome sequences of *Sinorhizobium meliloti* 1021 and *Pseudomonas aeruginosa* PAO1, Philippot *et al.* (2011) have shown that approximately $1/3^{rd}$ of the these denitrifying bacteria possessing the *nir* gene do not have the N_2OR enzyme, so that denitrification terminates at stage of N_2O emission (Philippot *et al.*, 2011). More comparative genomic analysis of prokaryotic denitrifiers revealed that out of 68 strains having either *nir*K or *nir*S genes, only 48 also possessed the *nos*Z gene (Jones *et al.*, 2008).These results collectively indicate that a significant fraction of the denitrifying population in soil does not possess the

genetic ability to produce N_2 as the end product of denitrification, and will end up producing N_2O.

The other observation of significance to N_2 production during denitrification is that *Desulfitobacterium hafniense* and *Anaeromyxobacte*r spp. found in soil and sediment do not possess either of the *nir* genes but are genetically capable of producing N_2OR (Sanford *et al.*, 2002; Simon *et al.*, 2004; Zumft & Kroneck, 2006). These denitrifiers with only *nos*Z genes are referred to as possessing atypical *nos* clusters and are common in bacteria and archea (44 % genome), either lacking other denitrifier genes (56 %) or performing DNRA (31 %). The significance of these atypical denitrifiers would be higher in soils producing higher rates of N_2O to reduce the N_2O to N_2.

Most denitrifying bacteria are facultative anaerobes, reducing NO_3^- only in the absence of O_2. However, it has recently been revealed that *Microvirgula aerodenitrificans* and several bacteria belonging to genera *Paracoccus*, *Thiobacillus*, and *Enterobacter*, isolated from soil and sediment are capable of carrying denitrification in the presence of O_2 (Patureau *et al.*, 2000). In these bacteria, only the N_2OR remains inactive during exposure to O_2, other denitrifying enzymes still carry on their function under aerobic condition (Morley *et al.*, 2008). Since the research for aerobic denitrification is recent, it is quite a debatable topic and assumed to be a major contributor to N_2O emission. More research is needed to substantiate this concept, but until then denitrification is considered as anaerobic process (Baggs, 2011).

Although bacteria are the major contributor to denitrification, a report by Shoun & Tanimoto (1991) suggests fungal denitrification also plays a substantial part in N_2O production in agricultural systems. The fungal denitrification consists up of NirK, and P450nor (cytochrome NO reductase) enzymes, although it lacks N_2OR (Shoun *et al.*, 2012). Under certain conditions such as acidification or excessive use of ammonical fertilizers, fungal denitrification could be the main source of N_2O production (Thomson *et al.*, 2012).

The fungal denitrification is mostly accompanied by a unique phenomenon of co-denitrification (Tanimoto *et al.*, 1992), during which NO_2^- combines with amines and imines and generates hybrid N_2 or N_2O species. Since fungi lack N_2OR, the co-denitrification is the only pathway to produce N_2 during fungal denitrification (Baggs, 2011). To date there is no direct measure for co-denitrification and thus its contribution to global warming remains uncertain. It has been reported that fungi may be responsible for more than 90 % of the N_2O produced in

grassland soils (Laughlin & Stevens, 2002). The magnitude of the fungal contribution to measured N_2O emission from arable soil is still unexplored (Herold *et al.*, 2012).

There are several requirements for denitrification to take place in agricultural soils: availability of substrates in non-limiting form like N oxides (NO_3^-, NO_2^-, NO, or N_2O) as terminal electron acceptors and soluble C as electron donors, presence of active denitrifiers; suitable anaerobic condition; and optimum soil pH and temperature for biochemical reactions to prolong to completion. Depending on the availability of suitable conditions, denitrification could be complete (when N_2 is the end product) or incomplete (when N_2O is the end product). Different soil and climatic factors affect the activities of denitrifiers and their enzymes in soil and hence affect the process of denitrification. These factors and their influence on denitrification are discussed in detail in the next section of this chapter. DEA and DR measured under various conditions in grassland soils in selected studies are given in Table 2.1, showing major variability among studies.

2.3 FACTORS AFFECTING DENITRIFICATION

2.3.1 Soil Factors

The conceptual model proposed by Firestone & Davidson (1989) and Davidson (1991), known as the 'hole in the pipe", represents interactions and controls on numerous factors involved in the release of gaseous N products during denitrification from soils. The model suggests the production of NO and N_2O is related to available resources in soils. The model describes three levels of regulation on the relative amounts of N_2O and N_2 produced during denitrification:

i) Factors regulating rate of N flowing through the system (fertilizer application rate, animal excretion)

ii) Factors regulating the ratio of intermediate gases and end products (SWC, pH, organic matter content, denitrifier population)

iii) Factors regulating the diffusion of gases from soil profile (soil texture, pore size, SWC).

Long-term cultivation severely affects the physical, biological, and chemical characteristics of agricultural soils. This ultimately affects the denitrification process through changes in microbial community composition and activity. The major proximal and distal factors influencing denitrification illustrated by Saggar *et al.* (2013) are discussed in the following sub-sections.

2.3.1.1 Soil water content (SWC) and aeration

One of the important factors controlling denitrification in soils is the presence of oxygen, which is strongly influenced by available soil water content. Increasing SWC may affect denitrification directly or indirectly: water fills soil pores and reduces O_2 diffusion to microsites; releases available C and N through wetting and drying cycles; provides suitable conditions for microbial growth and activity; and provides a medium through which substrates and products can diffuse to soil microorganisms (Aulakh *et al.*, 1992). The proportion of anoxic spaces in soils increases with SWC (Groffman & Tiedje, 1991; Sexstone *et al.*, 1985) and favours denitrification. Several field studies have shown elevated denitrification rates (DRs) when SWC is high (Bolan *et al.*, 2004b; de Klein & Van Logtestijn, 1994a; Ledgard *et al.*, 1999; Luo *et al.*, 2000). Rainfall, irrigation, and urination events by grazing animals might increase SWC on a grazed farm and increase N_2O and N_2 productions.

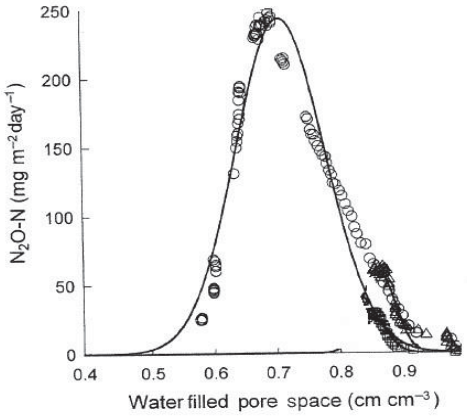


Figure 2.2 Nitrous oxide flux as a function of water filled pore space (Castellano *et al.*, 2010).

Anoxic conditions in soils also depend on their texture. In a fine-textured soil anoxic microsites occur at lower moisture contents than in coarse-textured soils (Groffman & Tiedje, 1991; Parton *et al.*, 1996). Under natural conditions soil aeration is also controlled by the partial pressure of O_2 and the respiratory activities of soil microorganisms (Tiedje, 1988). Parkin & Tiedje (1984) reported a 1.5 to 5 times increase in denitrification activities as O_2 partial pressure decreased from 20 to 5 % and speculated that large increases in the denitrification rate (DR) occur when O_2 partial pressure is below 0.5 %. In the absence of oxygen, denitrifiers use NO_3^- as a final electron acceptor and reduce it to N_2O or N_2, thus O_2 depletion leads to increased denitrification. Increased microbial activity in soil also reduces the availability of O_2 and thus increases the demand for other terminal electron acceptors and hence accelerates denitrification (Baggs *et al.*, 2000; Garcia-Montiel *et al.*, 2003; Miller *et al.*, 2008; Paré, 2011).

In addition to influencing total denitrification, SWC or aeration also controls the proportion of N_2O in the total denitrification product. Soil aeration alters the diffusion of O_2 as well as intermediate gaseous N products of denitrification (Stange *et al.*, 2013). When SWC increases, the proportion of N lost in denitrification as N_2O decreases (Fig. 2.2) and N_2 becomes the chief denitrification product (Bateman & Baggs, 2005; Weier *et al.*, 1993a). The increase in N_2 production or decrease in N_2O production occurs through two mechanisms. First, the rate of

diffusion of N_2O from the site of production is decreased at higher SWC (Petersen & Andersen, 1996), and slow diffusion leads to longer residence time for N_2O to be reduced to N_2. Second, since N_2OR activity is O_2 sensitive, higher SWC increases N_2OR activity by reducing its exposure to O_2, and leading to N_2 as the principle product of denitrification (Weier *et al.*, 1993a).

In studies of denitrification, water-filled pore space is the variable most widely used to estimate soil moisture content in different soil types (Farquharson & Baldock, 2008). This critical soil water content, termed the threshold soil water, varies with soil type and is considered to be close to 60 % WFPS or field capacity in most soils (de Klein & van Logtestijn, 1996). Davidson (1991) has reported the maximum amount of N_2O emission through denitrification to be at 80 % WFPS, with a further increase in SWC, the N_2O produced is reduced to N_2, and at 100 % WFPS the chief denitrification product N_2 is released through denitrification (Fig. 2.2).

The increased DR with increasing SWC or decreasing O_2 content in soils is indirectly due to the enhanced abundance and activity of denitrifying organisms in favourable condition. The continued anaerobic conditions in wetlands and high nutrient loadings in terms of reactive N and C lead to high denitrification activities that are generally associated with the diverse denitrifier community (Reyna *et al.*, 2010; Tiquia *et al.*, 2006) and more abundant denitrifiers (Chon *et al.*, 2011) as compared with aerobic soils (Graham *et al.*, 2010). There is limited information on the direct or indirect effect of SWC on the denitrifier community structure or denitrifier abundance in soils. Liu *et al.* (2012) have reported *nar*G and *nos*Z gene abundances in the paddy soils significantly correlated with their water contents during drying process of the wetting drying cycles. Similarly, Mergel *et al.* (2001a) found that denitrifiers were less abundant in summer that in other seasons due to drier soils in summer than other seasons.

Apart from an increase in SWC, another factor that controls the soil aeration in grazed grasslands is compaction. On farms, the use of heavy machinery and treading by grazing animals can lead to soil compaction. With soil compaction, the total pore volume of soil decreases. Smaller soil pores are either moved downwards by compaction or get filled with water and larger pores get eliminated and hence WFPS increases and oxygen availability decreases, which causes an increase in denitrification (McTaggart *et al.*, 1997; Van Groenigen *et al.*, 2005b).

2.3.1.2 Availability of soil organic carbon

Since denitrification is driven by heterotrophic microbes, C availability is one of the key factors affecting the process (Knowles, 1982). The availability of organic C provides energy and stimulates heterotrophic microbial activity. Several studies have reported higher DRs from soils treated with organic amendments such as manure, composts, and plant residues when compared with unamended or mineral-N treated soils (Dambreville *et al.*, 2006a; 2006b; Miller *et al.*, 2008, 2009a). Slurry application generally enhances denitrification in soils due to the increase in readily available C and N, which fuel microbial growth and stimulate denitrification activity through the consumption of O_2 by microbes (Thangarajan *et al.*, 2013). DRs may also vary with the type of C substrate from simple compounds such as glucose to complex crop residues (Aulakh *et al.*, 1991b; Weier *et al.*, 1993b).

The high denitrification rates that occur in grazed pastures are related to moderate to high levels of available C, which are added to the soil through: the decomposition of plant litter, root exudates, grazing animal faeces, and the solubilisation of organic matter in the urine of grazing animals or in the water contained in slurries of farm organic wastes (Carran *et al.*, 1995). The addition of C to grazed pastures through animal excretion in the form of dung or urine often leads to higher denitrification rates and to spatial and temporal variabilities in denitrification (Saggar *et al.*, 2004a). The surface layers (0–30 cm) of soils have higher denitrification potentials due to their higher organic C contents. Dhondt *et al.* (2004) have found greater denitrification enzyme activity (DEA) in upper soil layers of riparian soils of mixed vegetation with higher organic carbon content than other deeper layers. Hernandez & Mitsch (2007a) have reported a significant linear relationship between the presence of organic matter content and denitrification potentials in the created wetlands. High availability of organic matter and water soluble C under anaerobic conditions in these wetlands with high NO_3^- loadings have influenced high denitrification activities.

Glucose addition increases denitrification due to a supply of readily available C for denitrifying organisms but with the addition of complex sources like straw, the denitrification depends on the C/N ratio of the source. When C/N ratio in the added organic matter is low, it leads to higher decomposition of residue that releases water soluble or dissolved organic C and NH_4^+ contents through mineralisation thus making conditions favourable for microorganisms to

produce N_2O (Huang *et al.*, 2004). The higher C/N ratio also favours reduction of N_2O to N_2 during denitrification and leads to completion of the process (Bhandral *et al.*, 2007).

Apart from influencing denitrification, C availability also affects the abundance of denitrifiers. The size of the soil bacterial denitrifier community increased after the addition of complex C sources such as manure (Miller *et al.*, 2009a) or plant residues (Henderson *et al.*, 2010; Miller *et al.*, 2008). Following the addition of crop residues to soils as a C source (250 mg C kg^{-1} soil), Miller *et al.* (2008) measured increased numbers of denitrifier genes (8.1×10^4 cnorB_p gene copies g^{-1} dry soil compared with 3.7×10^4 g^{-1} dry soil in the soil with no added residue. Similarly, Tatti *et al.* (2013) reported an addition of 10 g of dry compost to soil resulted in addition of 2.6×10^6 and 1.4×10^6 of *nir*K and *nir*S gene copy numbers g^{-1} dry soil.

The type of C substrate added to the soil also affects its denitrifier gene abundance. Studies have found that compared with glucose, the addition of liquid manure to soil increases *nos*Z gene abundance and hence enhances denitrification. This difference is thought to occur because manure provides both labile and complex forms of C to the soil organisms (Paul & Beauchamp, 1989; Van Kessel & Reeves Iii, 2000). Dambreville *et al.* (2006a) found that composted pig manure applied soil had a more diverse denitrifier community and higher DEA and denitrification than the control soil.

2.3.1.3 Availability of mineral N

The application of synthetic N fertilizers, animal wastes, and biological N-fixation are various means by which N reaches the soil (Seitzinger *et al.*, 2006). Among these N sources the chief source of mineral N addition on grazed farms is animal urine. It has been estimated that N_2O production within 24 hours of urine deposition by animals accounts for nearly 8 % of the annual N_2O emission on grassland (Williams *et al.*, 1999). Although synthetic fertilizer application also provides inorganic N to the farm, when same amount of urine-N, urea-N or ammonium-N was applied on soil N_2O production from urine application was higher than other N sources (Sherlock & Goh, 1984). Higher N_2O production from urine patches compared with fertilizer N addition is hard to explain. The addition of N and its transformation to NO_3^- in soil (form most suitable for denitrification) will enhance N_2O and N_2 production through denitrification. The availability of NO_3^- in soil is affected by the nitrification rate, rate of leaching, and the rate of NO_3^-

consumption by plants and non-denitrifying microorganisms (Abbasi & Adams, 1998, 2000; Luo *et al.*, 1999a; Tiedje, 1988). The NO_3^- content of soil is one of the major factors affecting both DRs and the proportion of N_2O in the final end product of denitrification (Bremner & Blackmer, 1978).

As NO_3^- reduction is the first step of denitrification. NO_3^- availability is the prime requirement for denitrifiers to initiate the process (Bolan *et al.*, 2004b). The presence of NO_3^- is essential for the activation of NAR under anaerobic condition (Bryan, 1981). Denitrifiers are facultative anaerobes: after depletion of O_2 in soil, NO_3^- is the most powerful reductant, and denitrifiers have a higher affinity for this ion than for any of the other nitrogen oxides, NO_2^-, NO, or N_2O, present in the system (Aulakh *et al.*, 1984). When other factors such as SWC and organic C are non-limiting, the addition of NO_3^- increases the DR in soils.

A number of studies have shown increased denitrification with the addition of mineral N in one form or another: manure, mineral fertilizer or through animal excreta in the field (Corre *et al.*, 1990; de Klein & Van Logtestijn, 1994b; Jarvis *et al.*, 1991a; 1991b; Ledgard *et al.*, 1999; Ryden, 1983; Saggar *et al.*, 2002; 2003; 2004c). Significantly higher cumulative DR (13.3 mg N_2O-N kg^{-1} soil) have been reported by Miller *et al.* (2008) in soils amended with 50 mg NO_3^--N kg^{-1} soil compared with the DR (< 2.5 mg N_2O-N kg^{-1} soil) in control soil. These results clearly indicated the role of NO_3^- addition in increasing denitrification.

Despite influencing DR, high NO_3^- availability in soils inhibits the activity of N_2OR. This favours N_2O production over N_2 as the end-product of denitrification (Bremner & Blackmer, 1978). Luo *et al.* (1996) have reported lower $N_2O + N_2$ production in the soil samples incubated with additional NO_3^--N content beyond 50 ug N g^{-1} soil and warned of the harmful effect of high concentrations of NO_3^- on denitrification activities. Senbayram *et al.* (2012) have also advocated the negative impact of higher NO_3^--N contents in soil on measured DRs.

Increased denitrification with NO_3^- addition is attributed to higher denitrifier gene abundance and denitrifiers activities. More gene copies of *nar*G (2.8×10^2 to 5.0×10^4 ng^{-1} DNA) corresponding to higher NO_3^- content in river sediments (12.9 mgL^{-1}) compared with *nar*G copies (1.0×10^2 ng^{-1} DNA) in river sediments with lower NO_3^- content (2.0 mgL^{-1}) (Reyna *et al.*, 2010).

2.3.1.4 Soil pH

Over the past few decades efforts have been made to understand the relationship between soil pH and denitrification (Wijler & Delwiche, 1954), with these results suggesting that neutral to alkaline (pH ranging from 6.5 to 7.5) conditions in soil are optimum for denitrification. Šimek & Cooper (2002) have extensively studied the effect of pH on DRs in soils, and report lower DRs in soils under acidic pH compared with neutral or alkaline soils (Bremner & Shaw, 1958; Bryan, 1981; Nägele & Conrad, 1990). However, denitrification may still continue at pH as low as 3.5 and thus is responsible for significant N losses even in naturally acidic soils (Dalal *et al.*, 2003; Weier & Gilliam, 1986).

Low (< 6) pH has an inhibitory effect on N_2OR formation and activity (Liu *et al.*, 2010). This is mainly due to an interference in the assembly of the enzyme rather than to the direct effects of low pH on enzyme activity (Bakken *et al.*, 2012). Acidic pH (< 5) impairs denitrification and increases the relative production of N_2O rather than that of N_2. Numerous studies have reported changes in intermediate denitrification products with changes in soil pH during the measurements (Firestone *et al.*, 1980; Koskinen & Keeney, 1982). Parkin *et al.* (1985) demonstrated a two-fold decrease in DR and a three-fold decrease in DEA with a 1.94 units decrease in soil pH from 6.02 to 4.08. In agricultural soils, continuous use of N fertilizer leads to soil acidification resulting in enhanced N_2O production (Guo *et al.*, 2010).

pH (6–8) is optimal for most bacterial denitrifiers. Soil pH is one of the most comprehensively studied factors affecting denitrification and is considered to be one of the key factors driving denitrifier community structure (Enwall *et al.*, 2005; Wakelin *et al.*, 2008) and denitrifier gene abundance (Bárta *et al.*, 2010; Ligi *et al.*, 2013; Liu *et al.*, 2010). Bru *et al.* (2011) evaluated the contribution of soil chemical properties to the spatial distribution of the abundance of denitrifiers at the landscape scale. The results showed that soil pH was the main driver of denitrifier abundance in soils. Using pure cultures of *Paracoccus denitrificans*, Thomas *et al.* (1994) have reported that under low pH N_2O reduction is more inhibited than NO_2^- reduction. The denitrifying population adapted to low pH environment can produce N_2O but has less capacity to reduce it to N_2 (Saleh-Lakha *et al.*, 2009a).

An additional complication to understanding the relationship between pH and denitrification is bacterial acclimatisation to the prevailing pH, which allows acclimated

microbes to maintain high rates of denitrification over a range of pH (Palmer *et al.*, 2010; Parkin *et al.*, 1985; Šimek *et al.*, 2002). Continuous use of mineral fertilizers may lead to acidification of agricultural soils and thus will enhance N_2O emission, while the adjustment of soil pH by liming to neutral would reduce N_2O emission. The relationship between soil pH and production or reduction of N_2O have been revealed under laboratory incubations (Šimek *et al.*, 2002); whether this relationship holds true under field condition is still unknown due to the methodological limitation of measurement of N_2.

2.3.1.5 Availability of metal ions for enzyme reductions

Reduction enzymes involved in denitrification contain metals as cofactors for electron transport or at catalytic centres as active sites. The conversion of NO_3^- to N_2O needs Fe, Cu, and Mo containing proteins, while the reduction of N_2O to N_2 needs only Cu containing protein (Glass & Orphan, 2012). Each of the four denitrifier reductase enzymes needs varying metal contents for its activation. NAR enzyme complex contains one Fe_3S_4 cluster, four Fe_4S_4 clusters and one molybdopterin (Mo) active site. The conversion of nitrite to nitric oxide is catalysed by either Fe (four Fe atoms) containing NirS or Cu containing NirK. Depending on its subunit, the NirK enzyme might possess either 6 or 24 Cu atoms. NOR in denitrifying bacteria contains three Fe atoms (two non-hemes and one heme). N_2OR is a homo-dimeric protein with two structurally distinct Cu co-factors Cu_Z and Cu_A per monomer that are crucial for its activity. The N_2OR enzyme contains 12 Cu atoms per homodimer. Reductase enzymes involved in denitrification have both Cu dependent and independent variants, but the N_2OR enzyme is completely Cu dependent for its activation. It is likely that the distinct biochemical dependence on Cu of denitrification pathways in different classes of denitrifying bacteria will lead to different denitrification phenotypes (Felgate *et al.*, 2012).

Laboratory incubations have demonstrated the importance of Cu availability for the denitrification process and, more importantly, for the reduction of N_2O to N_2 by denitrifying bacteria (Granger & Ward, 2003; Iwasaki & Terai, 1982; Twining *et al.*, 2007). The requirement of Cu by N_2OR could play a critical role in the proportional emission of N_2O during denitrification (Signor & Cerri, 2013). Providing additional Cu to Cu-limited cultures not only enhanced the denitrifying bacterial growth but also increased the conversion of N_2O to N_2

(Granger & Ward, 2003). There is limited information on the effect of Cu application on denitrifier enzyme activities in soils and sediments (Wang *et al.*, 2013). Soils with high organic matter are likely to have low Cu availability (Bolan *et al.*, 2004a; Lair *et al.*, 2006; 2007), which is probably due to the formation of stable chelate complexes between Cu and organic matter in soils. Lower Cu availability in soils will affect N_2OR activity and enhance N_2O production in soils. Insufficient metal ions such as Fe and Mo will affect NAR, NIR, and NOR activities and thus will lead to lower N_2O production (Richardson *et al.*, 2009).

2.3.1.6 Denitrifier community structure and abundance of denitrifying organisms and reductase enzyme activity

Denitrifier abundance is an important factor that affects N_2O emissions through denitrification (Spiro, 2012). If all the denitrifying bacteria (with differential potentials to carry out each step of denitrification) in a soil performs equally well, the NO and N_2O production is believed to be negligible and available NO_3^- will directly be transformed to benign N_2 (Bakken *et al.*, 2012). Advancement in molecular technologies has enabled us to relate microbial communities and soil biochemical processes. Recent work on the ecology of denitrifying organisms has focused on their identification by analysis of *nir*K, *nir*S, and *nos*Z genes. These genes are targeted because they catalyse key steps in the process, are widespread among the taxonomically diverse denitrifiers, and are unique to organisms that denitrify (Braker *et al.*, 2000; Henry *et al.*, 2004; Philippot, 2002).

In order to understand various soil conditions leading to denitrification in soils, studies have simultaneously examined denitrifier community structure and/or denitrifier abundance with N_2O, and (N_2O+N_2) production in soils (Chen *et al.*, 2012a; Chon *et al.*, 2011; Dambreville *et al.*, 2006b; Enwall *et al.*, 2005; Miller *et al.*, 2008, 2009a; Wolsing & Priemé, 2004). These studies carried out on wide variety of soils, environmental conditions, and management practices have reported contrasting results. The measurements carried out represent varied experimental conditions and lack comparative measurements under consistent environment. The comparisons of denitrifier community structure and abundance among various studies are difficult due to the various soils, methods, and environmental parameters evaluated over varying time periods.

Studies have related changes in denitrifier community structure with denitrification, although strong relationships are not always observed (Cavigelli & Robertson, 2000; 2001; Chèneby *et al.*, 1998; Mergel *et al.*, 2001a). Enwall *et al.* (2005) have found limited influence of denitrifier community composition and denitrifier abundance on the measured denitrification rate. On the other hand, Philippot *et al.* (2009) have shown that the spatial distribution of the relative abundances of denitrifiers with the genetic capacity to reduce N_2O to N_2 is linked to areas with high denitrification rates and low N_2O emissions.

Attempts to predict N_2O emissions based on denitrifier *nos*Z gene abundance and expression revealed an incongruity between the predicted and the actual N_2O emissions, suggesting the existence of an unaccounted N_2O sink (Henderson *et al.*, 2010; Morales *et al.*, 2010; Smith *et al.*, 2007). Bacteria lacking other denitrifying genes but containing the *nos* gene are known to possess atypical *nos*Z gene and the bacteria containing the *nos* gene along with the other denitrifying genes are known to possess typical *nos*Z genes. The atypical *nos*Z genes are abundant in terrestrial environment (Sanford *et al.*, 2012) and previous studies reporting typical *nos*Z targeted primers do not include the atypical *nos*Z diversity.

Denitrifier community structure is subject to change depending on soil condition such as changes in pH, availability of C, NO_3^-, and NH_4^+ contents (Jones & Hallin, 2010). A strong seasonal shift in denitrifier community composition has been suggested (Braker *et al.*, 2010). Consequently, the denitrification rate can vary with a change in the composition of a denitrifying community in soils. Thus prediction of the N_2O flux or denitrification rate becomes difficult without considering community differences along with gaseous measurements. Different techniques, such as most probable number (MPN), polymerase chain reaction (PCR), cloning and sequencing genes, and studying reductase enzymes, have been used to characterise and quantify denitrifiers in soils (Rosch *et al.*, 2002). Therefore, to have overall understanding of factors affecting denitrification in a particular system, along with studying other factors, it becomes very important to study the microbial community contribution. Cavigelli & Robertson (2000) have recommended including community composition in the models to calculate denitrification.

The community structure and abundance of denitrifiers may influence N_2O emissions through denitrification and may be key factors to manipulate in order to reduce N_2O emissions to the atmosphere. Soils lacking denitrifiers with the *nos*Z gene could be critical factors in

determining N_2O emissions. Inoculating soils with *Agrobacterium tumefaciens* lacking N_2OR, Philippot *et al.* (2011) illustrated the reduction of N_2O (produced by *Agrobacterium tumefaciens*) to N_2 by the indigenous denitrifier community originally present in soil.

For most ecosystems denitrification is positively correlated with nitrogen supply and carbon availability and influenced by rainfall and temperature shifts. Although the process is generally modelled without reference to the population of denitrifying bacteria, physiological differences among these bacteria suggest that an understanding of their diversity and ecology may help explain differences in denitrification rates.

2.3.2 Climatic Factors

2.3.2.1 Soil Temperature

Temperature regulates denitrification by either increasing or decreasing the microbial activity in soils. Soil temperature may also affect the availability of O_2, NO_3^-, N_2O, and C in soil and thus indirectly affect denitrification. Exponential relationships have been reported between soil temperature and N_2O flux when soil temperature ranged from 11 ^oC to 26 ^oC in a maize field study (Song & Zhang, 2009). In a recent review of N_2O emissions from 8 sites in Ireland with varying soil type and management regime, Rafique *et al.* (2011) reported that the N_2O flux at 17 ^oC was five times greater than the N_2O flux at 5 ^oC. Higher N_2O emission at higher temperature is likely to be stimulated by higher microbial growth and activity (Sierra, 2002). Similar to other microbial processes, denitrification increases with increasing temperature, until the temperature reaches 30 ^oC; a subsequent increase in temperature leads to the denaturation of enzymes, resulting in decreasing denitrification (Bolan *et al.*, 2004b). There is no well-defined temperature range for the denitrification process to occur as the denitrifying bacteria involved in the process are able to adapt to a broad range of temperature conditions (5–25 ^oC) in soil (Malhi *et al.*, 1990; Powlson *et al.*, 1988), so optimum temperature for denitrification could differ with prevailing soil condition. The highest rates of denitrification have been reported at temperatures 60–70 °C (Bremner & Shaw, 1958; Keeney *et al.*, 1979), although non-biological reactions (such

as chemodenitrification) probably contribute to denitrification at these high temperatures (Firestone, 1982).

As microbial activity is accelerated in soils at higher temperatures, this increases cellular demand for O_2, which promotes anoxic conditions that are favourable for denitrification (Baggs *et al.*, 2002). Several studies report the effect of temperature on denitrification rates in soil. Within a limited temperature range of 15 ^{o}C to 35 ^{o}C, the temperature coefficient of denitrification (Q_{10}) was reported by Stanford *et al.* (1975) to be about 2. This value translates to a two-fold increase in denitrification for every 10 ^{o}C increase in temperature. In many field experiments concentrations of N_2O in soil solution and soil air increased with rising temperature (Heincke & Kaupenjohann, 1999). Soil temperature can either directly influence the activity of existing denitrifiers community, or it can result in potential changes to denitrifier communities, modify denitrification activities, and thus increase denitrification rate with increasing temperature (Schimel & Gulledge, 1998).

Soil temperature affects the activation energy of any bio-chemical reaction occurring in soil. It has been observed that the activation energy of N_2O reduction is higher than N_2O production (Holtan-Hartwig *et al.*, 2002). At low soil temperature, therefore, there is higher N_2O production and increases in the N_2O/N_2 product ratio as N_2O reductase activity slows (Avalakki *et al.*, 1995; Keeney *et al.*, 1979). A number of studies in New Zealand pasture soils have shown higher denitrification losses in winter, with soil temperatures as low as 10 ^{o}C (Luo *et al.*, 2000; Ruz- Jerez *et al.*, 1994). Higher denitrification reported in winter is the result of high moisture content and low oxygen content in the soils. Prolonged lower temperature in the temperate grasslands in New Zealand might have encouraged denitrifier populations that are either cold-tolerant or cold-adapted that can continue to denitrify at low soil temperatures (Dorland & Beauchamp, 1991).

2.3.2.2 Rainfall

Higher denitrification rates have been observed with increasing rainfall, which is attributed to increased SWC, nutrient solubility, lower oxygen content, and high denitrifier activity (Ellis *et al.*, 1998). Peak denitrification rates are generally found following rainfall (de Klein *et al.*, 1999; Velthof *et al.*, 1996). High N_2O emissions have been measured following high rainfall events

and continue for 24–48 hours until emissions return to background levels (Corre *et al.*, 1990). The topography of an area also changes the effect of rainfall on denitrification. A high correlation (r = 0.73) was observed between N_2O emission and the amount of rainfall on the sloped area compared with a flat area (r = 0.57). The length of time N_2O emissions took to return to background level was higher (48 h) in sloped area than in flat area (24 h) (Corre *et al.*, 1990). Higher rainfall in winter in New Zealand is one of the chief factors responsible for higher DRs, even in lower soil temperature (Luo *et al.*, 2000). Another rainfall effect rainfall is the wetting of dry soil with sudden and intense rain. Heavy rain after a long dry spell intensely increases the DR (Luo *et al.*, 2000), which is reportedly due to the sudden increase in microbial activity in soil with the onset of anoxic conditions.

2.3.3 Management Factors

2.3.3.1 Animal Grazing

Animal grazing plays an important role in affecting DRs in agricultural systems especially grasslands. Grazing animals only use a small proportion of the nutrients they ingest, with the remaining 60–95 % returned to soil in the form of animal excreta. Deposition of excreta in the form of dung and urine by grazing animals leads to very high concentrations of NO_3^- in soil; under anaerobic conditions when soil C is non-limiting this leads to higher DRs (Luo *et al.*, 1999b). Creation of these hot-spots results in very high spatial and temporal variability of DRs. Several studies have reported herigh denitrification in grazed pastures than in ungrazed pastures throughout the season (de Klein *et al.*, 2001; Luo *et al.*, 1999b; Ryden, 1986).

Increase in denitrification with grazing is the result of increased reductase enzymes activities and changes in the community composition of denitrifiers (Frank & Groffman, 1998; Frank *et al.*, 1998; 2000). Patra *et al.* (2005) found higher DEA in intensively grazed land compared with lightly grazed land. The effect of grazing on rate of denitrification is more evident in a wet winter season compared with a dry summer season when soil moisture is lower and not encouraging denitrification. Higher denitrification has been observed from urine patches compared with dung patches (Van Groenigen *et al.*, 2005a), which is likely due to the higher N and water contents of animal urine than of dung. Urea is the main component of urine and

accounts for over 70 % of urine-N content (Oenema *et al.*, 1997). In the process of urea hydrolysis a large quantity of aqueous ammonia is released, which causes root scorching and increases denitrification (Shand *et al.*, 2002).

Animal grazing induces treading, which leads to soil compaction and promotes anaerobic conditions that favour denitrification (Hansen *et al.*, 1993; Menneer *et al.*, 2005; Ruz- Jerez *et al.*, 1994; Yamulki & Jarvis, 2002). With soil compaction the total pore volume of soil decreases. Smaller soil pores are either moved downwards by compaction or fill with water, and larger pores are eliminated, hence WFPS increases and oxygen availability decreases, which causes increase in denitrification (McTaggart *et al.*, 1997; Van Groenigen *et al.*, 2005b). It has been suggested that the application of urine-N in pastures may lead to a five-fold increase in denitrification and, in combination with soil compaction denitrification, might increase up to 8-fold (Van Groenigen *et al.*, 2005b). Restricted grazing on farms, especially during high rainfall periods and application of urease or nitrification inhibitor, might reduce N_2O production through denitrification (Saggar *et al.*, 2013).

2.3.3.2 Animal Waste Management

Intensification of pastoral farming leads to increased effluent generation each year in New Zealand (Longhurst *et al.*, 2000). It has been estimated by Saggar *et al.* (2004d) that a typical dairy shed in New Zealand releases 70 million m^3 of effluent annually. These values for pig farm and meat processing plants are 4 million m^3 and 50 million m^3 effluent respectively. Increased denitrification has been reported to occur after the application of dairy, piggery and meat effluents to land (Russell *et al.*, 1993; Whalen, 2000). With the application of farm effluents, the soil is enriched with available C, NH_4 and NO_3-N, and water, which increases soil biological oxygen demand (BOD) by reducing oxygen diffusion rate through water-filled pores (Barton & Schipper, 2001) and thus increases the process of denitrification. To reduce N_2O production from animal waste addition on farms, decreasing total quantity of animal excreta deposition, reducing total N content in the excreta, and avoiding soil conditions leading to elevated N_2O production, are needed.

Table 2.1 Summary of selected studies measuring denitrification in grasslands using acetylene inhibition technique

Country	Soil Texture	Condition of measurement	DEA (mg N_2O-N kg^{-1} soil h^{-1})	DR (µg N_2O-N kg^{-1} soil h^{-1})	Length of study	Reference
New Zealand	Fine loam	Up-slope	0.79	0.59	24 hours	Deslippe *et al.* (2014)
New Zealand	Fine loam	Mid-slope	0.84	0.69	24 hours	Deslippe *et al.* (2014)
New Zealand	Fine loam	Toe-slope	0.99	12.2	24 hours	Deslippe *et al.* (2014)
Ireland	Sandy loam	June 2009	NM	85.12	5 days	Baily *et al.* (2012)
Ireland	Sandy loam	Sep 2009	NM	93.22	5 days	Baily *et al.* (2012)
Ireland	Sandy loam	March 2010	NM	13.22	5 days	Baily *et al.* (2012)
UK	Silty clay and Silty clay loam	Grazing grasslands	0.01	NM	8 hours	Sgouridis *et al.* (2011)
UK	Silty clay and Silty clay loam	Fritillary Meadow	0.05	NM	8 hours	Sgouridis *et al.* (2011)
UK	Silty clay and Silty clay loam	Buffer Zone	0.006	NM	8 hours	Sgouridis *et al.* (2011)
UK	Silty clay and Silty clay loam	Hay Meadow	0.005	NM	8 hours	Sgouridis *et al.* (2011)
USA		SW (112 kg N ha^{-1})	NM	0.6 to 1.7 µg N_2O-N m^{-2} h^{-1}	2 years	Sullivan *et al.* (2005)
USA		AN (112 kg N ha^{-1})	NM	0.3 to 1.5 µg N_2O-N m^{-2} h^{-1}	2 years	Sullivan *et al.* (2005)

NM = not measured in the study; SW = Swine waste; AN = Ammonium nitrate

Table 2.1 Continued

Country	Soil Texture	Condition of measurement	DEA (mg N_2O-N kg^{-1} soil h^{-1})	DR (µg N_2O-N kg^{-1} soil h^{-1})	Length of study	Reference
Canada	Sandy loam	Glucose addition	NM	14.2	24 hours	Murray *et al.* (2004)
UK	Coarse sandy loam	Box incubation	NM	1.0	48 hours	Jarvis *et al.* (2001)
UK	Coarse sandy loam	Jar incubation	NM	7.9	48 hours	Jarvis *et al.* (2001)
UK	Sandy loam	Grass	0.08	3.36	8 months	Šimek *et al.* (2004)
UK	Sandy loam	Clover	0.09	2.50	8 months	Šimek *et al.* (2004)
China	Sandy loam	GI (0 to 5.33 sheep h^{-1})	NM	0.14	2 years	Xu *et al.* (2008)
Denmark*	Loamy sand	AS (52.9 g N m^{-2})	NM	1.1 µg N_2O-N m^{-2} h^{-1}	4 days	Carter (2007)
Denmark*	Loamy sand	AU (52.9 g N m^{-2})	NM	21.0 µg N_2O-N m^{-2} h^{-1}	4 days	Carter (2007)
France	Sandy	GI: light to intensive	0.65	NM	24 hours	Patra *et al.* (2005)
New Zealand	Silt loam	Control site	NM	0.87		Luo *et al.* (2000)
New Zealand	Silt loam	Grazed site	NM	1.75		Luo *et al.* (2000)
New Zealand	Silt loam	Control	0.311	1.99	1 year	Watkins *et al.* (2013)
New Zealand	Silt loam	DCD (33 kg ha^{-1} yr^{-1})	0.368	3.99	1 year	Watkins *et al.* (2013)

*Use of ^{15}N technique; GI = Grazing intensity; AS = Ammonium solution; AU = Artificial urine; KN = Potassium nitrate

Table 2.1 Continued

Country	Soil Texture	Condition of measurement	DEA (mg N_2O-N kg^{-1} soil h^{-1})	Cumulative Denitrification (mg N_2O-N kg^{-1} soil)	Length of study	Reference
New Zealand	Silt loam	Control	0.83	0.2	56 days	Zaman & Nguyen (2010)
		CU (400 kg N ha^{-1})	0.83	9.1	56 days	(Zaman & Nguyen, 2010)
		KN (400 kg N ha^{-1})	0.83	4.2	56 days	(Zaman & Nguyen, 2010)
New Zealand	Silt loam	Control	0.78	5.88	28 days	Zaman *et al.* (2008c)
		KN (200 kg N ha^{-1})	0.78	16.99	28 days	Zaman *et al.* (2008c)
		KN+Lime	0.78	19.16	28 days	Zaman *et al.* (2008c)
		KN+Zeolite	0.78	18.39	28 days	Zaman *et al.* (2008c)
Belgium	Clay	Incubation duration	0.66	NM	4 days	D'Haene *et al.* (2003)
		Incubation duration	0.52	NM	7 days	D'Haene *et al.* (2003)

KN = Potassium nitrate

2.4 METHODS OF MEASURING DENITRIFICATION

Denitrification in soils depends on the outcomes of complex interactions between soil properties, soil micro-organisms, climatic factors, and management practices. There are various ways of tracing denitrification in a system, such as measuring the gaseous emissions, tracking the appearance and disappearance of chemical substances, and targeting the organisms or genes involved in various transformations during denitrification. Prediction and estimation of DRs and N_2O emissions from agricultural soils are challenging. These limitations include restrictions and difficulties in quantifying the dominant end product N_2 against its high ambient concentration in the atmosphere, problems in tracking high spatial and temporal variability of the DRs, and the complex interactions of the factors driving the process (Braker *et al.*, 2010).

As a consequence of the problems associated with obtaining accurate measurements of denitrification, the actual amount of N loss by denitrification is often lacking in N cycling studies. Therefore, although measurements of denitrification and N-losses have continued for many decades, there is still scope for development of more accurate and precise techniques to measure denitrification under both field and laboratory conditions (Aulakh *et al.*, 1991a; Groffman *et al.*, 2006). This section of the review discusses the most widely used techniques and a selection of the most promising new methods that are currently available to measure and calculate denitrification in wide variety of ecosystem. The advantages and disadvantages of using various available methods to measure denitrification are listed in our review paper (Saggar *et al.*, 2013).

2.4.1 Acetylene inhibition technique (AIT)

The most commonly used method for estimating DR and DEA from environmental samples involves measuring the production of N_2O in the presence of acetylene (C_2H_2), also known as the AIT. The presence of C_2H_2 gas in the soil atmosphere (preferably > 1 ml L^{-1}) inhibits N_2O reduction to N_2 (Fedorova *et al.*, 1973) and makes the easily detected N_2O the sole product of denitrification (Aulakh *et al.*, 1991a; Balderston *et al.*, 1976; Yoshinari & Knowles, 1976). Soil samples are incubated in the presence and absence of C_2H_2, and the difference in N_2O emitted

during the incubation is used to calculate the amount of N_2 produced and the product ratio N_2O/ N_2 or N_2O molar ratio [N_2O/ (N_2O+ N_2)] during denitrification.

AIT is the most commonly used and affordable method of measuring denitrification. However, concerns have been raised regarding this method. The various issues that demonstrated the underestimation of denitrification by using AIT (Felber *et al.*, 2012) are:

- under prolonged incubations denitrifiers could adapt and use C_2H_2 as a source of C resulting in over-estimation of DR (Barton *et al.*, 1999; Seitzinger *et al.*, 1993; Tiedje *et al.*, 1989)
- Mahmood *et al.* (1999) pointed that the N_2O produced from the soil cores during prolonged incubations becomes increasingly trapped within the soil cores, which is difficult to measure
- the addition of acetone as a stabilizer in C_2H_2 cylinders may act as additional source of C and could also lead to the over-estimation of DR (Tiedje, 1988). On the other hand, the incomplete purification of C_2H_2 and the presence of acetone in cylinders can affect microbial activity and the DR (Aulakh & Doran, 1990)
- under aerobic conditions C_2H_2 may also catalyse oxidation of NO, which is a precursor of N_2O, and may lead to underestimation of denitrification (Bollmann & Conrad, 1997)
- the C_2H_2 present in the incubation system may at times suppress the overall microbial respiration (Zhang *et al.*, 2009). However, past studies have suggested C_2H_2 does not affect microbial respiration (Aulakh *et al.*, 1991a)
- using a low concentration of C_2H_2 (1 %) can also block nitrification by many nitrifiers, thus eliminating NO_3^- from the system and reducing the natural substrate for denitrification (Mosier, 1980; Seitzinger *et al.*, 1993)
- the incomplete diffusion of C_2H_2 into the soil cores may lead to incomplete inhibition of N_2O reduction, C_2H_2 also inhibits nitrification, and
- a narrow time-frame during which N_2O production and consumption can be monitored before the C_2H_2 runs out of action (Groffman *et al.*, 2006; Ostrom & Ostrom, 2012).

A large number of reviews have been published describing improvements to the method itself and identifying its limitations (Aulakh *et al.*, 1992; Felber *et al.*, 2012; Groffman *et al.*, 1999; 2006; Nieder *et al.*, 1989; Payne, 1991; Qin *et al.*, 2013; 2014; Revsbech & Sorensen, 1990;

Tiedje *et al.*, 1982; 1989; von Rheinbaben, 1990). Numerous modifications have been made over the years, particularly in late 1970s and 1980s when a large number of research projects were undertaken to the original method proposed by (Ryden *et al.*, 1979a; 1979b). These modifications include:

- use of CaC_2 granules (coated and uncoated) to generate C_2H_2 gas within soil eliminating the inconvenience of using welding grade C_2H_2 gas cylinders (Aulakh *et al.*, 1991a)
- scrubbing welding grade C_2H_2 through sulphuric acid to remove mixed impurities (Aulakh & Doran, 1990)
- use of gas recirculation method which involves amending the atmosphere of soil cores with C_2H_2, and at the same time controlling the level of oxygen (Parkin *et al.*, 1984; Robertson & Tiedje, 1984; Sexstone *et al.*, 1985).
- use of "static cores" (usually intact) collected from the field and placed in jars for incubation. Use of un-perforated and perforated PVC tubings to hold the soil cores intact during the incubation (Groffman *et al.*, 1999; Ryden & Skinner, 1987; Sorensen *et al.*, 2006; Tiedje *et al.*, 1989)
- addition of C_2H_2 to soil cores in a sealed atmosphere and incubation for N_2O measurement for up to 24 hours (Groffman *et al.*, 1999; Ryden & Skinner, 1987; Tiedje *et al.*, 1989)
- addition of C_2H_2 in the chambers in field instead of laboratory incubation, developed by John Ryden and colleagues (Ryden *et al.*, 1979a; 1979b; Ryden & Dawson, 1982) to overcome some of the variability inherent in soil studies.

AIT has also been used to measure denitrification enzyme activity (DEA) during which soil samples supplied with excess moisture, C and NO_3^- together with chloramphenicol are incubated in the presence of C_2H_2 in anaerobic conditions (N_2 atmosphere) (Tiedje *et al.*, 1989). The N_2O emitted gives an estimate of potential denitrification of soil and the ambient reductase enzyme activity. Thus DEA is a traditional and widely used method (Čuhel & Šimek, 2011; Firestone & Tiedje, 1979; Luo *et al.*, 1998; Martin *et al.*, 1988; Murray *et al.*, 1995; Parsons *et al.*, 1991; Tiedje *et al.*, 1989) to determine potential denitrification in soils.

Various studies have measured DEA or potential denitrification rate in soils or sediments using AIT (Cavigelli & Robertson, 2000; Hénault *et al.*, 1998; Rich & Myrold, 2004). This

potential denitrification rate measured under optimal condition is regarded as the maximum rate of the microbial reduction of N_2O or N_2OR activity in soils (Burns *et al.*, 2013; German *et al.*, 2011; Wallenstein & Weintraub, 2008). Inconsistencies in the duration of the incubation of samples and in the amount of chloramphenicol used for inhibition of *de novo* enzyme synthesis in DEA assay by various researchers, poses concerns about the optimum amount of chloramphenicol required to completely inhibit the *de novo* enzyme synthesis without affecting the existing enzyme activity. Improvement in the existing methodology for measuring denitrification is therefore needed both for precise measurement and to improve our understanding of this complex biological process.

Recent studies have suggested the inhibition of soil N_2OR activity by C_2H_2 during DEA measurement is incomplete (Yu *et al.*, 2010). Qin *et al.* (2013) have reported N_2 production in C_2H_2-treated soils in addition to N_2 production in non-C_2H_2 treated soils, indicating incomplete inhibition of N_2OR activity by AIT (11.2 % of produced N_2O was reduced to N_2 in the presence of C_2H_2). Qin *et al.* (2014) have suggested using direct N_2 quantification techniques to measure DEA.

AIT has also been applied in studies that have led to better understanding of the spatial and temporal variability of denitrification (Groffman *et al.*, 1999). Despite previously mentioned limitations, AIT is easy and relatively inexpensive, and sometimes the only available method to measure denitrification. In systems with high NO_3^- concentrations such as grazed grassland it is one of the best methods to measure denitrification.

Only a few studies compare AIT and ^{15}N labelled in parallel field trials. (Rolston *et al.*, 1982) reported good correspondence between both N_2O and N_2 fluxes measured with AIT and isotope labelling technique. In the absence of any other useful alternative, AIT will continue to be widely used in the foreseeable future (Groffman *et al.*, 2006). The method should, however, be standardised before being applied to assess the spatial variability and differences in DRs across the sampling areas.

2.4.2 Isotope tracer technique

The labelled isotope, ^{15}N is the basis of a number of methods, including isotope fractionation, isotope dilution, and determining a ^{15}N mass balance, to be used to measure denitrification in soil, sediments, and water. The ^{15}N tracer technique is the most frequently used method for direct estimation of the ^{15}N labelled gas that is generated following addition of $^{15}NO_3^-$ and $^{15}NH_4^+$ to intact soil cores, sediments, and ground and surface water (Hauck & Melsted, 1956).

Earlier quantification of denitrification was determined by addition of $^{15}NO_3^-$ to the soil and then measuring the production of ^{15}N labelled N_2 (Hauck & Melsted, 1956). However it was recognized that the addition of $^{15}NO_3^-$ alone would not detect denitrification that occurred as a result of the nitrification–denitrification coupled reaction and might lead to underestimation of the process. To overcome this underestimation in subsequent incubations both $^{15}NO_3^-$ and $^{15}NH_4^+$ are added simultaneously to the soil under investigation (Hauck *et al.*, 1958; Nishio *et al.*, 1983). Although, small additions of labelled NH_4^+ and NO_3^- do not alter the coupled nitrification/denitrification processes in fertilised agricultural soils, the ^{15}N method may cause over-estimation of denitrification in N-limited agricultural systems.

During this technique the simultaneous analysis of $^{15}N_2$ and $^{15}N_2O$ is critical to distinguish the relative contribution of different processes to complex N transformations and N oxide emissions (Hsu & Kao, 2013). Several soil studies have successfully quantified both $^{15}N_2$ and $^{15}N_2O$ from the system (Bergsma *et al.*, 2001; Stevens *et al.*, 1993). The analysis of ^{15}N in soils and $^{15}N_2O$ and $^{15}N_2$ gases produced during denitrification by isotope-ratio mass spectrometry (IRMS) can be fully automated (Stevens *et al.*, 1993). Thus the fluxes of N_2 and N_2O can be measured simultaneously in laboratory and field experiments by using the ^{15}N stable isotope labelling method.

Recent developments in engineering technologies based on the principle of laser cavity ring down spectroscopy (CRDS) have enabled field-deployable instruments to measure N_2O isotopic ratios (Crosson *et al.*, 2011). This technique would be helpful in measuring the isotopic abundance of labelled $^{14}N^{15}N^{16}O$ and $^{15}N^{14}N^{16}O$ relative to $^{14}N^{14}N^{16}O$ in N_2O. Using the labelled substrates and isotopic assembly of terminal and central nitrogen atom will make it possible to distinguish the N_2O formed via the hydroxylamine oxidation pathway and that of the NO_3^-

reduction and would be equally effective in distinguishing between fungal and bacterial N_2O production.

When compared with the AIT, the ^{15}N labelling method is considered to be better for direct quantification of denitrification. However, its application is often limited because of the high cost of ^{15}N-substrates and ^{15}N analyses, the expensive IRMS required, and the laborious procedures involved in sample preparation. For these reasons relatively few researchers having IRMS facilities have used the ^{15}N tracer technique rather than AIT to investigate denitrification. Overall, the ^{15}N tracer technique had been useful in generating reliable estimates of denitrification rate in a range of environments. Use of this technique in conjunction with C_2H_2 assay can provide improved estimates of denitrification rates in soils.

Another method of determining denitrification uses stable isotopes of five elements: hydrogen, oxygen, nitrogen, carbon, and sulphur (H,O,N,C,S). When these stable isotopes are used simultaneously it is possible to trace all the reactions and measure the various N oxides that are formed during the entire process of denitrification. The stable isotope ratio is expressed in delta notation "δ" and denitrification can be estimated from the measured correlation between $\delta^{15}N$ and $\delta^{18}O$. Studies of stable isotopes can provide both source-sink (tracer) and process information in ecosystems (Peterson & Fry, 1987). In a study of denitrification in ground water (Aravena & Robertson, 1998), $\delta^{15}N$ and $\delta^{18}O$ were used to provide information on the role of denitrification in attenuating nitrate, and $\delta^{34}S$, $\delta^{18}O$, and $\delta^{13}C$ were used both to provide information on the role of reduced sulphur and carbon as electron donors for nitrate reduction and to demonstrate the dominance of carbon as electron donor.

2.4.3 Direct N_2 quantification

Measurement of N_2 emitted directly from soil is a major challenge, since it is difficult to quantify small changes in N_2 concentrations against the high natural background atmospheric concentration. For directly measuring N_2, intact soil cores or sediments are kept in air-tight systems with the air inside replaced by an inert gas such as He or Ar, and the background N_2 concentration decreased from 79 % N_2 to approximately 1 % N_2 (Seitzinger *et al.*, 1980; Stefanson & Greenland, 1970; Wickramasinghe *et al.*, 1978). The N_2 produced during denitrification within the air-tight container is then analysed by gas chromatography.

In general, two types of incubation systems are used for direct N_2 measurements: i) continuous air flow with He + O_2 but no N_2, and changes in N_2 gas concentration in the outlet used for calculating N_2 emission (Butterbach-Bahl *et al.*, 2002; Cárdenas *et al.*, 2003); ii) a closed system with the changes in the composition of gases in the headspace measured by periodically sampling small amounts of gases and replacing the sampled volume with He (Liu *et al.*, 2010). In both systems it is necessary to remove all N_2 present in the test soil aggregates or cores before the measurements begin. The continuous flow incubation system has been improved by the development of a technique to measure N_2 and N_2O emissions simultaneously (Butterbach-Bahl *et al.*, 2002; Cárdenas *et al.*, 2003; Khalil & Richards, 2011; Scholefield *et al.*, 1997).

A major advantage of this method is that no addition of substrate (labelled or otherwise) or inhibitor is required. Thus, there is no interference with soil microbial processes, including those involved in denitrification, during the measurements. However, the incubation system used is complex. Since it is not possible to measure very small quantities of N_2 gas, this method is not able to detect small changes in denitrification products, and the method only appears to be useful for measuring denitrification in highly fertilised agricultural and grassland soils (Scholefield *et al.*, 1997). However, in spite of its limitations this method is increasingly being used for quantification of N_2 emissions in both aquatic and terrestrial ecosystems (Butterbach-Bahl *et al.*, 2002). This method has also been used to measure O_2, NO, N_2O, and N_2 simultaneously from incubated pure cultures of bacteria such as *Agrobacterium tumefaciens* and *Pseudomonas denitrificans* by Molstad *et al.* (2007). Due to easy measurements of these gases in these incubations it has become easier to trace the activities of denitrifying bacteria real time without using expensive molecular techniques.

2.4.4 Mass balance approaches

The mass balance technique has for some time been used for estimating N fluxes emitted at field scale (Allison, 1955) and more recently at stream reach, watershed, and regional scales (David & Gentry, 2000; Pribyl *et al.*, 2005). In this method denitrification is computed mathematically, with the difference between N inputs and outputs being the estimate of denitrification. The underlying assumption is that the ecosystem in which denitrification is being estimated is a

closed system so that only inputs and outputs need to be considered. With the known amount of material entering and leaving a system, mass flows can be identified that might otherwise have been unknown or difficult to measure. In terrestrial systems the major inputs are application of N fertilizer, biological N_2 fixation, atmospheric N deposition, animal excretion, and organic matter deposition. The outputs are crop or biomass harvest and export, animal consumption, leaching, and runoff N losses. A mass balance calculated at field scale, however, is unlikely to include small denitrification changes and, more seriously, it cannot describe the spatial and temporal dynamics of denitrification (McIsaac & Hu, 2004). Overall, this technique has a limited ability to provide accurate estimates of denitrification rate, especially when a large range of inputs is contributing to denitrification.

In this study AIT was used for measurement of denitrification and denitrification enzyme activity in various soils.

2.5 MOLECULAR APPROACHES IN UNDERSTANDING DENITRIFICATION

Traditional methods of measuring DRs in soils are based on the biochemical transformation of substrates, measured through substrate depletion or product formation over time. These techniques provide insight to the biogeochemical changes during denitrification in soils, but do not provide a view of the microbial contribution to these biochemical transformations (Sharma *et al.*, 2006). Agricultural activities such as cropping, fertilization, organic waste deposition, and pesticide application may lead to changes in denitrifier community structure and denitrifier abundance in soils. The soil microbial communities are characterised according to their phenotypic and genetic diversity and the methods to measure microbial diversity in soils have been categorised into two groups: biochemical techniques and molecular techniques. In the past, denitrifier community has been characterised by using most probable number (MPN) count (Lensi *et al.*, 1995) and DEA assay (Martin *et al.*, 1988). Most recently, molecular techniques have been used to examine denitrifier community structure, abundance, and activity.

Before the development of molecular techniques, it was difficult to relate microbial community structure to soil processes due to our inability to identify unculturable organisms (Forney *et al.*, 2004), which may comprise up to 99 % of the total microbial population in soils (Amann *et al.*, 1995). This has limited our knowledge of the microbial ecology of soils. The

development of molecular techniques have enabled the monitoring of microbial genes directly from environmental samples (Olsen *et al.*, 1986). For example, Saleh-Lakha *et al.* (2009a) and Saleh-Lakha *et al.* (2009b) studied the effect of pH, temperature, and nitrate availability on *nir*S, *cnor*B, and *nos*Z expression and denitrification activity of *Pseudomonas mandeli* and Chen *et al.* (2012b) studied the long-term effect of fertilization on the *nir*K microbial community.

2.5.1 Soil DNA Extractions

The first step in applying the molecular techniques to study soil denitrifiers is to extract DNA from the soil samples. Since their origin, there have been developments and improvements in DNA extractions and, apart from some non-kit-based protocols, almost all DNA extractions are currently performed using commercially available kits (Ínceošlu *et al.*, 2010; van Elsas & Boersma, 2011). In both the non-kit and commercial kit protocols, DNA extraction consists of microbial cell lysis, and nucleic acid purification. The first step in DNA extraction involves cell lysis (Roose-Amsaleg *et al.*, 2001; Trevors, 1992), which is accomplished through chemical or enzymatic disruption of cell membranes, mechanical lysis using freeze-thaw cycles (Kuske *et al.*, 1998; Luna *et al.*, 2006), bead-beating, freeze-grinding in liquid nitrogen, or sonication (Robe *et al.*, 2003).Various reviews analyse the advantages and disadvantages of these techniques for DNA extraction (Gabor *et al.*, 2003; Leff *et al.*, 1995; More *et al.*, 1994; Robe *et al.*, 2003). Soil contains a mixture of phenolic compounds, which makes DNA extraction from soil difficult compared with DNA extraction from pure microorganism cultures (Saano *et al.*, 1995). As these organic compounds in soil inhibit the polymerase chain reaction (PCR) amplification of target DNA, the extracted DNA from soil samples should be purified by removing inhibitory compounds (Zhou *et al.*, 1996).

The detailed DNA extraction method includes placing a soil sample in a tube containing glass beads and subjecting the sample to rigorous shaking in an attempt to break the cells. The sample is further subjected to chemical cell lysis to release all the cell content efficiently into solution. After lysis sample undergoes series of extractions to remove PCR inhibitory compounds. Finally, the extract is cleaned, the extracted genetic material is concentrated, and the resulting extract is then used for further molecular analysis.

Following an appropriate DNA extraction procedure is also critical for obtaining a high quality representative DNA sample of the microbial community present in soil (Carrigg *et al.*, 2007). Studies evaluating different extraction protocols mostly focussed on optimising the maximum total DNA yield, and DNA quality (Bürgmann *et al.*, 2001; Kuske *et al.*, 1998; Miller *et al.*, 1999). In a comparative study of various DNA extraction procedures and the use of commercially available kit (DNA Isolation Kit MoBio, Carlsbad, CA, USA) Maarit Niemi *et al.* (2001) have shown that the yield and amplification of DNA using the commercial kit was better than with the other DNA extraction protocols available.

2.5.2 PCR dependent techniques

A denitrifying ability is one of the unique properties of microorganisms. Denitrifying organisms do not belong to a specific taxonomic group and thus total bacterial phylogeny-based approaches are not suitable to study denitrifiers. Therefore, the existing techniques to study the ecology of denitrifier community are based on functional genes (those are responsible for N transformations during the process) or their transcripts as molecular markers to trace this process (Hallin *et al.*, 2007; Philippot, 2006; Philippot & Hallin, 2006).

Most culture-independent techniques used to study denitrifiers are based on PCR (Mullis & Faloona, 1987). PCR, comprises cyclic reactions under controlled temperature conditions that lead to the extremely efficient and sensitive amplification of a specific gene region of target DNA (van Elsas & Boersma, 2011). Target DNA is amplified using two primers (reverse and forward) either universal (e.g. 16S rRNA) or specific (e.g. *nir*S, *nir*K, *nos*Z in this study) containing sequences complementary to the target region that anneal to opposite ends of the template. DNA polymerase binds to the primer sites and transcribes to the target gene. Repeated temperature cyclings lead to exponential amplification of the target region of DNA. The amplification generates a mixed pool of amplicon when degenerate primers are used or when the target gene is polymorphic, which reflects the composition of the target genes in the studied sample (Philippot & Hallin, 2006).

PCR is subject to various biases such as DNA extraction procedures, primer selection, and PCR conditions. The choice of appropriate DNA extraction method will determine the quality and quantity of the nucleic acid used for further amplification. Inefficient lysis of cells in

soil samples or improper extraction of DNA will not be representative of the total microbial community present in the sample. Failing to carry out a proper clean up or to remove all the inhibitory compounds will lead to improper or no PCR amplification of the target DNA. Appropriate primer design is crucial for achieving a specific product in order to amplify a wider range to cover as many versions of gene as possible. Appropriate selection of PCR condition, such as annealing temperature, elongation temperature, length of each PCR cycle and number of PCR cycles, is essential for efficient amplification of the target DNA.

Some of the PCR-based community profiling techniques generally used to study denitrifier community structure are described below.

2.5.2.1 Determination of denitrifier community structure

Denaturation/ temperature gradient gel electrophoresis (DGGE and TGGE):

DGGE and TGGE methods were originally developed to detect point mutation in the DNA sequences; however, these have now been adapted to study microbial ecology (Muyzer *et al.*, 1993; 1999). TGGE methods are not as popular as DGGE, which have been extensively used to study denitrifier community diversity in environmental samples (Throbäck *et al.*, 2004). After PCR amplification, DGGE separates gene fragments of same size on the polyacrylamide gel with a gradient of increasing concentration of the denaturants urea and formamide. Due to its convenience as a rapid, reproducible, and inexpensive method, DGGE allows the analysis of a large number of samples, making it possible to follow changes in the denitrifier communities over time or in response to treatments. DGGE bands can be excised, purified, PCR-amplified, and sequenced so that sequencing of clones can be reduced.

Restriction Fragment Length Polymorphism (RFLP)

This method relies on DNA polymorphism to study denitrifier diversity, also known as amplified ribosomal DNA restriction analysis (ARDRA). In this method PCR-amplified DNA is digested with 4-base pair cutting restriction enzyme (Liu *et al.*, 1997). The choice of restriction enzyme is crucial for success of the analysis. After the fragments are cut the fragment lengths are detected using agarose or non-denaturing gradient polyacrylamide gels electrophoresis for community analysis (Tiedje *et al.*, 1999). This method is useful for detecting structural changes in microbial

communities but not as a measure of diversity or detection of specific phylogenetic groups (Liu *et al.*, 1997). Since a single species may have 4–6 restriction fragments, it becomes too complex to analyse species in the banding pattern of diverse communities (Tiedje *et al.*, 1999).

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

This method follows the same principal as RFLP, except for the incorporation of a fluorescently labelled PCR primer on the 5' end with a fluorescent dye such as Tetrachlorofluorescein (TET) or *6-carboxyfluorescein (*6 FAM). The mixture of amplified genes, known as amplicons, is then subjected to a restriction reaction, in which an enzyme is used to cut the amplicons at a specific recognition sequence. This sequence results in a mixture of gene fragments that can be separated based on their length and thus produce a "fingerprint" of the community composition. The fingerprint can be statistically analysed to produce metrics of the community structure. Individual bands or peaks are considered "operational taxonomic units" (OTUs), and do not necessarily correspond to a single species. T-RFLP has been widely used to study denitrifier community composition in soils (Braker *et al.*, 2001; Rich & Myrold, 2004; Rösch & Bothe, 2005). T-RFLP allows for rapid analysis of the community structure and diversity of functional genes like *nir*S, *nir*K and *nos*Z in different samples.

2.5.2.1 Determination of denitrifier gene abundance

The abundance of denitrifier genes can be measured using immunological and microarray techniques, but very accurate estimates of gene abundance can be achieved through PCR. In quantitative (or real-time) PCR, gene abundance is measured based on the detection of fluorescence signals corresponding to the synthesis of PCR amplicons (Heid *et al.*, 1996) allows for accurate estimation of number of copies of PCR amplicons by extrapolation of the amplicon accumulation curve (Yoshida *et al.*, 2009). The number of copies of the target DNA is determined by comparison with a standard curve prepared using DNA of a sample of known concentration (Yoshida *et al.*, 2009). The two types of commonly used PCR for quantification of denitrifier genes are competitive PCR (cPCR) and quantitative PCR (qPCR).

The cPCR is based on simultaneous amplification of the target DNA and a known concentration of control DNA known as competitor. The competitor molecule must have the

same priming sites but a different size from the target gene, making a co-amplification possible during PCR and detection of target DNA by electrophoresis (Sharma *et al.*, 2007). The calculation of the amplification of target DNA is based on the ratio of target to competitor PCR product by agarose gel electrophoresis. cPCR has been successfully used to quantify *nir*S and *nir*K genes in marine, stream sediments and biofilm samples but not been tested for soil (Cole *et al.*, 2004; Michotey *et al.*, 2000; Qiu *et al.*, 2004).

The key feature of qPCR is that the amplified DNA is detected as the reaction progresses in real time. The quantification at the exponential phase of the PCR when the efficiency is recognised to be the highest is one of the greatest advantages of the qPCR compared with cPCR. Despite numerous alternative probes for qPCR, the most commonly used detectors are non-specific intercalating dyes (e.g. Syber Green) (Giglio *et al.*, 2003). The dye used in PCR binds extensively to double stranded DNA (dsDNA) causing fluorescence of the dye. The increase in the formation of dsDNA product during PCR increases the fluorescence of dye and thus the quantification of the DNA content. However, the dye used in PCR may also bind non-specific PCR product including primer-dimers, therefore analysis of the dissociation curve of the samples known as melting curve analysis of the samples must be performed after the PCR amplification (Sharma *et al.*, 2007). qPCR has been used extensively to study the influence of soil environmental factors such as O_2 status, pH, temperature, nutrient availability on denitrifier gene abundance (López-Gutiérrez *et al.*, 2004; Miller *et al.*, 2008; Philippot, 2005; 2006; Philippot & Hallin, 2006; Philippot *et al.*, 2009).

2.5.3 PCR independent techniques

2.5.3.1 Fluorescent in situ *hybridization (FISH)*

FISH allows visualization of the collective microbial community so that phylogeny, morphology, localization, and abundance can be measured. Nucleic acid hybridization using Fluroscent probes binds to the region of complimentary sequence in the chromosome of extracted DNA and RNA or pure cultures cells. FISH utilizes oligonucleotide probes specific to the 16S rRNA, with the degree of conservation of the probe target sequence governing the level of taxonomic depth that

can be discerned by the probe (Amann *et al.*, 1995). Alternatively, functional genes can be targeted, allowing visualization of all cells contributing to a function of interest. The FISH and its variants have been successfully used to study denitrifier communities in environmental samples (Ginige *et al.*, 2004; Zhang & Chen, 2011). A different approach to obtain broader insight into the phylogenetic affiliation of members of denitrifier communities in the environment is to use a special variant of fluorescence in situ hybridization (FISH) called recognition of individual genes (RING)-FISH and to combine it with subsequent cell sorting (Pratscher *et al.*, 2009). Compared with the results obtained with conventional FISH using singly labelled oligonucleotide probes, the use of multiple-labelled probes increased the intensity of the fluorescence signal and thus the sensitivity of RING-FISH between 10- and 50-fold. The disadvantage of using the FISH technique is a lack of sensitivity unless sequences are present in high copy numbers (10^3–10^4 cells per ml) (Pernthaler *et al.*, 2002). Due to high secondary and tertiary structure or binding of ribosomal proteins, the target sites of rRNA might not be accessible to the probe, thus leading to low detection of cells (Fuchs *et al.*, 1998). The FISH method does not allow detection of cells with low ribosome contents, therefore slow-growing or starving cells may not be detected by this method (Amann *et al.*, 1995).

2.5.3.2 Immunological assays

This technique, which allows access to the active denitrifier community, involves targeting proteins instead of nucleic acids. Antibodies have been used to identify isolated denitrifiers: both NIR enzymes (*Nir*K and *Nir*S) were targeted by Coyne *et al.* (1989) and Ward *et al.* (1993), but the technique has rarely been applied to environmental samples (Philippot & Hallin, 2006). In order to study active denitrifiers possessing *nir*S genes, Metz *et al.* (2003) designed an antibody specific for Cu NIR. Using flow cytometry, the antibody-labelled cells are sorted and phylogenetic affiliations determined using 16S rRNA oligonucleotide probes. Although the presence of reductase enzymes in the environmental samples indicates the corresponding activity, the intensity of the activity might vary among the different species of denitrifier and also among the conditions in which they are active. The lack of information about stability of enzymes after the disappearance of its substrate might lead to overestimation about the activity of the denitrifiers present in the system (Correa-Galeote *et al.*, 2013).

2.5.3.3 Nucleic acid hybridization arrays

Hybridization arrays (microarray, beadarray, and phylochips) utilize specifically designed probes attached to a solid surface that fluoresces when bound to target DNA.The target DNA are fluorescently dyed and complementary to the probes. The labelled DNA originating from the sample can then be assigned a phylogeny or function based on the information known about the original probes. Arrays rely on oligonucleotide probes of 16S rRNA from specific groups of organisms to discern phylogeny (Sagaram *et al.*, 2009; Sessitsch *et al.*, 2006) and as such can define common composition or function. Microarray-based, whole-genome hybridization has been used to identify microorganisms in environmental samples (Wu *et al.*, 2004). DNA microarray is one of the latest technologies to detect denitrifier communities in soils using the well-established DNA-DNA hybridisation principle. For denitrifying bacteria, oligomer microarrays of different molecular sizes have been developed for assessing *nar*G, *nir*K, *nir*S and *nos*Z gene distribution and diversity (Cho & Tiedje, 2002; Mergel *et al.*, 2001b; Taroncher-Oldenburg *et al.*, 2003; Wu *et al.*, 2001). However, due to the heavy cost of analysis and the advent of low costing next generation sequencing, researchers are now losing interest in this technique.

It is difficult to suggest one best technique to study denitrifier community structure or diversity. In order to obtain the broadest picture and the most information the most useful technique would be to use a variety of tests with different endpoints and degrees of resolution.

2.5.4 Sequencing

The introduction of high throughput sequencing technologies has allowed more environmental samples to be analysed to a greater depth, revealing much higher microbial diversity than previously possible and generating robust inferences between environments. DNA sequencing is used to study the composition of entire microbial communities without isolating or cultivating individual organisms. Earlier Sanger sequencings (Sanger *et al.*, 1977) of 16S rRNA gene libraries were made by cloning 16S rRNA amplicons into E. coli (Eckburg *et al.*, 2005). In 2005 a breakthrough in DNA sequencing led to the so-called “next generation” of sequencing. With

this latest technology large communities can be studied based on phylogeny or their function. The platforms most commonly applied in environmental microbiology studies include 454 pyrosequencing (Roche Applied Science, Basel, Switzerland), Illumina/Solexa Genome Analyzer (Illumina, San Diego, CA, USA) SOLiD (Applied Biosystems, Foster City, CA, USA), the HeliScope Single Molecule Sequencer (Helicos BioSciences, Cambridge, MA, USA), and the Single Molecule Real Time technology (SMRT, Pacific Biosciences, Menlo Park, CA, USA).

High throughput sequencing has revealed a much higher microbial diversity than previously understood (Benson *et al.*, 2010; Dowd *et al.*, 2008; Sogin *et al.*, 2006; Sul *et al.*, 2011). A commonly used approach to characterize microbial communities is 16S rRNA gene sequencing (amplicon sequencing); another approach, "Genomics", uses whole genome sequencing (shotgun sequencing, high throughput sequencing) to characterise the entire genome of bacteria in an environmental sample. These techniques are laborious in terms of laboratory and computational work, especially when dealing with large number of samples

The ongoing advancement in molecular techniques such as genome sequencing and metagenomic projects will provide new denitrification gene sequences, which would, as recently demonstrated, aid in designing new primers (Jones *et al.*, 2013).

In this study DNA was extracted from soils using PowerSoil® DNA Isolation Kit MoBio, Carlsbad, CA, USA. T-RFLP was used to determine denitrifier community structure using *nir*S, *nir*K and *nos*Z genes. Measurement of genes abundance (*rpo*B, *nir*S, *nir*K and *nos*Z) was done using qPCR.

2.6 MITIGATION TECHNIQUES TO REDUCE DENITRIFICATION

Nitrogen transformation processes are inevitable in nature, but despite following the best management practices, it is hard to eliminate all the N_2O production from denitrification in soils or sediments. Some mitigation options that have been proposed to target denitrification include the application of Cu fertilizer, SOM management, and the application of lime to regulate pH and enhance the efficiency of N_2OR enzyme. A more efficient way to mitigate N_2O is proposed to be strategies targeting both nitrification and denitrification simultaneously, preferably by controlling the amount of reactive N released in soil (Baggs, 2011). Some of the possible

mitigation techniques to reduce denitrification or N_2O production through denitrification are discussed in this section of review chapter.

2.6.1 Managing N application and transformations on farm

The traditional methods for reducing N_2O emissions include restricting the application of inorganic N fertilizers on farms or increasing the N-use efficiency of the applied fertilizer. The N-use efficiency has been enhanced by performing the following practices.

A major source of anthropogenic N_2O emission is the use of nitrogen fertilizers in agriculture. As a substantial proportion of applied fertilizers are emitted in the form of N_2O, better targeted fertilizer applications, which reduce the availability of nitrogen to microorganisms, can substantially decrease N_2O emissions. Possible strategies should include reducing the amount of fertilizer and applying it at the cropping time when demand for N is highest and preferably in dry conditions when leaching losses would be minimal. Using slow-release fertilizers and avoiding N in NO_3^- form, which is likely to produce large emissions through denitrification especially under high rainfall condition, would also be useful. Similarly, improved land drainage and better management practices to limit anaerobic conditions in soils (for example, land compaction and excessive wetness) could reduce denitrification rates and, thus, N_2O emissions.

The use of NH_4^+ based fertilizer rather than NO_3^- under mild, moist conditions to restrict the supply of NO_3^- for denitrification could lower N_2O emission from denitrification. The use of nitrification inhibitor to slow down the oxidation of NH_4^+ to NO_3^- might also have the potential to reduce N_2O emission (Ledgard & Luo, 2008). Dobbie & Smith (2003) reported that DCD was effective in reducing N_2O emissions when it was applied with urea and ammonium sulphate from an intensively managed grassland site in the UK.

The practice of restricted grazing has been proposed as one of the options to reduce N_2O emissions on farm. Studies in New Zealand farms have demonstrated nearly 60 % reduction in N_2O emission due to less excretal deposition and lower soil compaction when grazing animals were kept in house during late-autumn-winter compared with year-round farm grazing (Chadwick *et al.*, 2002; de Klein *et al.*, 2006; Luo *et al.*, 2008b).

2.6.2 Biochemical changes in soil

One of the options for mitigating N_2O released through denitrification is by enhancing the reduction of N_2O to N_2 (Richardson *et al.*, 2009). The advancement in the biochemical and molecular sciences has developed our understanding of the enzymology, chemistry, and microbiology of the denitrification process. This advancement may enable us to develop mitigation techniques by manipulating the physiochemical conditions of soil, thus influencing the physiology and activity of denitrifying bacteria ensuring as far as possible the reduction of N_2O to N_2 (Thomson *et al.*, 2012). The inactivity of N_2OR enzyme under high O_2, low pH or lower availability of Cu ions results in high N_2O production during denitrification.

One of the possible strategies to mitigate denitrification in soil could be the application of biochar. It has been observed that biochar application facilitates the reduction of N_2O to N_2 by both its liming effect and by enhancing the electron availability to denitrifiers, which helps completing the denitrification process to emit N_2 as the end product (Cayuela *et al.*, 2013). With biochar application, decreases in N_2O emission by 10 % to 90 % have been observed in 14 different agricultural soils.

A set of management practices is needed that could either lower the N_2O emission from soil or increase the N_2O reduction to N_2. Management practices such as the application of crop residue, liming, increasing Cu availability, high SWC, biochar application, may enhance N_2O reduction to N_2. Higher N_2O production from some soils may be due to its poor capacity of reduce N_2O to N_2.

2.6.3 Plant breeding and genetic engineering

One of the options to reduce the application of active N to agricultural soils is growing cereal or pasture crops to fix atmospheric N_2 to sustain growth and yield. Beatty & Good (2011) have discussed the prospects of engineering cereal crops and bacteria to establish conditions in the roots of plants to support the activity of either the symbiotic or free living bacteria to fix atmospheric N_2 and to increase the affinity of bacteria to the engineered plant roots. Another approach is incorporating nitrogenase directly into the plant by engineering the plant organelle to

provide anaerobic conditions to support the activity of this enzyme. In a similar approach, greater reduction of N_2O to N_2 has been achieved by inoculating soybean plants with rhizobium bacteria possessing *nos*Z gene to reduce N_2O to N_2 (Sameshima-Saito *et al.*, 2006). In that case, boosting the soils' capacity to reduce N_2O has been proposed as a tool to mitigate N_2O. Sameshima-Saito *et al.* (2006) have demonstrated that the *Bradyrhizobium japonicum* USDA110 carrying *nos*Z gene has the capacity to reduce even a low concentration of N_2O. Inoculating the legume plants with this bacterium might enhance its capacity to reduce N_2O to N_2 (Hénault & Revellin, 2011).

2.7 CONCLUSIONS

Denitrification is the dissimilatory respiratory reduction of NO_3^- to N_2 if incomplete it leads to production of harmful greenhouse gas N_2O. Denitrification is widely spread in terrestrial and aquatic ecosystems. Generally it is carried out by phylogenetically diverse groups of organisms including bacteria, fungi, archaea, protozoa, etc. Denitrifiers are facultative anaerobes, thus NO_3^- reduction only occurs in the absence of O_2. Denitrification rates and the proportion of N_2O and N_2 produced during the denitrification process depends on various factors such as soil properties (SWC, NO_3^--N, organic C, pH), environmental conditions (rainfall, temperature), agricultural practices (fertilization, irrigation, grazing, organic waste management), and the presence of active denitrifiers in the system. The effect of individual parameters on DRs has already been established; however, we need to develop our understanding of the influence of interaction of physical, chemical, and microbial factors with DRs and the relative emission of end products.

There has been considerable development in the approach to quantification of N_2O and N_2 produced during denitrification. Although the ongoing modifications in the methods used in the past together with development of the latest technologies have enhanced our understanding of the process, there are very few studies in New Zealand pasture soils that measure denitrification under various soil and climatic conditions. Most of the research in the New Zealand agriculture system is focussed on measuring N_2O production through nitrification and the application of mitigation techniques to reduce N_2O produced during nitrification. None of past the denitrification-based studies in New Zealand have accounted for the denitrifier

community structure and its abundance, which has led to variable DRs or production of N_2O through denitrification.

Denitrification leads to the loss of available soil N, which in turn causes inefficiency in resource allocation and pollutes the atmosphere. An understanding of the role of microorganisms in regulating total denitrification is needed to ascertain the DRs under various farm situations, including urine deposition by grazed dairy cattle. This involves considering the complex interaction of microorganisms and biotic and abiotic factors, to study denitrification. There is a need for extensive study of denitrification both globally and in New Zealand pasture systems, given the associated production of N_2O. A complete understanding of the process would help formulate efficient and cost-effective mitigation techniques to regulate this challenging process.

The subsequent chapters of this thesis describe the experiments conducted with the aim of exploring the variations in DEA and DR among the pasture soils collected from various farms in New Zealand and relating these variabilities to physicochemical characteristics, denitrifier community structure, and denitrifier gene abundance in those soils. Chapter 3 describes the experiments performed to optimise the AIT, T-RFLP, and qPCR used during the research. The key findings of various incubations to relate soil properties with DRs, denitrifier community structure, and denitrifier gene abundance are presented in chapters 4 to 7.

Chapter 2

Chapter 3

Experimental techniques and protocols for quantification of denitrification enzyme activity, denitrification rate, and denitrifier genes in soils

3.1 INTRODUCTION

There are numerous direct and indirect methods to measure the denitrification rate and emissions of nitrous oxide (N_2O) and dinitrogen (N_2) during denitrification. However, all the existing methods have limitations, as detailed by Groffman *et al.* (2006) and reviewed in section 2.4. The most commonly used method, the acetylene (C_2H_2) inhibition technique (AIT) for estimating denitrification rate (DR) and denitrification enzyme activity (DEA) from soils, involves measuring the production of N_2O in the presence of (C_2H_2). The addition of C_2H_2 gas in the soil atmosphere (preferably > 1 ml L^{-1}) inhibits N_2O reduction to N_2 and makes the easily detectable N_2O the sole product of denitrification (Aulakh *et al.*, 1991a; Balderston *et al.*, 1976; Yoshinari & Knowles, 1976). Soil samples are incubated in the presence and absence of C_2H_2 gas, and the difference in N_2O emitted during the incubation is used to calculate the amount of N_2 produced and the product ratios N_2O/N_2 or $N_2O/(N_2O+N_2)$ during denitrification. Due to possible nitrification occurring in non-C_2H_2treated jars that can directly produce both N_2O and more NO_3^- available for the denitrification calculation of N_2, N_2O/N_2 or $N_2O/(N_2O+N_2)$ ratios with AIT might not provide the correct estimate.

The chemical or gaseous measurement methods provide insight into the biochemical process of denitrification in soils, but they do not provide an overview of the microbial contribution to these biochemical transformations (Sharma *et al.*, 2006). The development of molecular techniques has enabled relationships among microbial communities to be explored, together with detailed studies of the soil biochemical processes and the factors affecting these processes. Various molecular techniques are used to examine the denitrifier community structure, and some of these techniques are reviewed in section 2.5. Among these techniques,

Polymerase Chain Reaction (PCR) based techniques are commonly used to determine the denitrifier community structure and abundance in a system. These data can be utilised to relate variation in gene abundance to the variations in biotic and abiotic factors and process rates (Sharma *et al.*, 2007).

At the start of this research an extensive literature research was conducted on the available methods for measurement of DEA, DR, and the estimation of denitrifier community structure and denitrifier gene abundance. Preliminary experiments were then conducted to optimise the methodology for such measurement. Some alterations were made to published methods to optimise the experimental conditions for measuring DEA and DR using the AIT (sections 3.2 & 3.3). Many manipulations were made to optimise the published protocols for molecular work based on various denitrifier genes (section 3.4). When measuring soil physiochemical characteristics, few optimisations were required and mostly existing laboratory protocols were followed (section 3.5).

3.2 DEVELOPMENT OF PROTOCOLS FOR MEASUREMENT OF DENITRIFICATION ENZYME ACTIVITY (DEA)

The acetylene inhibition technique (AIT) used to measure DEA was originally proposed by Smith & Tiedje (1979). Soil samples supplied with excess moisture (for complete saturation) to make a slurry added with C, NO_3^-, and chloramphenicol (to inhibit the *de novo* synthesis of reductase enzymes) are incubated in the presence of C_2H_2 in anaerobic conditions (N_2 atmosphere) (Tiedje *et al.*, 1989). The N_2O produced from slurry gives an estimate of the potential denitrification rate of soil and the ambient reductase enzyme activity.

The addition of water and flushing with N_2 gas in the flasks create anaerobic conditions for denitrifiers in the slurry that might activate reductase enzymes and new enzyme synthesis might occur (Firestone, 1982). The synthesis of new reductase enzymes would invalidate the interpretation of DEA as a measure of ambient reductase activity. Chloramphenicol, a broad-spectrum antibiotic that inhibits new protein synthesis by binding to ribosomes, is added during the DEA assay to inhibit the synthesis of new N reductase enzymes. A few concerns have been raised regarding this method, including the use of high concentrations of chloramphenicol, which may also inhibit the activity of existing denitrifier reductase enzymes during the DEA assay

(Dendooven *et al.*, 1994; Murray & Knowles, 1999). However, it has been reported that in pure cultures of *Pseudomonas denitrificans* and *Flexibacter Canadensis* chloramphenicol, when used in higher concentrations, inhibits enzymes responsible for NO_3^- reduction and NO reduction respectively (Wu & Knowles, 1995).

The original DEA method described by Smith & Tiedje (1979) has been extensively modified in a number of studies (Carter *et al.*, 2006; Dendooven & Anderson, 1994; Luo *et al.*, 1996). Therefore, laboratory tests were conducted to determine the optimum level of chloramphenicol for quantification of DEA. The objectives were to assess the available methodologies and to develop protocols to use in the current research.

The source of C_2H_2 used in the incubations can be important because impurities such as acetone might inhibit microbial activity (Nunez *et al.*, 2009). Preliminary laboratory incubations conducted to learn the AIT showed significantly higher N_2O emission in incubations with added C_2H_2 (441.27 µg N_2O-N kg^{-1} soil hr^{-1}) compared with incubations without C_2H_2 (281.52 µg N_2O-N kg^{-1} soil hr^{-1}) (T-test $P < 0.05$). These results indicated the welding grade C_2H_2 used in this study is appropriate to use for measurements of DEA and DR, and the adopted purification procedure is adequate to remove impurities that might otherwise have inhibited microbial activity.

It was also observed that N_2O emissions during measurements of DEA in incubated soil samples varied with variations in incubation temperature at the time of measurement. The measured N_2O emission was significantly lower (280 µg N_2O-N kg^{-1} soil hr^{-1}) at 17 ^oC than at 21 ^oC (387 µg N_2O-N kg^{-1} soil hr^{-1}) (T-test $P < 0.05$). It was therefore decided to keep the incubation temperature constant (25 ^oC) as also suggested by Tiedje *et al.* (1979).

Once the incubation conditions (as described above) had been established, DEA incubations were monitored for 12 hours to trace the changes in N_2O production with time. It was observed that after 6 hours of incubation the rate of increase of N_2O production in the flasks was constant (Fig. 3.1). One of the reasons for constant N_2O production rate with increasing incubation time could be a limitation of available resources, especially carbon. Available C in soil is used both by denitrifiers and non-denitrifiers, making it utilised rapidly over incubation time. It was reported by Smith & Tiedje (1979) that by 4–8 hours of incubation the denitrifying bacteria attain their maximum capacity to denitrify. After this point denitrification activity will only increase with an additional supply of organic carbon. Similarly, in the reported experiment,

with the consumption of available carbon, denitrification activity did not increase beyond 6 hours as no significant change in the N_2O production in soils was measured, probably due to the consumption of added nutrients during incubation. The length of incubation for the DEA assay was therefore restricted to 6 hours for this study.

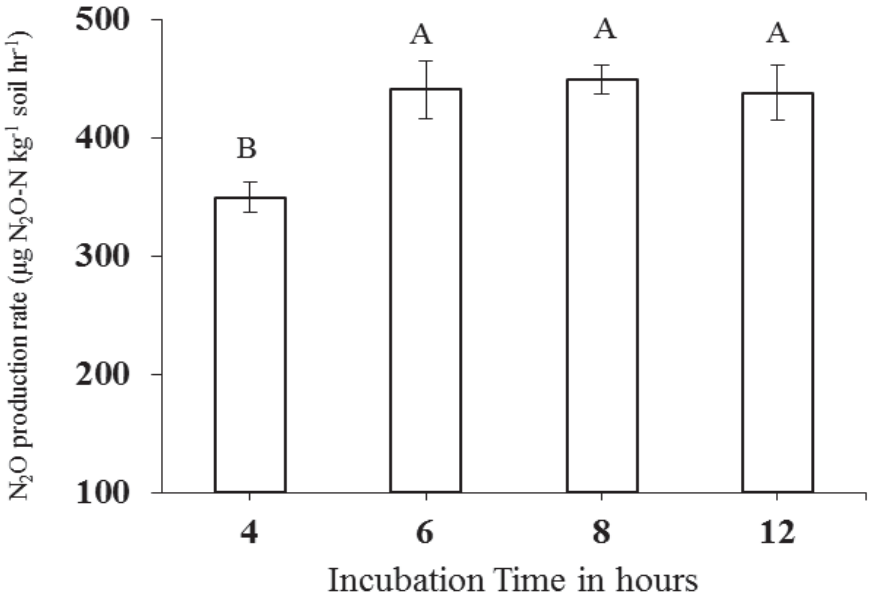


Figure 3.1: Nitrous oxide production at various time-points during denitrification enzyme activity assay in Tokomaru silt loam soil incubated for 12 hours. Each column represents mean ± standard error of mean (n = 4). Columns with same letter values are not significantly different (P < 0.05). The test represents differences in means using 1-way analysis of variance (ANOVA).

Objective: To test the effect of different chloramphenicol concentrations added during the DEA on N_2O production rates in samples.

3.2.1 Materials and methods

3.2.1.1 Collection of soil samples

The soil used for DEA measurements was Tokomaru silt loam, collected from Massey University No. 4 Dairy Farm (40º 22' S, 175º 36' E) in April 2010. Representative soil samples were obtained by avoiding the farm entrance, water trough areas, and obvious dung patches.

Twenty-five soil cores (25 mm dia × 100 mm long) were collected from four randomly selected areas in the farm (25 × 4 = 100 cores). The collected soil cores were taken to the laboratory, pooled, sieved to 2 mm and stored at 4 ^{o}C until used for analyses. The composite sample was further split to generate 4 replicates for analytical procedures. Subsamples of the collected soil were used for determination of soil water content and mineral N (NO_3^- and NH_4^+) content.

3.2.1.2 Description of treatments

The incubations were conducted in replicates of four to assess the effects of various concentrations of chloramphenicol on production of N_2O production during measurements of DEA.

The treatments refer to the concentrations of chloramphenicol in final slurry: T1 = No Chloramphenicol; T2 = 2 mg L^{-1}; T3 = 10 mg L^{-1}; T4 = 20 mg L^{-1}; T5 = 50 mg L^{-1}, and T6 = 100 mg L^{-1} Chloramphenicol.

3.2.1.3 Incubation of soil samples

Samples (10 g) of field-moist soil were placed in 125 ml Erlenmeyer flasks (Kimax®). To this flask 20 ml of deionised water and 8 ml of a solution containing 0.45 g KNO_3 L^{-1}, 0.77 g D-glucose L^{-1}, and variable concentrations of chloramphenicol (0–100 mg L^{-1}) were added to make slurries. This solution provided 2.2 mg NO_3^- (35 µmoles NO_3^-) and 2.5 mg C (208 µmoles C) in each flask. The flasks were sealed using rubber septa Suba-Seal® (Sigma-Aldrich). Air was flushed from the flasks with N_2 gas to create anaerobic conditions. Ten percent of the headspace volume (10 ml) of the flasks was replaced with purified (acetone-free) C_2H_2. The C_2H_2 was purified by passing the welding-grade C_2H_2 gas through concentrated H_2SO_4 followed by water to remove the impurities present in the C_2H_2 gas (Aulakh *et al.*, 1991a). The purified C_2H_2 gas was collected in a rubber bladder (Gilbert® rugby ball bladder) after initially flushing the bladder with purified C_2H_2 several times. This purified C_2H_2 gas was injected into the incubation flasks. The flasks were shaken for 1 minute to mix the contents with the C_2H_2 gas.

3.2.1.4 Collection of gas samples

Gas samples (5 ml) for time 0 (T0) were taken immediately and replaced with equal quantity of N_2 with a 25-ml polypropylene syringe fitted with a 3-way stopcock and transferred to 12-ml evacuated glass vials (Exetainer®, Labco, UK). The flasks were then placed on an orbital shaker (set at 125 rpm) and incubated at constant temperature (25 ^{o}C) for 6 hours (hrs). A 5-ml gas sample was taken after 1, 2, 4, and 6 hrs of incubation from each flask and each time, the same amount of N_2 gas was replaced in the flasks. The collected gas samples were diluted to 25 ml by adding 20 ml of N_2 gas and transferred to 12-ml evacuated glass vials. Dilution of the gas samples was required as the increased N_2O concentrations with time, exceeded the detection limit (19200 ppb N_2O) of the gas chromatograph (GC). The over-pressurized gas vials were placed on an auto-sampler connected to a GC for analysis of N_2O production in each flask. The over-pressurized gas samples in each vial ensured collection of gas by the needle of the auto-sampler connected to the GC (Shimadzu GC 17A Japan equipped with a back flush system). This GC had a sample loop, a ^{63}Ni- electron capture detector (ECD) operating at column, injector and detector temperatures of 55, 75 and 330 ^{o}C respectively. Nitrogen (99.99 % purity) was used as the carrier gas and a makeup gas of 5 % methane in argon for ECD.

3.2.1.5 Calculations of DEA

The N_2O production rate in flasks was determined by the slope of the linear regression of the N_2O in samples over the incubated time (0–6 hrs). The GC provides N_2O production in each flask as ppb that is read as μL-N_2O L^{-1} gas. The measured volumetric N_2O concentrations were used to calculate the amount of N_2O contained in the water and gas phases as follows:

$$N_2O \text{ flux} = \frac{N \times DF \times \left(V_g + V_l \times \alpha\right)}{W} \quad \text{(Eq 3.1)}$$

where: N_2O flux = rate of N_2O produced (μL-N_2O kg^{-1}soil hr^{-1}) during the incubation period; N = N_2O production rate in each flask (μL-N_2O L^{-1} gas hr^{-1}); DF (Dilution factor) = (5ml sample+ 20 ml N_2 gas)/(5-ml sample) = 25/5 = 5 at all the sampling times ; V_g = volume (L) of gas phase in the incubation flasks; V_l = volume (L) of liquid phase (soil + water) in the incubation flasks; α (the Bunsen absorption coefficient) = 0.544 (Groffman *et al.*, 1999); W= weight of soil (kg).

$$DEA = N_2O \text{ flux} \times \rho \quad \text{(Eq 3.2)}$$

where: DEA is the denitrification enzyme activity (μg N_2O-N kg^{-1} soil hr^{-1}); N_2O flux = rate of N_2O produced (μL-N_2O kg^{-1} soil hr^{-1}) during the incubation period; ρ = the density of N_2O-N (1.145 μg N_2O-N μL^{-1}) at normal temperature (25 ^oC) and pressure (1 atm).

3.2.1.6 Statistical Analysis

The effect of various chloramphenicol concentrations on N_2O yield from soil incubations was assessed by analysis of variance (ANOVA) using Minitab 16 software by keeping N_2O emissions as the response variable, and chloramphenicol concentrations as the factor. Subsequently, a Tukey's studentized Range (HSD) test at $\alpha = 0.05$ significance level was used *post hoc* to reveal significant differences among treatment means.

3.2.2 Results

3.2.2.1 Effect of chloramphenicol concentrations on DEA in soils

The measured DEA varied from 188 to 550 μg N_2O-N kg^{-1} soil hr^{-1} depending on the concentration of chloramphenicol used (Fig. 3.2). The highest DEA was measured for T1 (0 mg L^{-1}) and the lowest (188 μg N_2O-N kg $soil^{-1}$ hr^{-1}) for T6 (100 mg L^{-1}). The DEA values for treatments with 2 and 10 mg L^{-1} of chloramphenicol were statistically similar, and there was no difference in DEA values from treatments receiving 20–100 mg L^{-1} of chloramphenicol.

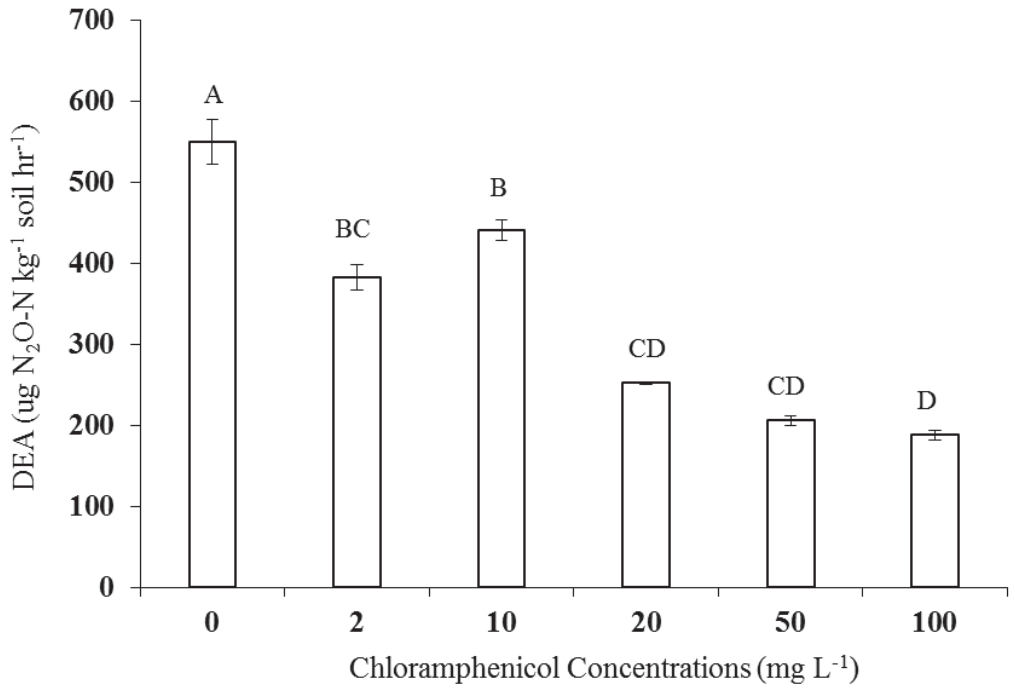


Figure 3.2 Denitrification enzyme activity of soil samples (DEA) (Tokomaru silt loam) incubated (for 6 hours) with varying chloramphenicol concentrations (0–100 mg L^{-1}). Each column represents mean (n = 4) ± Standard error of mean. Columns with same letter values are not significantly different ($P < 0.05$). The test represents differences in means using one way analysis of variance (ANOVA).

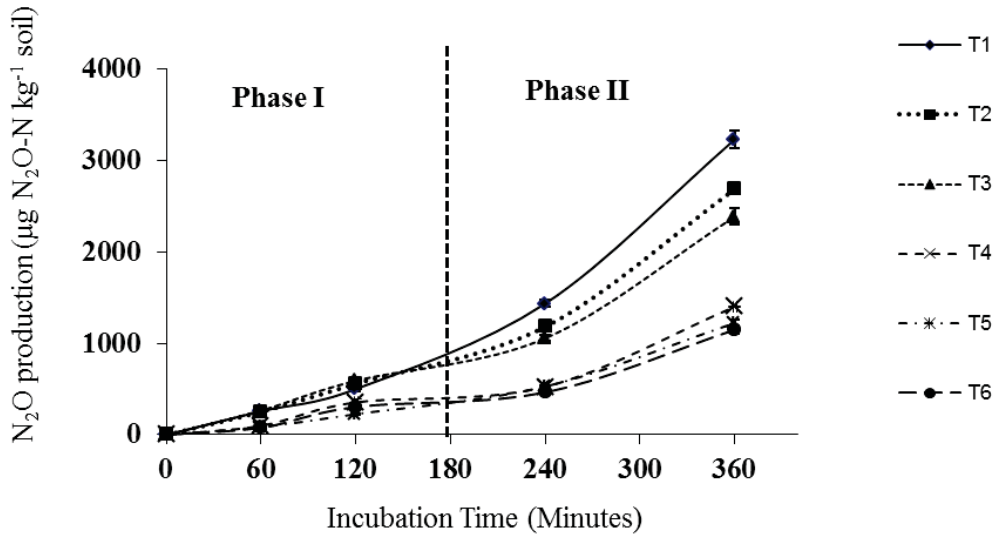


Figure 3.3: N_2O production during denitrification enzyme activity assay in soil (Tokomaru silt loam) incubated with varying chloramphenicol concentrations (T1 = 0, T2 = 2, T3 = 10, T4 = 20, T5 = 50, T6 = 100 mg L^{-1}) at different incubation times. Data are means (n = 4) ± standard error of mean.

DEA decreased with increasing concentrations of chloramphenicol and was highest in the control treatment. The decline in DEA with an increase in chloramphenicol is similar to that observed by Firestone *et al.* (1979) and Pell *et al.* (1996). These researchers reported that addition of chloramphenicol, even at the concentration of 20 mg L^{-1}, inhibited the DEA, and with increasing concentration of chloramphenicol beyond 20 mg L^{-1} the inhibition effect increased. They found a 17–42 % decrease in DEA at a chloramphenicol concentration of 1 g L^{-1} compared with incubations without chloramphenicol. The decreased DEA at higher chloramphenicol concentrations signals the decrease in synthesis of new enzymes and also the activity of existing enzymes (Firestone *et al.*, 1979; Pell *et al.*, 1996).

DEA is a short incubation assay divided into two phases (Smith & Tiedje, 1979). In phase I (1–3 hours) existing enzyme activity results in production of N_2O, and in phase II (3 to 6 hours) new enzymes are synthesized and the N_2O production is increased at an ascending rate. Smith & Tiedje (1979) suggested that addition of chloramphenicol does not inhibit denitrification in phase I but inhibits the increase in denitrification rate in phase II. In contrast,

Pell *et al.* (1996) observed that higher concentrations of chloramphenicol affected denitrification in both the phases and suggested that if chloramphenicol was not used judiciously in DEA analysis it may upset the activity of resident denitrifying enzymes in soil. This hypothesis is evident from the lower DEA at higher chloramphenicol concentrations ($\geq$ 20 mg L^{-1}) in the current experiment.

In the absence of chloramphenicol (T1) in the current study, there would have been no restriction of *de novo* synthesis of reductase enzymes and this appeared to result in a non-linear increase in N_2O emission towards the end of the 6 hours incubation (Fig. 3.3). The higher N_2O emission in phase I at 2 and 10 mg L^{-1} levels than 20 mg L^{-1} and beyond corroborates the findings of earlier studies that at lower concentrations chloramphenicol do not restrict the resident enzyme activity. When used in higher concentrations (20, 50, and 100 ppm) chloramphenicol starts inhibiting the existing enzyme activity along with *de novo* enzyme synthesis in soils, as evident from lower N_2O production in the samples with higher chloramphenicol in phase I as well as phase II of incubation. In phase II at 2 and 10 mg L^{-1} levels of chloramphenicol a slight reduction in N_2O emission was observed, suggesting an inhibitory effect of chloramphenicol on the production of new enzymes. These results revealed that chloramphenicol concentrations of 10 mg L^{-1} had no adverse effect on DEA and therefore this concentration was used in the subsequent measurements of DEA.

3.2.3 Final DEA protocol to be followed during the research

Based on the results described above the final protocol followed for DEA measurement was as follows:

Soil samples (10 g) were placed in 125-ml flasks. Slurries were prepared by adding 25 ml solution containing 2.2 mg NO_3^- (35 µmoles NO_3^-) as KNO_3^-, 2.5 mg C (208 µmoles C) as D-Glucose, and 10 mg chloramphenicol. The flasks were sealed using Suba-Seal® septa (Sigma-Aldrich). Air was flushed from the flasks with N_2 gas to create anaerobic conditions. Ten percent of the headspace volume (approx. 10 ml) of the flasks was replaced with purified (acetone-free) C_2H_2. Gas samples (5 ml) for time 0 (T0) were taken immediately and replaced with an equal quantity of N_2. The flasks were then placed on an orbital shaker (set at 125 rpm) and incubated at

25 ^{o}C for 6 hrs. A 5-ml gas sample was taken after 2, 4, and 6 hrs of incubation from each flask, and each time the same amount of N_2 gas was replaced in the flasks.

3.3 DEVELOPMENT OF PROTOCOLS FOR MEASUREMENT OF DENITRIFICATION RATE (DR)

The original method of measuring DR as described by Ryden *et al.* (1979a) has been extensively modified over time. These modifications include the type and amount of C_2H_2 (welding grade, CaC_2 generated, coated CaC_2) used for inhibition, the incubation time, the weight of soil used, and how the soil sample is processed (use of intact cores, reconstructed cores, sieved soils) (Aulakh *et al.*, 1992; Luo *et al.*, 2000; Mahmood *et al.*, 1997; 1999).

A laboratory incubation experiment was conducted to address the concerns regarding C_2H_2 inhibition incubation times and evaluate the method of soil processing for DR measurements. Pre-processing of soil is needed as a balance between achieving high enough C_2H_2 throughout the soil for inhibition of N_2O reduction to N_2 and not enhancing O_2 concentrations which could reduce denitrification.

Objectives: To assess the effect of soil pre-processing after collecting soil cores on denitrification rate and to determine the optimal time of incubation for measuring denitrification rate.

3.3.1 Materials and methods

3.3.1.1 Collection of soil samples

Two soils used for development of protocol include Tokomaru silt loam (used in the DEA measurement described earlier) and Manawatu fine sandy loam, from Longburn dairy farm (40°22'S, 175°32'E). In each farm 30 randomly located plots (100 m^2) (Fig. 3.4) were established and six soil cores each 25 mm diameter and 100 mm long were collected from 0–100 and 100–200 mm depths from each plot in July 2010 to provide field replicates. The six cores collected

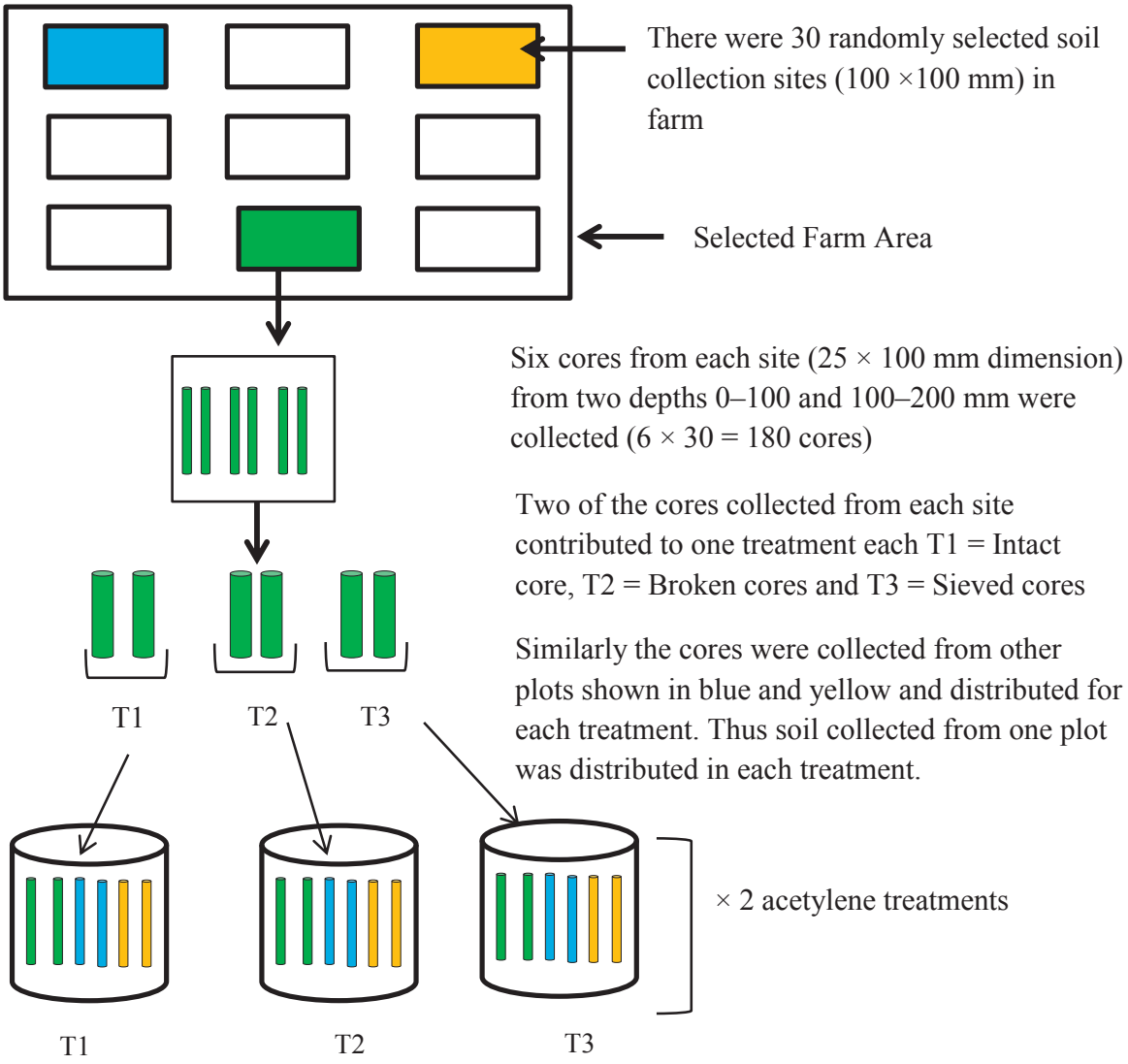
from each location and each depth were bulked together and stored as one sample in polythene bags (30 plots × 2 depths). The pasture from the top of the cores was removed for all the three treatments (intact, broken, and sieved) by cutting off the top 20-mm portion with a sharp knife. Two cores from each bag were used for the treatments: T1: Intact soil cores; T2: Broken cores; T3: Sieved (2 mm) soil. The pairs of soil cores separated for each treatment from 5 of the randomly located plots were then combined to give six field replicates, each with 10 cores per replicate. These were then divided into two equal samples for incubation with and without C_2H_2 for gas measurements. Subsamples of the collected soils were used for determination of soil water content and mineral N (NO_3^- and NH_4^+) content.

C_2H_2 inhibits both the conversion of N_2O to N_2 and nitrification, therefore N_2O produced in C_2H_2-treated jars is produced only by denitrification. The initial intention was to measure N_2O from non-C_2H_2 treated jars and N_2O+N_2 from C_2H_2-treated jars. These measurements would be used to calculate the $N_2 = [(N_2O+N_2)-N_2O]$ and $N_2O/(N_2O+N_2)$ ratio. However, due to the possibility of nitrification in the non-C_2H_2 jars these estimates would be unreliable; it was therefore decided to use the N_2O from non-C_2H_2 treated jars as N_2O produced by nitrification and denitrification (N_2O-NA) and N_2O from C_2H_2-treated jars as N_2O produced only from denitrification (N_2O-A). N_2O-A is also referred to as denitrification rate (DR) in this thesis.

3.3.1.2 Incubation of soil samples

The soil samples were transferred to 1-L glass preserving jars (5 cores per jar × 6 replicates × 3 treatments × 2 depths) with and without C_2H_2 to measure N_2O produced by nitrification and denitrification (N_2O-NA) and N_2O produced only by denitrification (N_2O-A) respectively. The head space volume (10 %) of half the jars was replaced with C_2H_2 by injecting 60 ml of purified C_2H_2. All the jars (3 treatments × 2 depths × 6 replicates × 2 C_2H_2 treatments = 72 jars) were incubated at 25 ^oC for 48 hours (Ryden & Skinner, 1987).

Treatments include T1: Intact soil cores; T2: Broken cores; T3: Sieved (2 mm) soil
Number of Replicates: 6 for each treatment for each soil
Total duration of incubation: 48 hours



Cores incubated in Agee jars. Each treatment had 5 jars each

6 cores × 2 acetylene treatments × 3 processing treatments × 5 replicates = 180 cores

Figure 3.4: The collection of soil cores for laboratory incubation.

3.3.1.3 Gas Sampling and analysis

The gas samples (25 ml) for time 0 (T0) were taken immediately and transferred to evacuated vials (12 ml capacity). At the same time, the same volume of ambient air was injected into the jars. During incubation a 10-ml gas sample was collected from each jar after 3, 6, 9, 12, 24, and 48 hours of incubation and an equal amount of ambient air was replaced to maintain the same gas pressure inside the jars. The collected gas samples were diluted to 25 ml by adding 15 ml of N_2 gas and transferred to evacuated glass vials. Gas samples were analysed by a gas chromatograph (GC) to measure the N_2O concentration. The amount of N_2O produced in the C_2H_2-treated jars corresponded to N_2O produced from denitrification (N_2O-A) or denitrification rate (DR) and N_2O produced in the non-C_2H_2-treated jars corresponded to N_2O produced from both nitrification and denitrification (N_2O-NA) in soils.

3.3.1.4 Calculations of Denitrification Rate and N_2O production

The N_2O production rate in flasks was determined by the slope of the linear regression of the N_2O concentrations in samples over the incubated time (0–48 hrs). The measured volumetric N_2O concentrations from the C_2H_2 treatments and non-C_2H_2 treatments were calculated from the amount of N_2O contained in the collected gas samples as follows:

$$\text{Total } N_2O \text{ produced} = \left(\frac{N \times V \times DF}{W}\right) \times \rho \qquad \text{(Eq 3.3)}$$

where: Total N_2O emitted (μg N_2O-N kg^{-1} soil hr^{-1}) = N_2O produced in each jar; N = rate of N_2O produced (μL-N_2O L^{-1} gas hr^{-1}) during the incubation period; DF (Dilution factor) = (10-ml sample + 15 ml N_2 gas)/ (10-ml sample) = 25/10 = 2.5 at all the sampling times; W= weight of soil sample (kg); V= volume of headspace in L; ρ is the density of N_2O-N = 1.145 μg N_2O-N μL^{-1} at normal temperature (25 ^oC) and pressure (1 atm); DR is the denitrification rate (μg N_2O-N kg^{-1} soil hr^{-1}).

N_2O-NA = Rate of N_2O-N production in non C_2H_2 treated jar (Eq 3.4)

N_2O-A Or DR = Rate of N_2O-N production in C_2H_2 treated jar (Eq 3.5)

3.3.1.5 Statistical Analysis

The ANOVA test for N_2O emitted during incubation for various treatments was performed using Minitab 16 software. A 2-way ANOVA procedure was applied to determine the effect of soils and soil processing on N_2O-NA and N_2O-A at each depth (0–100 mm and 100–200 mm) separately. The N_2O productions were taken as the response variable, with soil type, and soil pre-processing as factors. Tukey's studentized Range (HSD) test at 5 % significance level was used to compare means of the treatments.

3.3.2 Results

3.3.2.1 Effect of soil processing on denitrification and establishing the length of incubation for gas measurements from soils

Denitrification rates (DR) (measured as N_2O produced from C_2H_2 treated soils and produced through complete denitrification) in surface soils (0–100 mm) ranged from 7.27 to 17.40 µg N_2O-N kg^{-1} soil hr^{-1} in Manawatu soil (MW) and from 5.62 to 13.98 µg N_2O-N kg^{-1} soil hr^{-1} in Tokomaru soil (TM) (Fig. 3.5). In the sub-surface soils (100–200 mm) the DR ranged from 1.71 to 2.64 µg N_2O-N kg^{-1} soil hr^{-1} in MW soil, and from 0.58 to 1.69 µg N_2O-N kg^{-1} soil hr^{-1} in the TM soil (Fig. 3.5). DR in the sub-surface samples of both soils was very low.

The 2-way ANOVA (Table 3.1 & Fig. 3.5) suggested that in surface samples (0–100 mm) the interaction of soil type, and pre-processing was not significant (at $\alpha = 0.05$) on DR, indicating that the pre-processing of samples had similar effects on DR in both soils. Both MW and TM soils had higher DR in intact cores than sieved soils (Fig. 3.5). The 2-way ANOVA (Table 3.1) in the sub-surface samples showed interaction of soil, and pre-processing was

significant (at $\alpha = 0.05$), indicating that soil pre-processing had different effects on DR in the two soils. DR in the TM soil intact cores was slightly higher than in the sieved samples but there was no significant difference in the DR between intact cores and broken cores and also between broken cores and sieved samples. In the MW soil there was no significant difference in the DR between the intact cores and broken cores. There was no significant difference in the DR between the intact cores and sieved samples. However, DR in the broken cores was significantly higher than the DR in the sieved samples (Fig. 3.5).

Similar to DR the 2-way ANOVA (Table 3.1 & Fig. 3.6) suggested that in surface samples (0–100 mm) the interaction of soil type, and pre-processing was not significant (at $\alpha = 0.05$) on N_2O production from nitrification and denitrification (N_2O-NA), indicating that the pre-processing of samples had similar effects on N_2O-NA in both soils. Both the MW and the TM soils had higher N_2O-NA production in intact and broken cores than in sieved soils (Fig. 3.6). The 2-way ANOVA (Table 3.1) in the sub-surface samples showed the interaction of soil, and pre-processing was significant (at $\alpha = 0.05$), indicating that soil pre-processing had different effects on N_2O-NA in the two soils. There was no significant difference in the N_2O-NA production in the three treatments in the MW soil. In the TM soil N_2O-NA was higher in the intact core but not significantly different in the broken cores and sieved soil in the sub-surface samples (Fig. 3.6).

The rate of N_2O-NA in surface soils (0–100 mm) ranged from 2.74 to 5.22 μg N_2O-N kg^{-1} soil hr^{-1} in the MW soil and from 1.43 to 4.57 μg N_2O-N kg^{-1} soil hr^{-1} in the TM soil (Fig. 3.6). In the sub-surface soils (100–200 mm) the N_2O-NA ranged from 1.51 to 1.77 μg N_2O-N kg^{-1} soil hr^{-1} in the MW soil and from 0.31 to 1.34 μg N_2O-N kg^{-1} soil hr^{-1} in the TM soil (Fig. 3.6). The rate of N_2O emission in both the soils was lower in the sub-surface samples than in the surface samples.

Table 3.1 F and *P* values of 2-way analysis of variance test of effect of two soils (Tokomaru silt loam and Manawatu fine sandy loam) and soil pre-processing (intact cores, broken cores and sieved soil) at each sampling depth (0–100 mm and 100–200 mm) on denitrification (N_2O-A) and nitrous oxide (N_2O-NA) emission from nitrification and denitrification

Source	**N_2O-A**		**N_2O-NA**	
0–100 mm	**F**	***P***	**F**	***P***
Soil	16.21	0.0001	8.96	0.041
Pre-processing	129.56	0.0001	15.06	0.0001
Soil × Pre-processing	1.65	0.2080	2.13	0.067
100–200 mm				
Soil type	69.28	0.0010	206.83	0.0001
Pre-processing	9.35	0.0001	29.75	0.0001
Soil type × Pre-processing	4.17	0.0250	18.07	0.0001

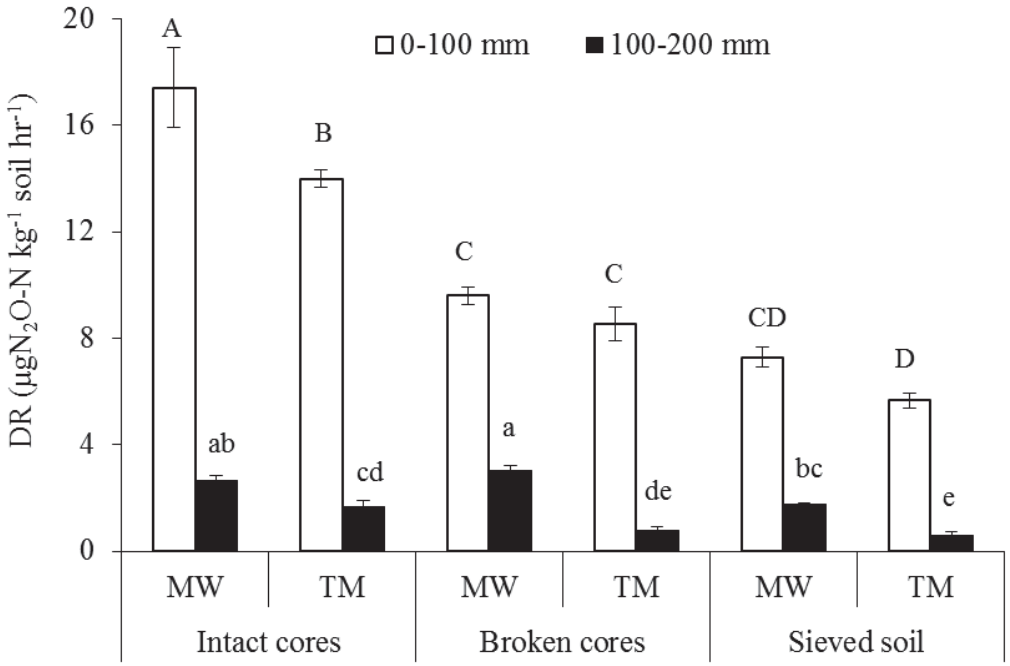


Figure 3.5: Denitrification rates (DR) in Tokomaru silt loam (TM) and Manawatu fine sandy loam (MW) soil collected from two depths (0–100 and 100–200 mm) and with different pre-processing of samples (intact cores, broken cores and sieved soil). Each column represents mean ± standard error of mean (n = 6). Columns with same letter values are not significantly different ($P < 0.05$). Letter values with the same case denote 2-way analysis of variance tests (ANOVA).

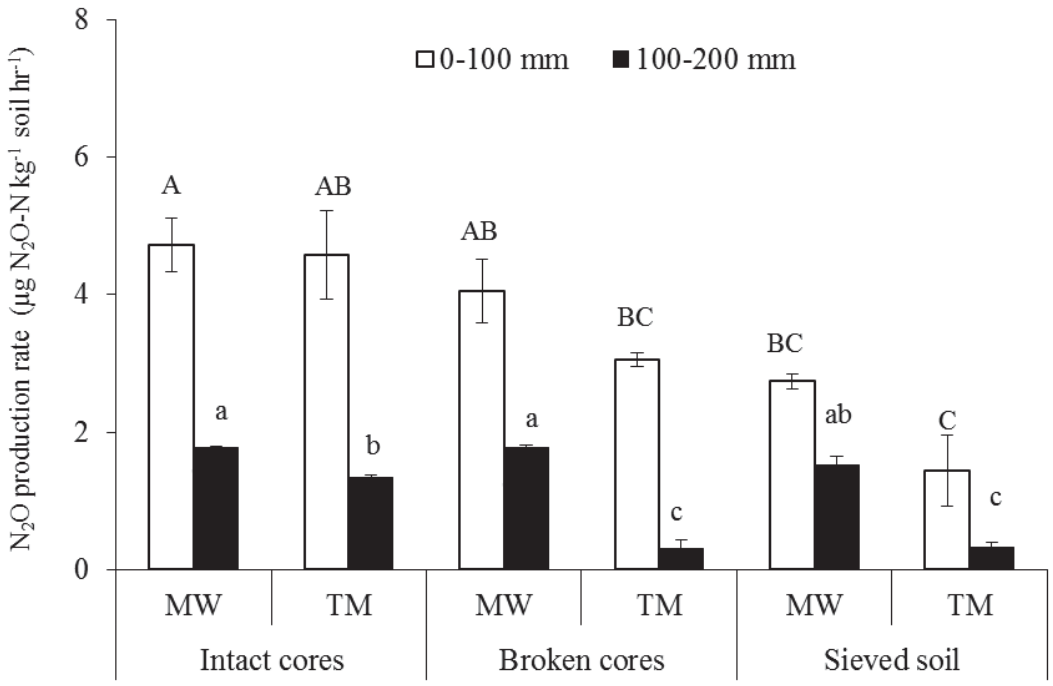


Figure 3.6: N_2O production rate from nitrification and denitrification in Tokomaru silt loam (TM) and Manawatu fine sandy loam soil (MW) collected from two depths (0–100 and 100–200 mm) and with different pre-processing of samples (intact cores, broken cores and sieved soil). Each column represents mean ± standard error of mean (n = 6). Columns with same letter values are not significantly different ($P < 0.05$). Letter values with the same case denote 2-way analysis of variance tests (ANOVA).

The higher DR in intact cores was probably due to the soil's compaction and more anaerobic conditions compared with the broken cores or sieved soil. Although the compacted soil in a core can enhance the rate of denitrification, the same conditions can also impair the diffusion of C_2H_2 into the soil and lead to underestimation of total denitrification. Denitrification could also be underestimated by entrapment of produced N_2O in the microsites in a core. Due to these reasons for underestimation of denitrification, Mahmood *et al.* (1999) favour the measurement of denitrification using broken cores rather than intact cores. They have reported an average of 40 % of the N_2O entrapped in the soil core, which led to underestimation of denitrification rates. Clough *et al.* (2000), using a ^{15}N enrichment technique, have also suggested underestimation of denitrification is the result of entrapment of N_2O in intact soil cores compared with broken cores.

The incubation period required optimising because the microorganisms converting N_2O to N_2 can become C_2H_2 tolerant with increasing duration of incubation (de Klein & Van Logtestijn, 1994a). These microorganisms may start decomposing the available C_2H_2 (Zaman *et al.*, 2008c), consequently interfering with the denitrification activity. Decomposition of C_2H_2 will make it less available for inhibition of N_2O reduction, showing no significant increase in the DR between 24–48 hours of incubation and 0–24 hours. The DRs in both the MW and the TM soils after 24 hours were not significantly different than the DRs after 48 hours. Within 24–48 hours denitrification rate became constant (Fig. 3.7) compared with the increasing rate within 12–24 hours of incubation (Fig. 3.8). The constant rate of denitrification could be due to lesser availability of resources (C & N) with increasing incubation time. Controlling the incubation at 24 hours for denitrification measurements seemed appropriate, considering the time needed by C_2H_2 to inhibit the conversion of N_2O to N_2 and also because denitrifiers start decomposing the added C_2H_2 for C source comparatively quickly.

If there was variable diffusion of C_2H_2 in intact cores then this would create variability in the inhibition of the N_2O reductase enzyme, and in the resulting N_2O emissions from the C_2H_2-treated soil cores. There could also be additional variability in N_2O emissions from intact soil cores through entrapment of released N_2O in soil. These variations in N_2O emissions from intact soil cores might introduce a measurement bias and lead to systematic underestimation of denitrification. The extent of this underestimation may vary among soils. Although intact soil cores produced more N_2O-NA and N_2O-A than sieved soils, conducting denitrification measurements on sieved soils was decided due to practical inconveniences with the use of intact cores. The nature of this study was to collect the samples from different sites and provide various treatments to soils – the breaking of soil cores during transportation and the non-homogenous distribution of treatments in intact cores lead to work with sieved soils instead of intact cores.

A number of difficulties were earlier described when applying the AIT to solid cores. In order to avoid the underestimation of denitrification, variability in measurements, and to simplify sample collection and transport from 10 New Zealand representative dairy farms situated in both North and South Islands, it was decided to conduct the denitrification measurement experiments in this study on sieved soils in order to get more uniform emissions from each soil replicate and treatment.

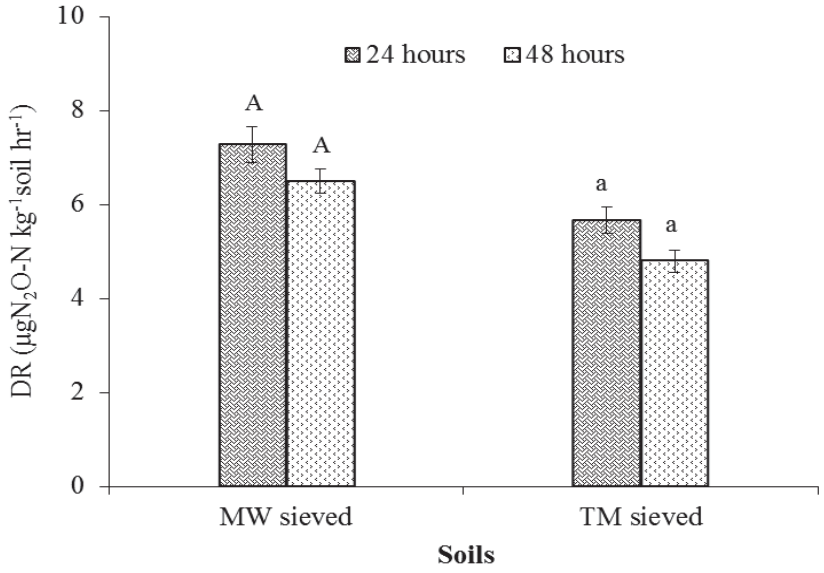


Figure 3.7: Denitrification rate (DR) in Manawatu fine sandy loam (MW) and Tokomaru silt loam soil (TM) sieved samples at variable incubation times (24 & 48 hrs). Each column represents mean ± standard error of mean (n = 6). Columns with same letter values are not significantly different ($P < 0.05$).

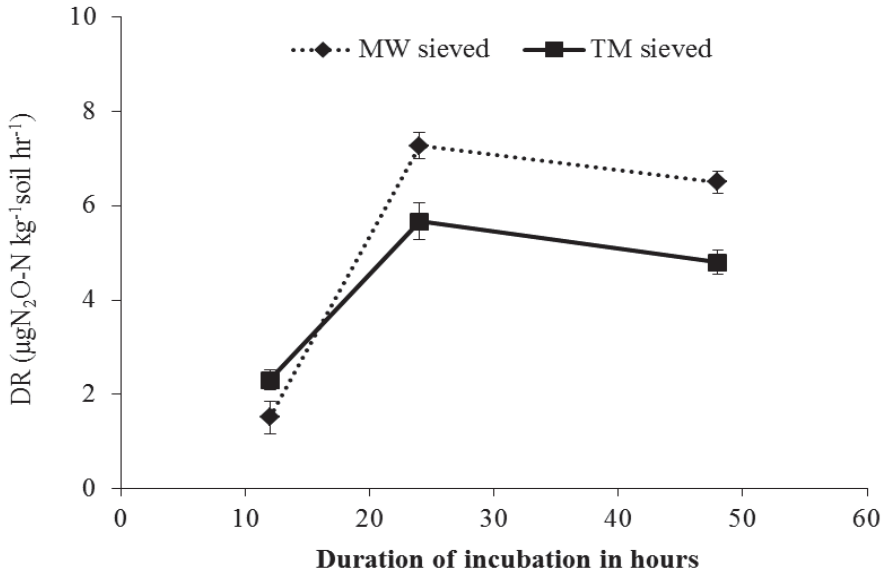


Figure 3.8: Denitrification rate (DR) in Manawatu fine sandy loam (MW) and Tokomaru silt loam soil (TM) sieved samples with increasing incubation time. Each data point represents mean ± standard error of mean (n = 6).

3.3.3 Final protocol for denitrification measurement followed during research

Replicated sieved soil samples were weighed in pairs into glass jars (1 L) with tight fitting lids. One jar from each pair was incubated as control (without C_2H_2) and the second jar had 10 % of the headspace replaced with purified C_2H_2. Both sets of jars were incubated at 25 ^{o}C for 24 hours. Gas samples were taken at time 0, 3, 6, 9, 12, and 24 hours with the gas sample replaced each time by the same volume of air to keep the atmospheric pressure inside the jars constant. Gas samples were analysed for N_2O in a gas chromatograph (GC).

3.4 MOLECULAR TECHNIQUES TO DETERMINE DENITRIFIER COMMUNITY STRUCTURE AND ABUNDANCE IN SOILS

The diverse soil transformation processes, including denitrification, are mediated by the interactions of microbial community structure and abundance in soil. To understand nitrogen cycling it is necessary to know the composition and abundance of the microbial community responsible for the process and its relationship to nitrogen dynamics in the environment (Philippot & Hallin, 2005; Taroncher-Oldenburg *et al.*, 2003). As very few microorganisms can be cultured in the laboratory (e.g. less than 1 % of soil microorganisms) (Forney *et al.*, 2004; Liu *et al.*, 1997), culture-independent molecular methods are increasingly used to study functional groups, including those carrying out denitrification (Torsvik & Øvreås, 2002). One of the popular culture-independent techniques is polymerase chain reaction (PCR) (Mullis & Faloona, 1987). Numerous PCR-based techniques are employed to study denitrifier community structure. These are reviewed in detail in section 2.5.2. These culture-independent methods are based on the extraction of total nucleic acids (DNA or RNA) from soil followed by targeted PCR-based analyses.

3.4.1 Soil DNA Extractions

Following an appropriate DNA extraction procedure is critical to obtaining a representative DNA sample of the microbial community present in soil (Carrigg *et al.*, 2007). Studies evaluating different extraction protocols mostly focussed on optimising the maximum total DNA yield, DNA quality and maximum diversity of detectable organisms (Bürgmann *et al.*, 2001; Kuske *et al.*, 1998; Miller *et al.*, 1999). In a comparative study of various DNA extraction procedures Maarit Niemi *et al.* (2001) have shown that the DNA yield and PCR amplification of extracted DNA using the soil DNA isolation kit (MO Bio Laboratories Inc., Carlsbad, CA, USA) was better than with the other DNA extraction protocols available. In order to obtain the most representative DNA sample with no inhibitory compounds for PCR amplification it was decided to opt for the PowerSoil™ MoBio DNA isolation kit for this research.

DNA was extracted from 0.25 g of 6 replicated soil samples (as received) (Manawatu fine sandy loam and Otorohanga silt loam) collected from 0-100 mm depth and sieved to 2 mm using a PowerSoil™ DNA Isolation Kit following the manufacturer's instructions (MO Bio Laboratories Inc., Carlsbad, CA, USA) (Appendix i). Soil water content was also

measured at the time of DNA extraction. The yield of DNA extracts was verified by 260/280 nm measurements using a Nanodrop spectrophotometer (Thermo Fisher). The quality of extracted DNA was verified by running a 5-µl aliquot of each extract on a 1 % TAE-agarose gel stained with Syber Safe Gel stain (Invitrogen) and visualized under UV light. DNA was stored at –20 ^{o}C until analysed. The recovery of the DNA from soils was checked by re-extracting the DNA from soils that had the highest and lowest DNA contents. The two sets of DNA extraction for both the soils were tested using the 2-sample T-test (Table 3.2), which revealed that for each soil the two sets of extractions were not significantly different, confirming the extracted DNA was representative of that soil.

Table 3.2 Amount of representative DNA extracted from soil samples. Data are mean ± standard error of mean (n = 6)

Soil	**Amount of DNA (µg g^{-1} dry soil)**		**T-test *P* value**
	Set 1	**Set 2**	
Manawatu Fine Sandy Loam	62.6 ± 13.7	55.2 ± 13.0	0.719
Otorohanga Silt Loam	14.66 ± 3.59	5.09 ± 2.50	0.117

3.4.2 Standardisation of Polymerase Chain Reactions for universal bacterial and denitrifier genes

PCR, the thermally controlled, polymerase-catalysed chain reaction, allows for the extremely efficient and sensitive enzymatic amplification of target DNA regions. Target DNA is amplified using universal or specific primers (short DNA fragments) containing sequences complementary to the target region. PCR-primers anneal to these regions of the target DNA allowing DNA polymerase to enable selective and repeated amplification.

Following previously published protocols, PCRs were performed to amplify denitrifying (*nir*S, *nir*K, *nos*Z) and universal bacterial genes (*rpo*B) from the DNA extracts. The PCR amplifications were tested with a number of available (published) primers for each gene (Table 3.3) until amplification of the specific base pair length was obtained for each gene. Thereafter primers mentioned in sections 3.4.3 and 3.4.4 was decided to be used in this study. The amplification of the genes using the published primers was performed according

to the protocols available in the respective literature. In the case of no amplification, modifications of denaturation temperature (ranging from 55 ^{o}C to 62 ^{o}C), numbers of cycles (ranging from 30 to 35), concentration of primers (from 0.3 to1.0 μM of each primer) or DNA templates (from 0.5 to 2 μl) were made until specific products were obtained. Finally, we decided to use the reaction mixture compositions described in sections 3.4.3 and 3.4.4 for various PCRs performed during the research.

Table 3.3 List of primers used for optimisation of PCR amplification of denitrifier gene

Genes	Primers tested	Primers used in research
*Nir*S	*nir*S-Cd3aF (5'-GTSAACGTSAAGGARACSGG-3') , R3cd(5'-GASTTCGGRTGSGTCTTGA-3') (Enwall *et al.*, 2010)	*nir*S-Cd3aF, R3cd
*Nir*K	*nir*K-F1aCU(5'-ATCATGGTSCTGCCGCG-3')-R3Cu(5'-GCCTCCATCAGRTTGTGGTT-3') (Hallin & Lindgren, 1999)	Copper-583F, 909R
	*nir*K-1F(5'-GGAATGGTGCCCTGGCA-3')-3R(5'-GAACTTGCCGGTGTCCAGAC-3') (Braker *et al.*, 1998)	
	*nir*K-1F(5'-GGAATGGTGCCCTGGCA-3')-5R(5'-GCCTCGATCAGATTGTGG-3') (Wolsing & Priemé, 2004)	
	*nir*K-2F(5'-GCGATCATGGTGCTGCC-3')-5R(5'-GCCTCGATCAGATTGTGG-3') (Dandie *et al.*, 2011)	
	*nir*K-2F(5'-GCGATCATGGTGCTGCC-3')-3R(5'-GAACTTGCCGGTGTCCAGAC-3') (Braker *et al.*, 1998)	
	Copper-583F(5'-TCATGGTGCTGCCGCGKGACGG-3')-909R(5'-GAACTTGCCGGTPGCCCAGAC-3') (Dandie *et al.*, 2011)	
*Nos*Z	*nos*Z-1F(5'-CGYTGTTCMTCCACAGCCAG-3')-1622R (5'-CGCRASGGCAASAAGGTSCG-3') (Throbäck *et al.*, 2004);	*nos*Z 2F, 2R
	*nos*Z-1F(5'-ATCCGCTTGTCACTCGACAGCCAG-3')-1R(5'-ATGTCGATCAAGCTGACGGTCAGTTCTTC-3')(Henry *et al.*, 2006);	
	*nos*Z-2F(5'-CAKRTGCAKSGCRTGGCAGAA-3')-2R(5'- CGCRACGGCAASAAGGTSMSSGT-3') (Henry *et al.*, 2006)	
*rpo*B	1698 F (5'-AACATCGGTTTGATCAAC-3')- 2041 R (5'-CGTTGCATGTTGGTACCCAT-3') (Dahllöf *et al.*, 2000)	1698 F, 2041 R
16S rRNA	27F(AGAGTTTGATCMTGGCTCAG) (Edwards *et al.*, 1989)- 519R(GWATTACCGCGGCKGCTG) (Turner *et al.*, 1999)	27F,519R

*Nir*S and *nir*K = nitrite reductase genes, *nos*Z = nitrous oxide reductase gene, *rpo*B = ribosomal polymerase gene

3.4.3 Determination of denitrifier community structure using PCR

Once the gene of interest is amplified from DNA by PCR, the resulting amplicons are separated in different ways to obtain a 'community profile' or 'fingerprint' of target organisms (denitrifiers in this study). Separation and resolution of amplicons is typically achieved by electrophoresis on acrylamide gels or by use of a capillary sequencer. The individual amplicons are separated with varying degrees of specificity, as bands on a gel, or as peaks on an electropherogramme. Individual bands or peaks are considered "operational taxonomic units" (OTUs), and do not necessarily correspond to a single species.

Several methods are available for the analysis of functional genes, including terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) or its variant temperature gradient gel electrophoresis (TGGE), and single stranded conformation polymorphism (SSCP). Community profiling methods have been reviewed in section 2.5.2. Of the above mentioned methods, T-RFLP and DGGE are particularly popular for the study of denitrifying communities in soils.

3.4.3.1 Protocol followed for T-RFLP during the research

The denitrifier genes encoding nitrite reductase *nir*S and *nir*K were amplified using labelled primers on the 5' end with the fluorochrome 6-FAM (6-carboxyfluorescein) *nir*S Cd3aF, R3cd and *nir*K Copper 583F, 909R. The PCR amplification was performed in a total volume of 25-µl reaction mixture containing 2.5 µl of 10×PCR buffer (1 mM $MgCl_2$), 0.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 1.25 U of *Taq* polymerase (Fisher *Taq*, Thermofisher Scientific® Inc.), 0.8 mg/ml Bovine Serum Albumin (BSA), 1.0 µM of each primer, and 10 ng DNA template per reaction. PCR was accomplished in a thermocycler (MaxyGene Thermalcycler, Axygen). The PCR amplification consisted of an initial denaturation of the DNA template at 94 ^{o}C for 30s, followed by 35 cycles of 20 s at 94 ^{o}C, 20 s at 56 ^{o}C, and 20 s at 68 ^{o}C. The reaction was completed by 10 min at 68 ^{o}C. The lower than usual extension temperature (68 ^{o}C) of the reactions (72 ^{o}C) in these reactions was based on the specifications of the *Taq* polymerase used for PCR amplification.

The amplification of *nosZ* gene was achieved with the 5' end labelled primer *nosZ* 2F, 2R. The PCR amplification was performed in a total volume of 25µl reaction mixture containing 10 µl of 2 × NEB Taq master mixes (New England Biolabs® Inc.) (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 % Glycerol, 0.08 % IGEPAL® CA-630, 0.05 % Tween® 20, 25 units/ml *Taq* DNA Polymerase, and pH 8.6 at 25 °C), 0.4 µM of each primer, and 10 ng DNA template per reaction. Due to the higher cost of Fisher *Taq*, it was decided to use NEB *Taq* for future PCR. PCR was accomplished in an Axygen Maxygene Thermocycler (Fisher Biotech, Australia). PCR consisted of an initial denaturation of the DNA template at 94 ^{o}C for 2 min, followed by 35 cycles of 30 s at 94 ^{o}C, 1 min at 56 ^{o}C, and 1 min at 72 ^{o}C. The reaction was considered complete after 10 min at 72 ^{o}C.

All PCR were accompanied by a negative (no DNA) control as well as a positive control reaction that targeted a 492 bp fragment of 16S rRNA using the forward primer 27F (Edwards *et al.*, 1989) and the reverse primer 519R (Turner *et al.*, 1999). The PCR product quality and yield were verified by gel electrophoresis on a 1.0 % w/v agarose gel in 1 × TBE buffer stained with SyberSafe gel stain and visusalized under UV light.

A 12 µl aliquot of each PCR product was combined with 0.5 µl of restriction endonuclease *Hha*I (New England Biolabs® Inc.), 2.0 µl 10 × NEB Buffer 4, and 0.2 µl BSA in a total volume of 20 µl and digested for 16 hours at 37 ^{o}C to produce a mixture of variable length, end-labelled fragments (Castro-González *et al.*, 2005). After the restriction enzyme digest, the DNA samples were purified to remove unused enzymes and other reagents, using an Axyprep™ PCR clean up kit (Axygen Biosciences) following the manufacturer's instructions (Appendix ii). Cleaned amplicons were stored at –20 ^{o}C. T-RFLP analysis was accomplished by capillary separation on the ABI3730 Genetic Analyzer (Applied Biosystems) at the Massey Genome Service (Massey University Palmerston North, NZ).

The data obtained after genotyping were analysed using Peak Scanner™ software (Applied Biosystems). The data were exported to MS Excel, and peaks were sorted based on the size of the fragments. To avoid inclusion of primers, fragments below 40 bp in length were discarded. To avoid the inclusion of errors due to contamination of T-RF signals with background fluorescence we discarded peaks smaller than 100 fluorescence units in height. A contingency table based on presence or absence of Terminal Restriction Fragments (T-RFs) was prepared. The total and average numbers of T-RFs were calculated for each soil sample.

3.4.3.2 Measurement of Richness and Diversity of denitrifier genes

There are many indices to measure richness and evenness proposed by Magurran (1988). In this thesis, evenness in the phylotype distribution of denitrifier genes has been calculated using Pielou's coefficient based on the Shanon index (H') due to its high discriminant ability, simple calculation and wide acceptance (Magurran, 1988). The total number of unique T-RFs per sample was used as a measure of genotype richness. The evenness of a community was represented by Pielou's evenness index (J) – where J is derived from the Shannon diversity index – and was calculated for each sample using the formulae in Eqs 3.6 and 3.7. J is constrained between 0 and 1. A higher value of J indicates the communities are more evenly distributed in a sample.

$$H' = -\sum_{i=1}^{n} Pi\ Ln(Pi) \qquad \text{(Eq 3.6)}$$

where: Pi = Relative abundance of each phylotype with respect to total number of phylotypes in a sample.

Pielou's coefficient of evenness (J) was calculated for each sample using the following formula:

$$J = H'/LnS \qquad \text{(Eq 3.7)}$$

where: S = Phylotype richness, i.e. sum number of phylotypes in a sample.

3.4.4 Determination of denitrifier gene abundance using qPCR

While T-RFLP is a useful tool for providing a qualitative view of microbial community structure and relative differences in the distribution of denitrifier phylotypes, the quantification of gene copy numbers is often desired for more accurate estimates of denitrifier abundance. The key feature of real-time qPCR is that the amplified DNA is detected as the reaction progresses in real time. In this thesis, SSoFast was used as double-stranded DNA (dsDNA) binding dye to monitor amplicons synthesis. It was preferred to labelled-probes for cost considerations as it can be used

for any reaction without sequence information. However, SYBR® green assays do not allow that the discrimination between amplicons sequences and false-positives may occur. Therefore, a post-dissociation curve analysis should be carried out to confirm that the fluorescence signal is generated only from target templates and not from the formation of non-specific PCR products or primer-dimers (Smith & Osborn, 2009).

In order to assess the abundance of denitrifying bacteria relative to the total bacterial community in the samples, quantification of a gene representing the universal bacterial community is done. Studies have reported the use of the 16S rRNA gene to quantify the total bacterial community ((Case *et al.*, 2007). However, the occurrence of multiple copies of 16S rRNA genes in some bacteria poses a difficulty in accurately assessing universal bacterial diversity. The gene coding for the beta sub-unit of the RNA polymerase *rpo*B has been used as an alternative marker for the microbial community studies. This gene is described as possessing the same characteristics as 16S r RNA (Dahllöf *et al.*, 2000) and, more importantly, the *rpo*B gene exists as a single copy in the bacterial genome (Mollet *et al.*, 1997) thus allowing for accurate estimation of abundance of bacterial communities in environmental DNA samples. In order to avoid the bias due to the use of 16S rRNA genes to describe total bacterial abundance the abundances of *rpo*B genes have been used in the current study.

3.4.4.1 Protocol followed for qPCR during the research

The quantification of bacterial *nir*S, *nir*K, *nos*Z, and *rpo*B genes in soil samples was assessed by qPCR using the *nir*S, *nir*K, and *nos*Z primers described above, and *rpo*B 1698 F (5'-AACATCGGTTTGATCAAC-3'), 2041 R (5'-CGTTGCATGTTGGTACCCAT-3') (Dahllöf *et al.*, 2000). qPCR was performed in a total volume of 10 µl of reaction mixture containing 5 µl of 2× Standard reaction SsoFast™ Evagreen® Supermix (Bio-Rad) as the detection system, and 0.3 µM of each primer and 5 ng DNA template per reaction. The reaction mixtures were placed in a PCR Lightcycler 480® (Roche Applied Science). The PCR amplification was carried out with an initial denaturation of the DNA template at 98 ^{o}C for 30s, followed by 40 cycles of 5s at 98 ^{o}C, 30s at 60 ^{o}C for *nir*S, *nir*K, *nos*Z, and 55 ^{o}C for *rpo*B and a continuous elongation from 65–95 ^{o}C. Melt curve analysis was performed for each reaction to confirm the specificity of the amplicon.

Duplicate assays were performed for each sample. All qPCR assays included negative control (without template DNA) and reactions containing between 10^3 and 10^9 standard DNA copies to generate standard curves and calculate amplification efficiencies according to Eq 3.10 (Pfaffl, 2004). DNA standards consisted of linearized plasmids (Hou *et al.*, 2010) with inserts containing cloned PCR products of *nir*S, *nir*K, *nos*Z and *rpo*B genes, as appropriate. Cloning reactions were carried out using a TOPO® TA cloning® kit (Invitrogen Life Technologies), following the manufacturer's instructions. Standards were sequenced to confirm the correct identity of the target. The sequencing was performed by Sanger sequencing using a capillary ABI3730 Genetic Analyzer (Applied Biosystems Inc.) at the Massey Genome Service (Massey University Palmerston North, NZ).

Amplification efficiencies of qPCR reactions for samples were within the expected range of values (E = 90–110 %) based on previous reports (McPherson & Moller, 2006). The gene copy numbers in the DNA template of each soil sample were calculated using Eq 3.8 (Lee *et al.*, 2008). The reactions were linear over 7 orders of magnitude and sensitive down to 10^2 copies, similar to the results reported in other studies (Bach *et al.*, 2002; Grüntzig *et al.*, 2001; Kolb *et al.*, 2003; López-Gutiérrez *et al.*, 2004; Stubner, 2002). PCR product quality and yield were verified by gel electrophoresis on a 1.0 % w/v agarose gel in 1 × TBE buffer and stained with SyberSafe gel stain and visusalized under UV light.

$$\text{Gene copy numbers} = \frac{AN \times D}{L \times 10^9 \times 650} \qquad \text{(Eq 3.8)}$$

Andrew Staroscik 2004 http://www.uri.edu/research/gsc/resources/cndna.html

where: AN = Avogadro's number (6.023×10^{23})

D = Amount of DNA template (the amount of DNA in standard sample in ng); L = DNA length (the length of amplified target DNA after PCR), 650 = weight of 1 mole of base pair in g.

The Lightcycler instrument records the maximum numbers of PCR cycles taken for a DNA sample to reach maximum amplification or accumulation of double stranded DNA in the form of a Cp value (crossing point). The number of gene copies that each of the serial dilutions of the standard sample contained was log transformed using Log-10, and the Cp values (the cycle numbers from lightcycler to initiate amplification in a DNA sample) obtained after real-time

qPCR plotted against the log-transformed gene copy number. This standard curve was used to calculate the reaction efficiency using Eq. 3.9 with gene copy numbers in DNA samples of various soils calculated using Eq. 3.11.

$$\text{Efficiency of the PCR (E)} = (\left(10^{-1/m}\right)-1)\times 100 \quad \text{(Pfaffl, 2004) (Eq 3.9)}$$

$$\text{No. of gene copies per sample} = 10^{(Cp-b)/m} \quad \text{(Lee } et\ al.\text{, 2008)} \quad \text{(Eq 3.10)}$$

where: Cp is the crossing point of the DNA sample, b is the y-intercept and m is the slope of the linear regression line fitted on the standard curve.

3.5 PROTOCOLS FOLLOWED FOR THE MEASUREMENT OF CHEMICAL CHARACTERISTICS OF SOIL DURING RESEARCH

3.5.1 Microbial biomass Carbon (MBC)

Microbial biomass carbon was determined using the chloroform fumigation-extraction technique described by Vance *et al.* (1987). The amount of C in the 0.5M K_2SO_4 extracts was determined by potassium dichromate oxidation (Jenkinson & Powlson, 1976) in which 8-ml aliquots of a 1:5 soil:extract solution were added to a mixture of sulphuric and orthophosphoric acids and refluxed at 150 ^oC for 30 min. The excess dichromate was then titrated with ferrous ammonium sulphate. Microbial biomass carbon content in the soil was calculated by subtracting the amount of C in the non-fumigated samples from that in the fumigated samples.

To check the recovery of the analytical procedure, three sets of glucose solutions were prepared with 25, 50. and 75 ppm C. We took 8-ml aliquot of these solutions and performed the steps mentioned above. After titration we checked the recovery of C in each solution by:

$$\%\ \text{C lost} = \left(\frac{C_{bd}-C_{ad}}{C_{bd}}\right)\times 100 \quad \text{(Eq 3.11)}$$

$$\%\ \text{C recovered} = 100 - \%\ \text{C lost} \quad \text{(Eq 3.12)}$$

where: C_{bd} is carbon added before digestion; C_{ad} is carbon determined after digestion and titration.

There was a 91 % per cent recovery of C in the 25 ppm solution, a 90 % in the 50 ppm, and an 85 % in the 75 ppm solution.

3.5.2 Soluble C

The soluble C in soils was the amount of C extracted in 0.5M K_2SO_4 (Ross, 1988, 1990). This corresponds to the amount of C in non-fumigated samples as described above.

3.5.3 Mineral-N

Soil NO_3^--N and NH_4^+-N were determined in fresh sieved soils by 1 hr soil extraction with 2 M KCl solution at a 1:5 soil:extract ratio, and subsequent colorimetric analysis of the filtrate using the automatic analyser method of Downes (1978).

3.5.4 Soil pH

Soil pH was measured in a 1:2.5 (w/w) soil to water mixture using a PHM 83 Autocal pH meter after leaving the mixture overnight to equilibrate.

3.5.5 Gravimetric soil water content

The gravimetric soil water content is the weight of water present in soil and calculated as

$$\text{SWC} = \left(\frac{\text{Weight of water (g)}}{\text{Weight of dry soil (g)}}\right) \times 100 \qquad \text{(Eq 3.13)}$$

Soil water content was determined gravimetrically by initially weighing fresh soils, oven-drying at 105 ˚C for 24 hours and again weighing the dried soil until constant weight was achieved.

3.5.6 Amount of water needed at field capacity (FC)

The amount of water needed to wet the soils to FC was determined using the suction plate method (Loveday, 1974). Soil samples (as received, sieved to 2 mm) were saturated with water on a suction plate and the plate was fitted to bubble tower with the suction pump set at a pressure of 0.1 bars. The assembly was kept undisturbed for 24 hours to pump out the excess water absorbed by soils. After 24 hours, the gravimetric soil water content was determined by drying

the wet soil at 105 ^{o}C for 24 hours. This soil water content corresponded to the amount of water in the soils at FC.

3.5.7 Water filled pore space percentage (WFPS %)

The WFPS of soil as percentage was calculated from equation:

$$\text{WFPS (\%)} = \left(\frac{\theta_m \times P_B}{TP \times \rho H_{2O}}\right) \times 100 \qquad \text{(Eq 3.14)}$$

where: θ_m is gravimetric water content (g/g), P_B is the soil bulk density (g cm^{-3}), ρH_2O is density of water (g cm^{-3}), and TP is the total porosity (Luo *et al.*, 2007).

$$TP = \left(1 - \frac{\text{Bulk Density}}{\text{Particle Density}}\right) \times 100 \qquad \text{(Eq 3.15)}$$

3.5.8 Total carbon (TC) and total nitrogen (TN)

Sub-samples of the collected soils were air dried, sieved (<2-mm size) and analysed for total carbon (TC), total nitrogen (TN) and Olsen P. The total C and N contents of the soil samples were determined by dry combustion on air-dry, finely ground soils using a Laboratory Equipment Corporation (LECO) CNS Analyser LECO FP-2 CNS Analyser Leco Corp, ST Joseph, MI, USA (Bremner, 1996; Nelson & Sommers, 1996).

3.5.9 Olsen P

Olsen P (Olsen *et al.*, 1954) was determined by the phosphomolybdate method of Murphy & Riley (1962) using a Spectrophotometer PU 8625 UV/VIS at 712 nm.

Chapter 4

Soil properties influencing denitrifiers and denitrification in New Zealand dairy-grazed pasture soils

4.1 INTRODUCTION

Denitrification, an anaerobic respiratory process of stepwise conversion of nitrate (NO_3^-) to dinitrogen (N_2), has been reported in a wide range of taxonomically diverse groups of bacteria and archaea (Ishii *et al.*, 2010; Philippot *et al.*, 2007; Tiedje, 1994) and also among some eukaryotes (Zumft, 1997). The process is mediated by four reductase enzymes: nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide (N_2O) reductase (N_2OR) (Zumft, 1997). These enzymes are encoded by *nar*/*nap*, *nir*, *nor*, and *nos* genes, respectively. Denitrifying bacteria are widely distributed in the environment (Graham *et al.*, 2014) and more than 60 genera of these bacteria have been identified so far (Chen *et al.*, 2012b).

Denitrifying bacteria may possess either the full set of four enzymes or only some of them (Dandie *et al.*, 2008; Wallenstein *et al.*, 2006). Denitrifiers such as *Paracoccus denitrificans* possess genes encoding all four reductase enzymes, and are thus able to transform NO_3^- directly to N_2. However, some other denitrifying bacteria like *Agrobacterium tumefaciens* lack N_2OR and will emit N_2O as the end product of denitrification (Wood *et al.*, 2001). Denitrifier community structure and abundance of particular denitrifying bacteria are important factors that determine denitrification in soil, and we require this knowledge to develop an understanding of the process in soils (Henry *et al.*, 2006). Our understanding of the denitrifier population in dairy pasture soils in New Zealand contributing to N_2O emissions is still limited.

Molecular studies have yielded conflicting results regarding the effect of environmental variables on denitrifier community structure and whether or not community structure is related to denitrification. Some studies have found significant correlations between denitrification as measured using the denitrification enzyme activity (DEA) assay and the diversity of denitrifier communities (Wertz *et al.*, 2009) or abundance (Enwall *et al.*, 2010; Hallin *et al.*, 2009);

however, other studies have reported that denitrifier diversity (Attard *et al.*, 2011) or abundance (Dandie *et al.*, 2008) is unrelated to DEA.

Several biotic and abiotic factors such as competition, predation, O_2 content, pH and substrate availability affect the diversity of denitrifying bacteria in soils (Franklin & Mills, 2003; Ladd *et al.*, 1996) and ultimately the rate of denitrification. The abundance of denitrifiers can have major impact on the rate of denitrification at a given place (Philippot & Hallin, 2005; Wallenstein *et al.*, 2006). Studies have shown that environmental variables affect microbial communities and their functions directly or indirectly (Boyle *et al.*, 2006; Mergel *et al.*, 2001a; Wolsing & Priemé, 2004).

Many studies of denitrification in New Zealand have related N_2O emissions with soil and environmental parameters using the DEA assay (Luo *et al.*, 1994a, b) or measurements of denitrification rate (DR) using acetylene inhibition technique (AIT) (Ruz- Jerez *et al.*, 1994; Zaman *et al.*, 2008b; Zaman & Blennerhassett, 2010). However, there remains a lack of understanding of the soil or environmental variables that drive the abundance and activity of denitrifying bacteria. This study measured the denitrification rate (DR), denitrification enzyme activity, and denitrifier genes distributions and abundances in New Zealand dairy pasture soils. The objectives of the study were to:

i) quantify denitrification rate, denitrification enzyme activity, denitrifier community structure and its abundance in 10 New Zealand dairy grazed pasture soils
ii) understand the relationship between soil physicochemical characteristics and denitrification activities (DEA and N_2O emissions) in New Zealand soils
iii) explore the relationship between soil physicochemical characteristics and denitrifier gene distributions and abundances in these soils
iv) relate denitrifier community richness and abundance with denitrification in soils.

4.2 MATERIALS AND METHODS

4.2.1 Description of soils sites

Pasture soils with varying physical and chemical characteristics were collected from 10 New Zealand dairy farms (Fig. 4.1). All the sites from which the soil samples were collected are commercially managed farms (Table 4.1). The farm sites contained mixed pasture, perennial ryegrass (*Lolium perenne*), and white clover (*Trifolium repens*). The annual fertilization varied on sites and consisted of application of 150–200 kg N ha^{-1}. TeK, HR, PL, and MF soils annually received 150 Kg N ha^{-1}, OH and MWEI received 160 kg N ha^{-1}; on the other hand, PS, LM, and MW received 200 kg N ha^{-1}. The soils were selected on the basis of their geographical location (North or South Island of New Zealand), variation in mineralogy non allophanic (MW, MWEI, PS, PL, TM, LM) to allophanic soils (HR, TeK, OH). These sites represented soil varying in physical characteristics from poorly drained (TM, TeK) to well drained (OH, HR, MW, MWEI) soils. Soil texture varied from stony silt loam (LM) to fine sandy loam (MW). The two Manawatu soils were collected from two adjoining paddocks; one had no irrigation, while the other had received effluent irrigation at the rate of 10 000 l ha^{-1} every 2 months for the past 4 years. The effluent application was 2 weeks before the collection of soil samples. There are two Paparua soils collected from dairy farms in Springston and Lincoln.

4.2.1.1 Soil origin and parent material: The 10 soils collected varied in their parent material and development. The TeK soils are fine textured and poorly drained, resulting from compact subsoil layers with slow permeability. Their parent material is fine rhyolitic alluvium subject to high fluctuating water tables, which have developed the pale gley subsoils and prominent mottles so characteristics of gleyed soils. Pockets of manganese and iron concretions can be found with the mottles in the sub-soil. There is no allophane in these soils, the gleying leads to the formation of kaolin type clays. The OH silt loam is central yellow brown loam developed from andesitic and rhyolitic terphra. These soils are strongly leached and have a high content of amorphous clay allophane. The HR silt loam is well drained with moderate permeability, sandy and gravelly soils of the broad levees. Parent material is coarse rhyolitic alluvium sufficiently weathered to exhibit the properties of volcanic loams.

The TM silt loams have formed on the high terraces on the south-eastern margins of the Rangitiki and Manawatu Rivers where the loess is thick and coarse. The morphology of this soil is quite striking, consisting of a relatively clay-rich mottled horizon above an impermeable fragipan of relatively unaltered silty loessial material that has cracked to form vertical columns.

The MW soil is formed from weakly weathered quartzo-feldspathic sediment washed down off the Ruahine and Tararua Ranges and deposited on the lowland plains as alluvium by the main river systems. Manawatu fine sandy loams are well drained and have moderate permeability throughout the profile. These soils have a high structural vulnerability and thus need to be well managed to maintain soil quality.

The LM soils are formed from gravelly glacial outwash with a variable depth of silty loess deposit at the surface. The soil is well drained and has moderate to rapid permeability. The LM soils are the best known example of the stony terrace soils of the Canterbury plains.

The detailed characteristics of these 10 soils are described in section 4.3.1.

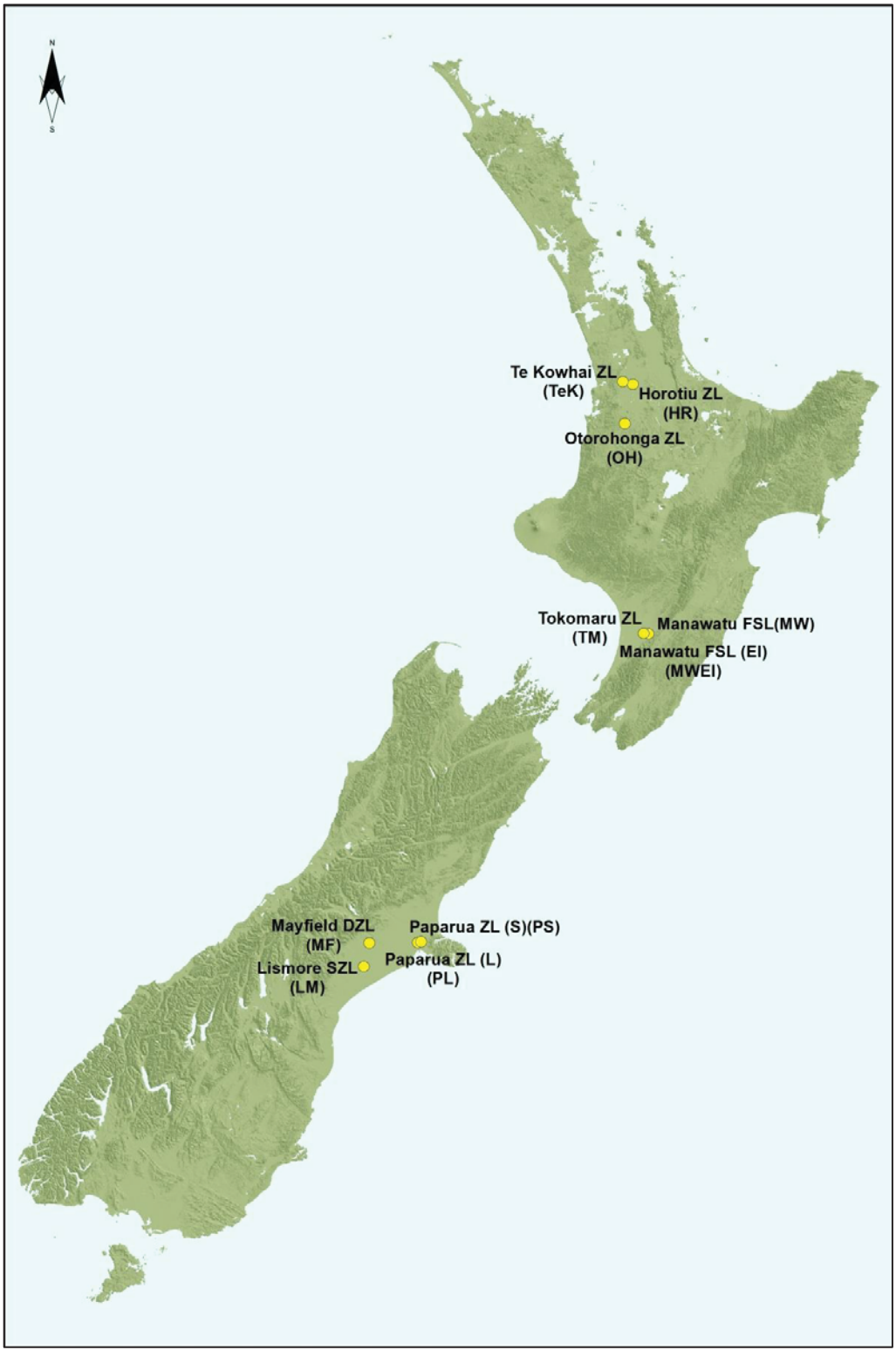


Figure 4.1 New Zealand map representing the location of the collected dairy-pasture soils. ZL = silt loam, FSL = fine sandy loam, DZL = Deep silt loam and SZL = stony silt loam.

Table 4.1 Description of soils collected for experiment

Soil	Location of the dairy farm	Geographical Location	NZ Soil Classification	USDA Soil Classification	Mineralogy Class	Date of sampling
Te Kowhai Silt Loam (TeK)	AgResearch Ruakura Waikato	37°44'57.55"S 175°10'27.06"E	Typic Orthic Gley	Typic Ochraqualf/Vintrandept	GlassyVolcanic , Kaolinitic	August 2010
Otorohanga Silt Loam (OH)	Tokanui Waikato	38°11'19.70"S 175°12'35.67"E	Typic Orthic	Typic Dystrandept Ochraqualf	Allophanic	August 2010
Horotiu Silt Loam (HR)	AgResearch Ruakura Waikato	37°46'30.80"S 175°18'23.27"E	Typic Orthic Allophanic	Typic Udivitrand Vitrandept	Allophanic	August 2010
Tokomaru Silt Loam (TM)	Massey University, Palmerston north	40°22'58.50"S 175°36'31.01"E	Argillic-fragic Perch-gley Pallic	Typic Fragiaqualf	Vermiculitic	September 2010
Manawatu Fine Sandy Loam (MW)	Longburn, Palmerston North	40°22'56.99"S 175°32'24.49"E	Weathered fluvial recent	Dystric Fluventic Eutrochrept	Illitic	November 2010
Manawatu Fine Sandy Loam (Effluent irrigated) (MWEI)	Longburn, Palmerston North	40°22'58.26"S 175°32'21.65"E	Weathered fluvial recent	Dystric Fluventic Eutrochrept	Illitic	December 2010
Paparua Silt Loam (Springston) (PS)	Springston, Christchurch	43°38'15.97"S 172°28'13.81"E	Weathered Orthic recent	Typic Dystrustepts	Illitic	December 2010
Paparua Silt Loam (Lincoln) (PL)	Lincoln, Christchurch	43°38'43.91"S 172°25'21.86"E	Weathered Orthic recent	Typic Dystrustepts	Illitic	December 2010
Lismore Stony Silt Loam (LM)	Ashburton, Canterbury	43°53'17.44"S 171°38'28.43"E	Pallic Orthic Brown	Typic Dystrustepts	Vermiculitic	December 2010
Mayfield Deep Silt Loam (MF)	Methven, Canterbury	43°38'30.12"S 171°43'47.28"E	No data	No data	No data	December 2010

4.2.2 Collection of soil samples

Twenty-five soil cores (25 mm diameter × 100 mm long) were collected from the 0–100 mm and 100–200 mm depths using a steel corer from six random locations of 100 m^2 area on each farm between August and December 2010 (10 sites × 6 replicates × 2 depth = a total of 120 samples). The 25 cores from each location were pooled but the 6 replicates from each farm were stored separately (n = 6). Field fresh soil cores were taken to the laboratory, sieved to 2 mm, and stored at 4 ºC in plastic bags. A sub-sample of each soil replicate was stored at –20 ^{o}C for molecular analysis. DEA and DR were measured within 2 days of collection, chemical and molecular analyses were carried out within 6 months of collection of soil samples. Since collected samples were either refrigerated or frozen until analysed no significant change in their biochemical or molecular characteristics during storage was expected.

4.2.3 Soil Characteristics

The methods used for the analyses of the soil samples collected above are described in chapter 3 of this thesis, therefore only a brief outline is given below. The field-moist sieved soils were analyzed for gravimetric soil water content (SWC), mineral N (NO_3^- and NH_4^+), total nitrogen (TN), total carbon (TC), pH, Olsen P, microbial biomass carbon (MBC), soluble C using the standard protocols followed in laboratory as given in section 3.5. Denitrification measurements (DEA and N_2O emissions from C_2H_2 and non-C_2H_2 treated jars) were conducted using AIT described in sections 3.2 and 3.3. Denitrifier community structure and abundances in the soils collected from 0–100 mm depth were measured using PCR based techniques T-RFLP and qPCR respectively following the protocols outlined in section 3.4. The total number of unique denitrifier gene T-RFs per sample was considered as measure of T-RF richness. Pielou's coefficient of evenness (J) was used as an indication of the evenness of denitrifier gene communities. Pielou's J is calculated from the Shannon diversity index (H') as described by Eqs 3.6 and 3.7.

4.2.4 Statistical Analysis

The data for soil chemical characteristics, gaseous emissions and denitrifier community structure were analysed using Minitab 16 software. Data normality was evaluated using the Shapiro-Wilk

normality test (Shapiro & Wilk, 1965). As the assumptions of normality of data were violated for some of the parameters, the data sets were transformed to normal using the Box-Cox transformations.

Since the soil chemical characteristics, and denitrification activities, were measured on 6 replicated samples, but pairs of the 6 replicates had been combined to give a total of 3 replicates for DNA analysis, the chemical characteristics, the average value of DEA and DR measurements were pooled in the same way to yield 3 replicates for statistical analysis. The pooling was done to minimise the cost of analysis and the amount of reagents required for molecular analysis. The effect of soil and sampling depth on the means of soil characteristics (pH, mineral N, TN, TC, Olsen P, MBC, DEA, DR) was assessed using a 2-way analysis of variance (ANOVA).

The differences in the means of soil characteristics such as pH, mineral N, TN, TC, Olsen P, MBC, DEA, DR, and molecular parameters number of T-RFs and gene copy numbers were assessed using a one-way analysis of variance test by keeping soil characteristics as the response and soil type as a factor. The Tukey's Studentized Range Test at $\alpha = 0.05$ significance level was used *post hoc* to reveal significant differences among means. The relationships among the soil chemical characteristics pH, mineral N, TN, TC, Olsen P, MBC, DEA, DR, number of denitrifier gene T-RFs, and abundance were determined using Pearson's correlation analysis.

The principal components analysis (PCA) was performed using the R computing environment v 2.15.3 (R Development Core Team, 2010). In order to examine the distribution of physiochemical characteristics of 10 soils, these soil parameters were used as factors for PCA. PCA is sensitive to relative scaling of the original variables. Therefore, the data were z-standardised (Manly, 2004). Similarly, the soil physiochemical characteristics were used as factors for PCA. The Eigenvalue and ratio of explained variances were obtained for PCA and are displayed in respective tables in Appendix iii. In order to illustrate the strength of correlation of factors with principal components projection plots (biplot) were prepared. A biplot uses points to represent the scores of the observations on the principal components, and it uses vectors to represent the coefficients of the variables on the principal components.

The cluster analysis was performed based on hierarchical distances to connect soils to form groups based on their Euclidean distances from one another using single, average, and Ward link methods. Based on the clusters, it was decided to illustrate the groupings based on average link method. The clusters were represented as dendograms to illustrate groupings of soils based on similar characteristics by using both the single and average link methods.

Multiple regression analysis on transformed data was performed following the stepwise backward elimination model at $\alpha = 0.10$. The model was selected based on the values of R^2, R^2 adjusted and the Mallow's Cp respectively. As the R^2 values were similar among the various models,

the model with Cp value close to total number of variables plus intercept or just smaller were selected.

4.3 RESULTS

4.3.1 Variations in soil characteristics with sampling depths

The two-way analysis of variance (Tables 4.2 & 4.3) indicated that the soil chemical characteristics significantly varied as a function of soil type and soil depth. The interaction of both soil and soil depth had significant effect on the soil characteristics; however, the effect of this interaction varied among various soil types. The differences in soil characteristics with depths showed that the TN, TC, and Olsen P contents were significantly higher in surface soils than sub-surface soils but in the TM these were same at both the depths. Soil pH did not differ among the two depths except in the OH and the PL soils. In the OH soil pH was lower at 0–100 mm depth and in the PL soil it was higher than pH at 100–200 mm depth. NO_3^--N content in soils was significantly higher in surface soils than in sub-surface soils, except in the PS soil in which NO_3^--N was the same at two depths. On the other hand, there was no significant difference in NH_4^+-N content in soils at two depths other than the OH and the PS soils: in both these soils NH_4^+-N content was lower in sub-surface samples. The MBC content and DEA were significantly higher in 0–100 mm depth than the 100–200 mm depth in all the soils. DR was significantly higher in all the soils at 0–100 mm depth than 100–200 mm depth except PL and LM in which DR was similar at both the depths.

4.3.2 The range of measured soil characteristics

Soils used in this study varied in their physical and chemical characteristics (Tables 4.4 and 4.5). In surface samples, the TC ranged from 36.2 to 82.6 g kg^{-1} soil, TN varied from 2.7 to 8.4 g kg^{-1} soil, both highest in the OH soil. Olsen P content in soils ranged from 22.7 to 60.7 mg kg^{-1} soil, with the highest P content in the MWEI soil. MBC ranged from 436 to 970 mg kg^{-1} soil and was the highest in the MW and MWEI soils. NO_3^--N content ranged from 5.4 to 58.7 mg kg^{-1} soil. The TM soil contained the least NO_3^--N and the MWEI soil had the highest NO_3^--N content. NH_4^+-N content in the soils varied from 0.9 to 13.4 mg kg^{-1} soil. The MWEI soil had the least amount of NH_4^+-N. Gravimetric SWC of field-moist, sieved soil samples ranged from 24 to 54 %. Water content was highest in the OH, HR, and MWEI soils.

In sub-surface samples the TC ranged from 24.8 to 59.4 g kg^{-1} soil, TN varied from 2.0 to 6.2 g kg^{-1} soil, both highest in OH soil. The Olsen P in soils ranged from 13.9 to 44.7 mg kg^{-1} soil, with the highest P content in the TM soil. MBC ranged from 210 to 510 mg kg^{-1} soil and was highest for TM soil. The NO_3^--N content ranged from 1.7 to 26.2 mg kg^{-1} soil. The TM soil contained the least NO_3^--N and the MWEI soil had the highest NO_3^--N content. The NH_4^+-N content in the soils varied from 0.7 to 10.4 mg kg^{-1} soil. The MWEI soil had the least amount of NH_4^+-N. Gravimetric SWC of field-moist sieved soil samples ranged from 23 to 55 %. Water content was the highest in the OH and HR soils.

Table 4.2 Two way ANOVA *P*-values of the chemical characteristics in different soils (S) used in this study collected from two sampling depths (0–100 mm and 100–200 mm) (D)

Source	Total-C	Total-N	pH	Olsen P	MBC	Soluble C	NO_3^--N	NH_4^+-N	SWC
S	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
D	0.0001	0.0001	0.8770	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
S × D	0.0001	0.0020	0.0001	0.0001	0.0001	0.0001	0.0020	0.1780	0.0001

ANOVA = analysis of variance, C = carbon, N = nitrogen, P = phosphorus, MBC = microbial biomass C, SWC = soil water content, NO_3^--N = nitrate –N, NH_4^+-N = Ammonical-N.

Table 4.3 Two way ANOVA *P*-values of the DEA, DR, and N_2O emissions in different soils (S) used in this study collected from two sampling depths (0–100 mm and 100–200 mm)(D)

Source	DEA	DR (N_2O-A)	N_2O-NA
S	0.0001	0.0001	0.0001
D	0.0001	0.0001	0.0001
S × D	0.0001	0.0001	0.0001

ANOVA = analysis of variance, DEA = denitrification enzyme activity, DR = denitrification rate (nitrous oxide produced in acetylene (C_2H_2) added jars, N_2O-A), and N_2O-NA = nitrous oxide produced in non-C_2H_2 added jars.

Table 4.4 Chemical characteristics measured in soils collected from 0 to 100 mm depth from 10 dairy-pasture farms in New Zealand. Data are mean (n = 6) ± Standard error of mean

Soil	Total C (g kg soil^{-1})	Total N (g kg soil^{-1})	pH (1:2.5, soil: water)	Olsen P (mg kgsoil^{-1})	MBC (mg kgsoil^{-1})	Soluble C (mg kgsoil^{-1})	Nitrate-N (mg kgsoil^{-1})	Ammo-N (mg kgsoil^{-1})	Gravimetric SWC (%)
MWEI	51.0 ± 2.0^{c}	5.2 ± 0.2^{c}	5.9 ± 0.05^{bc}	60.7 ± 6.0^{a}	970 ± 74^{a}	53 ± 13^{e}	58.7 ± 3.5^{a}	0.9 ± 0.1^{e}	52.8 ± 1.0^{a}
MW	44.6 ± 1.2^{d}	4.4 ± 0.1^{d}	6.3 ± 0.15^{a}	58.1 ± 5.9^{a}	836 ± 42^{a}	82 ± 7^{cd}	21.2 ± 4.0^{c}	3.8 ± 0.3^{d}	36.1 ± 1.4^{bc}
TM	36.2 ± 2.0^{e}	2.7 ± 0.1^{f}	5.7 ± 0.08^{cd}	50.5 ± 5.5^{bc}	645 ± 28^{b}	68 ± 5de	5.4 ± 0.3^{e}	5.6 ± 1.4^{bcd}	39.1 ± 3.4^{b}
TeK	25.87 ± 1.5^{f}	2.7 ± 0.1^{f}	5.6 ± 0.01^{d}	23.9 ± 1.4^{d}	548 ± 48^{bcd}	96 ± 4^{c}	13.5 ± 0.9^{d}	13.4 ± 1.8^{a}	39.9 ± 0.6^{b}
OH	82.6 ± 1.1^{a}	8.4 ± 0.4^{a}	5.6 ± 0.02^{d}	22.5 ± 0.5^{d}	464 ± 53^{cd}	259 ± 10^{a}	12.6 ± 0.9^{d}	11.6 ± 0.3^{a}	54.5 ± 0.7^{a}
HR	62.7 ± 0.8^{b}	6.4 ± 0.1^{b}	5.8 ± 0.02^{bcd}	24.1 ± 0.4^{d}	542 ± 20^{bcd}	194 ± 16^{b}	10.5 ± 1.6^{d}	12.8 ± 1.0^{a}	54.3 ± 1.3^{a}
PS	38.6 ± 0.6^{e}	3.5 ± 0.1^{e}	6.0 ± 0.06^{b}	37.0 ± 5.3^{c}	592 ± 48^{b}	102 ± 17^{c}	32.8 ± 3.1^{b}	4.5 ± 0.4^{cd}	33.5 ± 1.3^{cd}
LM	38.2 ± 0.7^{e}	3.7 ± 0.1^{e}	5.7 ± 0.08^{cd}	55.8 ± 5.9^{b}	658 ± 52^{b}	83 ± 4^{cd}	10.6 ± 1.5^{d}	12.9 ± 0.9^{a}	31.7 ± 1.6^{d}
MF	43.6 ± 1.8^{d}	4.4 ± 0.2^{d}	4.8 ± 0.08^{e}	53.2 ± 8.6^{b}	436 ± 76^{d}	212 ± 12^{b}	8.1 ± 0.8^{d}	8.1 ± 0.1^{b}	24.3 ± 0.6^{e}
PL	37.0 ± 1.1^{e}	3.0 ± 0.5^{f}	6.4 ± 0.04^{a}	28.8 ± 1.9^{c}	593 ± 23^{bc}	81 ± 3^{cd}	34.4 ± 4.0^{b}	6.7 ± 0.3^{bc}	30.5 ± 1.3^{d}

Letters denote 1-way ANOVA test. Values sharing the same letter are not significantly different. MWEI = Manawatu fine sandy loam (effluent irrigated); MW = Manawatu fine sandy loam; TM = Tokomaru silt loam; TeK = Te Kowhai silt loam; OH = Otorohanga silt loam; HR = Horotiu silt loam; PS = Paparua silt loam (Springston); LM = Lismore stony silt loam; MF = Mayfield silt loam; PL = Paparua silt loam (Lincoln); C = carbon; N = nitrogen; P = phosphorus; MBC = microbial biomass carbon; Ammo-N = ammonium N; SWC = soil water content.

Table 4.5 Chemical characteristics measured in soils collected from 100-200 mm depth from 10 dairy-pasture farms in New Zealand. Data are mean (n = 6) ± standard error of mean

Soil	Total C (g kg soil^{-1})	Total N (g kg soil^{-1})	pH (1:2.5, soil: water)	Olsen P (mg kgsoil^{-1})	MBC (mg kgsoil^{-1})	Soluble C (mg kgsoil^{-1})	Nitrate-N (mg kgsoil^{-1})	Ammo-N (mg kgsoil^{-1})	Gravimetric SWC (%)
MWEI	24.8 ± 1.09^{f}	2.6 ± 0.13^{c}	6.0 ± 0.03^{ab}	31.7 ± 2.2^{bc}	470 ± 50^{ab}	43 ± 10^{ef}	26.2 ± 2.3^{a}	0.7 ± 0.0^{e}	38.5 ± 0.7^{b}
MW	20.4 ± 1.30^{g}	2.1 ± 0.17^{a}	6.2 ± 0.12^{a}	22.7 ± 2.1^{b}	200 ± 40^{c}	72 ± 2de	7.7 ± 1.6^{c}	2.5 ± 1.7^{de}	24.1 ± 0.8^{d}
TM	31.6 ± 1.60^{c}	2.6 ± 0.10^{e}	6.0 ± 0.08^{ab}	44.7 ± 5.4^{a}	510 ± 31^{a}	63 ± 5^{bc}	1.7 ± 0.2^{d}	6.6 ± 0.8^{bc}	36.3 ± 2.3^{b}
TeK	16.5 ± 0.14^{h}	2.0 ± 0.02^{e}	5.7 ± 0.03^{c}	13.9 ± 1.0^{d}	262 ± 29^{cd}	90 ± 7^{cd}	7.7 ± 0.4^{c}	10.1 ± 1.1^{a}	26.2 ± 1.9^{cd}
OH	59.4 ± 1.05^{a}	6.2 ± 0.10^{a}	6.0 ± 0.02^{ab}	19.1 ± 2.4^{b}	253 ± 65^{cd}	252 ± 12^{a}	7.9 ± 0.8^{c}	8.0 ± 0.8^{ab}	54.6 ± 0.8^{a}
HR	40.3 ± 1.2^{b}	4.3 ± 0.13^{b}	5.7 ± 0.14^{c}	20.1 ± 1.4^{b}	207 ± 30^{d}	154 ± 8^{a}	5.0 ± 0.4^{cd}	9.6 ± 1.4^{ab}	49.7 ± 2.3^{a}
PS	29.2 ± 0.49^{cd}	2.3 ± 0.31^{cd}	5.8 ± 0.08^{bc}	24.7 ± 3.3^{bc}	414 ± 40^{b}	71 ± 3^{fg}	23.9 ± 4.1^{a}	4.0 ± 0.4^{cd}	29.3 ± 0.6^{c}
LM	19.4 ± 0.58^{g}	2.2 ± 0.08^{cde}	5.8 ± 0.06^{bc}	27.2 ± 3.3^{bc}	319 ± 37^{c}	54 ± 3^{g}	5.0 ± 0.4^{cd}	9.6 ± 1.4^{ab}	23.9 ± 0.9^{d}
MF	25.3 ± 1.29^{ef}	2.5 ± 0.09^{cd}	4.6 ± 0.09^{d}	25.6 ± 1.8^{cd}	203 ± 40^{d}	133 ±10^{b}	2.0 ± 0.3^{d}	7.9 ± 1.1^{ab}	22.9 ± 0.5^{d}
PL	27.8 ± 0.65^{de}	2.4 ± 0.07^{cd}	6.1 ± 0.06^{a}	19.6 ± 1.8^{cd}	425 ± 32^{ab}	51 ± 5^{g}	18.3 ± 1.6^{b}	4.0 ± 0.4^{cd}	25.4 ± 2.5^{cd}

Letters denote one way ANOVA test. Values sharing same letter are not significantly different. MWEI = Manawatu fine sandy loam (effluent irrigated); MW = Manawatu fine sandy loam; TM = Tokomaru silt loam; TeK = Te Kowhai silt loam; OH = Otorohanga silt loam; HR = Horotiu silt loam; PS = Paparua silt loam (Springston); LM = Lismore stony silt loam; MF = Mayfield silt loam; PL = Paparua silt loam (Lincoln); C = carbon; N = nitrogen; P = phosphorus; MBC = microbial biomass carbon; Ammo-N = ammonium N; SWC = soil water content.

DEA varied from 173.4 to 3738.2 μg N_2O-N kg^{-1} soil hr^{-1}, and was lowest in the OH surface soil and highest in the PS soil (Tables 4.6). In sub-surface samples, DEA varied from 1.4 to 435.1; the highest DEA being in the PS soil. Among these dairy pasture soils DR ranged between 2.9 1.08 and 21.8μg N_2O-N kg^{-1} soil hr^{-1} in surface soils and from 1.08 to 5.50 μg N_2O-N kg^{-1} soil hr^{-1} in sub-surface samples with the highest value in the TeK surface sample and the lowest in the TeK sub-surface soil.

Table 4.6 Denitrification enzyme activity (DEA), and denitrification rate (DR) of in soils collected from 0 to 100 mm depth from 10 dairy-pasture farms in New Zealand. Data are mean (n = 6) ± standard error of mean

	DEA ($\mu gN_2O–N\ kg^{-1}\ soil\ hr^{-1}$)		DR (N_2O-A) ($\mu gN_2O–N\ kg^{-1} soil\ hr^{-1}$)	
Soil	**0–100 mm**	**100–200 mm**	**0–100 mm**	**100–200 mm**
MWEI	2533.32 ± 378.9^{ab}	48.71 ± 10.93^{c}	7.31 ± 0.3^{c}	5.50 ± 0.11^{a}
HR	924.10 ± 137.4^{c}	170.93 ± 42.18^{b}	17.47 ± 3.3^{b}	2.76 ± 1.11^{bcd}
OH	173.37 ± 27.1^{d}	1.37 ± 0.32^{c}	6.85 ± 0.8^{cd}	1.96 ± 0.26^{cde}
MF	1026.46 ± 119.8^{c}	40.50 ± 8.41^{c}	4.62 ± 0.41^{cde}	3.26 ± 0.29^{bc}
MW	1130.24 ± 243.9^{c}	10.54 ± 2.57^{c}	5.33 ± 0.31^{cde}	3.82 ± 0.19^{b}
TM	608.41 ± 105.1^{cd}	7.72 ± 1.98^{c}	19.10 ± 1.8^{ab}	2.51 ± 0.92^{bcde}
TeK	180.35 ± 67.1^{d}	33.13 ± 12.08^{c}	21.83 ± 1.5^{a}	1.08 ± 0.13^{e}
PS	3738.23 ± 277.8^{a}	435.10 ± 85.11^{a}	4.72 ± 0.04^{cde}	3.31 ± 0.36^{bc}
LM	469.90 ± 100.6^{cd}	38.44 ± 7.49^{c}	2.90 ± 0.43^{e}	1.68 ± 0.43^{de}
PL	1930.32 ± 119.5^{b}	236.05 ± 65.84^{b}	3.31 ± 0.5^{de}	2.23 ± 0.31^{cde}

Letters denote one way ANOVA test. Values sharing same letter are not significantly different in the column they are present in. MWEI = Manawatu fine sandy loam (effluent irrigated); MW = Manawatu fine sandy loam; TM = Tokomaru silt loam; TeK = Te Kowhai silt loam; OH = Otorohanga silt loam; HR = Horotiu silt loam; PS = Paparua silt loam (Springston); LM = Lismore stony silt loam; MF = Mayfield silt loam; PL = Paparua silt loam (Lincoln). N_2O-A = nitrous oxide produced in C_2H_2 added jars.

4.3.3 Denitrifier community structure in New Zealand dairy-grazed pasture soils

Since the soil characteristics, particularly DEA and DR, were significantly lower in sub-surface samples, molecular analyses were performed on the surface samples only. Since both *nir*S and *nir*K genes encode for the same reductase enzyme (nitrite reductase) and these do not co-occur in same bacteria (Heylen *et al.*, 2006), for analysis we summed up the distribution and abundance of these two genes in each soil and report the added values here.

The richness of *nir*S gene varied significantly among the studied soils (Table 4.7). MW (24), HR (16), LM (16), and MF (16) soils had higher *nir*S gene richness than other soils and the PL soil (5) had the least *nir*S gene richness. The richness of *nir*K gene was significantly the highest in the MWEI (31) and the TM (26) soils, and *nos*Z gene richness was the highest in the MWEI soil (27). The richness of all three denitrifier genes (*nir*S, *nir*K, and *nos*Z) was lowest in the PL soil (5, 5, and 12 respectively). The evenness of the *nir*S community was lowest in the PL soil (0.41). As the HR soil had only a few dominant T-RFs of *nir*K genes, and the evenness of this gene was low (0.23), this gene is most evenly distributed in the MWEI, MW, and TM soil (0.86, 0.82, and 0.75 respectively). The diversity of *nir*S gene T-RFs was the least in the PL soil (1.61). The diversity of *nir*K gene was higher in the MWEI, MW and the TM soils than the other soils. Diversity of *nos*Z gene was not significantly different among most of the studied soils.

4.3.4 Total bacterial and denitrifier gene abundance in New Zealand dairy-grazed pasture soils

The abundances of bacterial genes measured as the gene copy numbers showed that the average gene copy numbers of total bacterial gene *rpoB* ranged from 3.5×10^8 to 1.6×10^9 g^{-1} soil, the *nos*Z 9.9×10^5 to 4.8×10^8 g^{-1} soil, the *nir*S 2.5×10^7 to 4.6×10^8 g^{-1} soil, the *nir*K 1.5×10^8 to 5.9×10^8 g^{-1} soil, and the *nir*S+*nir*K gene copy numbers varied from 2.6×10^8 to 7.5×10^8 g^{-1} soil (Figure 4.2). The genes encoding nitrite reductase (*nir*S+*nir*K) were more abundant than those encoding the final step of denitrification (*nos*Z). The relative abundance of *nos*Z, *nir*S, *nir*K, and *nir*S+*nir*K genes with respect to *rpo*B genes varied from 0.27 to 6.2 %, 3.22 to 55.3 %, 17.12 to 74.18 %, and 46.66 to 91.10 %, respectively. The proportion of *nir*K/*nir*S and *nir*(K+S)/*nos*Z ranged from 0.46 to 25.10 and 12.01 to 337.31, respectively (Table 4.8). The smallest relative abundance of *nos*Z genes to total bacterial gene were in PL,

OH and HR soils (0.27–0.64 %) (not significantly different in three soils) and highest in PS, MW, MWEI and TeK (4.79–6.23 %) (not significantly different in the four soils). The proportion of *nir*S+K to *rpo*B genes was smaller in OH, HR, MF (46.66–54.25 %) (not significantly different in three soils) and comparatively higher in PL and MW (85.08–91.10 %).

Table 4.7 Richness, evenness, and diversity of denitrifier gene terminal restriction fragments (T-RFs) in soils. Data are mean (n = 3) ± standard error of mean

	Richness			Evenness			Diversity		
Soil	***nir*S**	***nir*K**	***nos*Z**	***nir*S**	***nir*K**	***nos*Z**	***nir*S**	***nir*K**	***nos*Z**
MWEI	15 ± 1^{b}	31 ± 4^{a}	27 ± 2^{a}	0.70 ± 0.01^{ab}	0.86 ± 0.03^{a}	0.85 ± 0.02^{a}	2.71 ± 0.04^{ab}	3.42 ± 0.12^{a}	3.28 ± 0.07^{a}
MW	24 ± 1^{a}	20 ± 2^{b}	20 ± 3^{bc}	0.82 ± 0.01^{a}	0.75 ± 0.03^{b}	0.77 ± 0.05^{ab}	3.19 ± 0.05^{a}	2.97 ± 0.11^{ab}	2.98 ± 0.18^{ab}
TM	11 ± 3^{c}	26 ± 3^{a}	21 ± 1^{b}	0.60 ± 0.06^{b}	0.82 ± 0.03^{ab}	0.79 ± 0.002^{ab}	2.34 ± 0.24^{bc}	3.26 ± 0.10^{a}	3.03 ± 0.02^{ab}
TeK	9 ± 1^{c}	7 ± 2^{cde}	17 ± 3^{bcde}	0.57 ± 0.04^{b}	0.46 ± 0.06^{de}	0.72 ± 0.06^{bcd}	2.22 ± 0.14^{bc}	1.84 ± 0.24^{cde}	2.76 ± 0.23^{bcd}
OH	13 ± 4^{c}	4 ± 2^{de}	16 ± 1^{bcde}	0.64 ± 0.07^{b}	0.30 ± 0.15^{e}	0.71 ± 0.01^{bcd}	2.48 ± 0.28^{ab}	1.19 ± 0.60^{ef}	2.75 ± 0.06^{bcd}
HR	16 ± 7^{ab}	3 ± 1^{e}	15 ± 1^{cde}	0.63 ± 0.17^{b}	0.23 ± 0.06^{f}	0.70 ± 0.01^{bcd}	2.44 ± 0.67^{ab}	0.92 ± 0.23^{f}	2.71 ± 0.04^{bcd}
PS	24 ± 1^{a}	9 ± 2^{cd}	14 ± 2^{de}	0.82 ± 0.01^{a}	0.55 ± 0.05^{d}	0.69 ± 0.03^{cd}	3.19 ± 0.03^{a}	2.16 ± 0.19^{bcd}	2.65 ± 0.12^{cd}
LM	16 ± 2^{ab}	11 ± 2^{c}	19 ± 1^{bcd}	0.71 ± 0.02^{ab}	0.60 ± 0.05^{c}	0.77 ± 0.01^{abc}	2.76 ± 0.09^{ab}	2.36 ± 0.18^{bc}	2.96 ± 0.05^{abc}
MF	16 ± 4^{ab}	5 ± 2^{cde}	21 ± 2^{b}	0.70 ± 0.06^{ab}	0.37 ± 0.10^{e}	0.79 ± 0.03^{ab}	2.72 ± 0.22^{ab}	1.46 ± 0.41^{def}	3.03 ± 0.10^{ab}
PL	5 ± 1^{d}	5 ± 1^{cde}	12 ± 2^{e}	0.41 ± 0.07^{c}	0.41 ± 0.07^{de}	0.64 ± 0.02^{d}	1.61 ± 0.26^{c}	1.61 ± 0.26^{cdef}	2.48 ± 0.09^{d}

Letters denote one way ANOVA test. Values sharing same letter are not significantly different in the column they are present in. MWEI = Manawatu fine sandy loam (effluent irrigated); MW = Manawatu fine sandy loam; TM = Tokomaru silt loam; TeK = Te Kowhai silt loam; OH = Otorohanga silt loam; HR = Horotiu silt loam; PS = Paparua silt loam (Springston); LM = Lismore stony silt loam; MF = Mayfield silt loam; PL = Paparua silt loam (Lincoln); *nir*S and *nir*K = nitric oxide reductase gene; *nos*Z = nitrous oxide reductase gene.

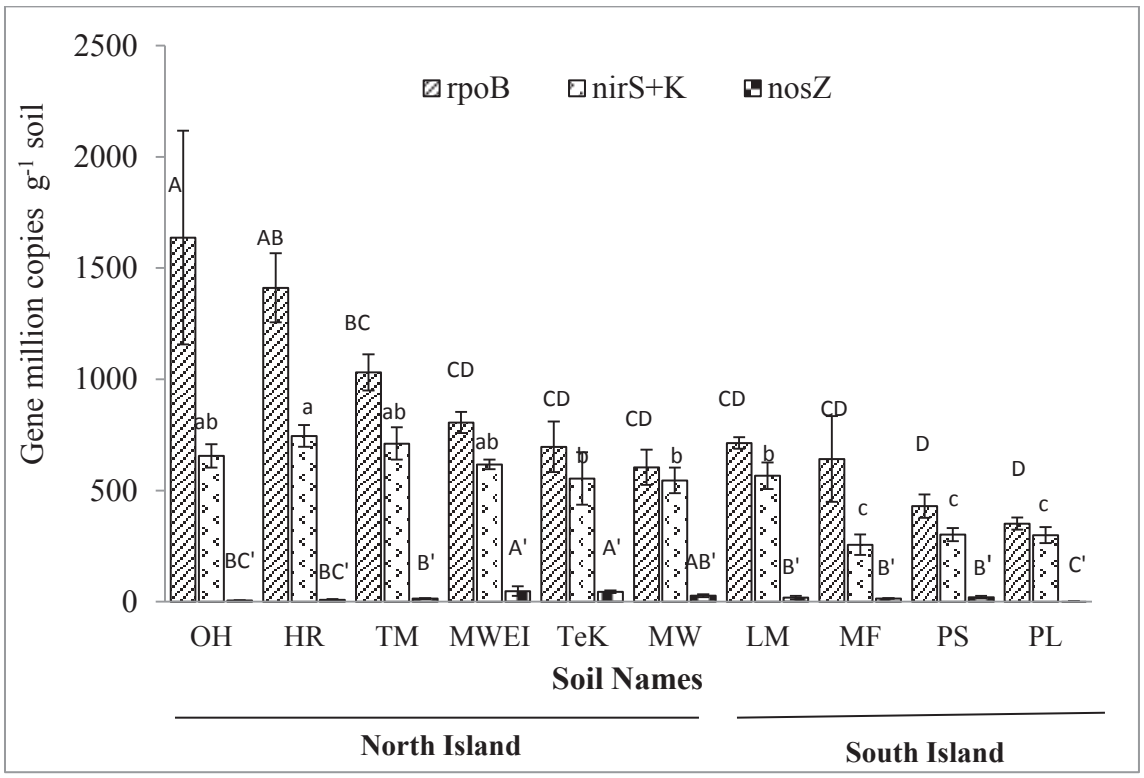


Figure 4.2 Total bacterial (*rpo*B) and denitrifier gene (*nir*S, *nir*K, *nos*Z) copy numbers in soils, error bars denote standard error of mean. Columns with same letter values are not significantly different. Letter values with same case or symbol denotes one test. OH = Otorohanga silt loam; HR = Horotiu silt loam; TM = Tokomaru silt loam; MWEI = Manawatu fine sandy loam effluent irrigated; TeK = Te Kowhai silt loam; MW = Manawatu fine sandy loam; LM = Lismore stony silt loam; MF = Mayfield deep silt loam; PS = Paparua silt loam (Springston); PL = Paparua silt loam (Lincoln).

Table 4.8 Ratio of relative abundance of denitrifier genes to each other and to *rpo*B gene. Data are mean (n = 3) ± standard error of mean

Soil	*nosZ*/*rpoB*%	*nir*K/*rpo*B %	*nir*S/*rpo*B%	*nir*S+K/*rpo*B%	*nir*K/*nir*S	*nir*S+K/*nosZ*
MWEI	6.23 ± 3.1^{ab}	74.18 ± 6.5^{a}	3.22 ± 0.73^{d}	77.14 ± 6.98^{abcd}	25.10 ± 4.66^{a}	17.94 ± 5.66^{c}
MW	4.83 ± 1.5^{abc}	42.14 ± 23.2^{bc}	48.96 ± 20.96^{ab}	91.10 ± 4.28^{a}	3.22 ± 2.91^{bc}	24.4 ± 8.93^{c}
TM	1.40 ± 0.3^{cd}	42.24 ± 7.4^{bc}	28.59 ± 8.90^{abcd}	70.84 ± 12.79^{abcd}	1.96 ± 0.88^{bc}	54.19 ± 11.36^{bc}
TeK	6.66 ± 0.4^{a}	23.11 ± 8.6^{c}	55.33 ± 17.30^{a}	78.44 ± 9.57^{abc}	0.57 ± 0.25^{c}	12.01 ± 2.09^{c}
OH	0.46 ± 0.19^{d}	17.12 ± 10.1^{c}	34.53 ± 10.77^{abc}	51.65 ± 20.0^{cd}	0.46 ± 0.22^{c}	118.52 ± 16.97^{b}
HR	0.64 ± 0.24^{d}	31.35 ± 2.09^{c}	22.91 ± 5.12^{bcd}	54.25 ± 7.19^{bcd}	1.51 ± 0.32^{bc}	101.91 ± 22.33^{b}
PS	4.79 ± 1.14^{abc}	36.65 ± 5.85^{c}	34.74 ± 7.34^{abc}	71.39 ± 6.88^{abcd}	1.17 ± 0.30^{bc}	16.01 ± 2.66^{c}
LM	2.55 ± 1.04^{cd}	37.74 ± 1.43^{c}	41.29 ± 4.99^{abc}	79.03 ± 5.87^{abc}	0.94 ± 0.11^{bc}	51.20 ± 27.94^{bc}
MF	2.79 ± 0.93^{bcd}	26.88 ± 9.88^{c}	19.78 ± 7.26^{bcd}	46.66 ± 15.98^{d}	1.65 ± 0.66^{bc}	17.94 ± 3.75^{c}
PL	0.27 ± 0.07^{d}	70.76 ± 2.34^{ab}	14.33 ± 5.13^{cd}	85.08 ± 6.20^{ab}	6.16 ± 1.82^{b}	337.31 ± 60.14^{a}

MWEI = Manawatu fine sandy loam (effluent irrigated); MW = Manawatu fine sandy loam; TM = Tokomaru silt loam; TeK = Te Kowhai silt loam; OH = Otorohanga silt loam; HR = Horotiu silt loam; PS = Paparua silt loam (Springston); LM = Lismore stony silt loam; MF = Mayfield silt loam; PL = Paparua silt loam (Lincoln); *rpo*B = RNA polymerase gene; *nir*S and *nir*K = nitrite reductase gene; *nos*Z = nitrous oxide reductase gene.

Table 4.9 Pearson's correlation coefficients ($P < 0.05$) between DR, DEA, and soil factors (0–100 mm)

	pH	**Olsen P**	**MBC**	**Soluble C**	**NO_3^--N**	**NH_4^+-N**	**SWC**
DEA	0.320	0.229	0.385	–0.239	0.688	–0.554	Ns
DR	ns	ns	ns	ns	-0.308	0.265	0.351
*nir*K richness	ns	0.371	ns	ns	0.414	–0.607	Ns
*nir*S+*nir*K richness	ns	0.429	0.578	ns	ns	–0.481	Ns
*nos*Z richness	ns	0.583	0.500	ns	ns	–0.319	Ns
*nir*K gene copies	ns	ns	0.532	ns	0.364	Ns	Ns
*nir*S+*nir*K gene copies	ns	ns	ns	ns	ns	Ns	Ns
*nir*S gene copies	ns	ns	ns	ns	-0.546	0.506	Ns
*nos*Z gene copies	ns	ns	0.483	ns	0.393	Ns	Ns
*rpo*B gene copies	ns	ns	0.445	ns	ns	Ns	Ns

DR = denitrification rate; *nir*K and *nir*S = nitrite reductase genes; *nos*Z = nitrous oxide reductase gene; *rpo*B = RNA polymerase gene; MBC = microbial biomass carbon; NO_3^--N = nitrate-nitrogen; NH_4^+-N = ammonical nitrogen; SWC = soil water content.

Table 4.10 Significant Pearson's correlation ($P < 0.05$) coefficients between soil N_2O emissions, denitrifier gene distribution and abundance

	Variable	Correlation Coefficient (r)
N_2O-NA	*nir*K gene copy numbers	0.375
	*nir*S+*nir*K gene copy numbers	0.554
	*nos*Z gene copy numbers	0.356
	*rpo*B gene copy numbers	0.356
DR(N_2O-A)	*nir*S+*nir*K gene copy numbers	0.497

DR = denitrification rate, N_2O-A = nitrous oxide produced in acetylene added jars, N_2O-NA = nitrous oxide produced in non-acetylene added jars, *nir*K and *nir*S = nitrite reductase genes, *nos*Z = nitrous oxide reductase gene, *rpo*B = RNA polymerase gene.

4.3.5 Influence of soil physicochemical characteristics on DR

Correlation Analysis

The correlation between soil chemical characteristics (TC, TN, pH, Olsen P, MBC, soluble C, NO_3^--N, NH_4^+-N, and SWC), gaseous emissions, and denitrifier gene richness and denitrifier gene abundance at 0–100 mm depth (Table 4.9) illustrated that DEA was significantly and positively correlated to pH, NO_3-N, MBC, and Olsen P contents of soils and negatively correlated to NH_4-N, soluble C, and N_2O emitted during denitrification measurements. DR was significantly correlated to SWC, NO_3^--N, and NH_4^+-N content.

The correlation analysis also showed that (Table 4.10) the T-RFs richness of *nir*K, *nir*S+*nir*K, and *nos*Z genes was significantly positively correlated to MBC and Olsen P and negatively to NH_4^+ content in the soils. *Nos*Z gene abundance was positively correlated to MBC, Olsen P, and soil NO_3-N content. The number of *nir*S gene copies was positively related to NH_4^+-N and negatively related to NO_3^--N contents of soils. On the other hand, a number of *nir*K gene copies were positively correlated to NO_3^--N content of the soils. The *nir*S+*nir*K gene copy numbers were positively correlated to MBC. The *rpoB* gene copy numbers were positively correlated to MBC, TN, and TC.

The correlation of gaseous measurements with molecular measurements suggested N_2O productions during these measurements were positively correlated to *nos*Z, *nir*S, *nir*K, *nir*S+*nir*K, and *rpoB* gene copy numbers (Table 4.10). DR was positively correlated only to *nir*S+*nir*K gene copy numbers.

4.3.6 Similarities among soils (0–100 mm depth) based on physicochemical characteristics

Principal Component Analysis (PCA)

The projection of variables on the factor plane (Fig. 4.3) revealed the strength of variance and relationship among soil variables with ordination axes. It showed that DEA, MBC, Olsen P, pH, and NO_3-N varied in the same direction and pointed towards MWEI, suggesting these properties are higher in the MWEI soil than in the other soils. Similarly, SWC, TC, and TN varied in same direction and towards allophanic soils (OH and HR), indicating these properties are higher in these soils.

The principal components analysis (PCA) of soil physicochemical characteristics (0–100 mm depth) generated 9 principal components (PCs). The Eigenvalue and the proportions of explained variances (Table 1, Appendix iii) showed the 1st and 2nd axes of PCs explained 37 and 21 % of the total variation (Fig. 4.4). To illustrate the explained variance in the data, only the first 3 components were considered; the subsequent PCs did not contribute a significant addition in variance.

The correlation coefficients and the relationship between PCs and variables (Table 2, Appendix iii) suggested that as PC1 increased, DEA, NO_3^--N, Olsen P, and MBC contents decreased. Most of the variation in PC 1 was due to NO_3^--N, NH_4^+-N, Olsen P, MBC, and DEA in soils. When PC2 increased, all the soil parameters but DEA decreased. Most of the variance in PC2 was due to TN, TC, and SWC.

Cluster Analysis

The hierarchical cluster analysis (Fig. 4.5) of the surface samples, based on their characteristics produced four clusters (Figs 4.4 & 4.5), which are described below as groups.

Group I: The group I is represented by replicates of the MWEI soil with negative PC1 and positive PC2 scores. The other 9 soils have higher DEA, NO_3^--N, Olsen P, and MBC contents than this soil.

Group II: This group is represented by two allophanic soils (HR & OH). This group has positive PC1 and PC2 scores, and the soil has higher SWC, TN, and TC contents than other the 8 soils.

Group III: With negative PC2 and slightly negative to positive PC1 scores, soils MW, LM, PS, PL, TeK, TM, and MF form one cluster. Three of the soils belong to the South Island, the

other four to the North Island of New Zealand. These soils have moderate to high SWC, DEA, Olsen P, and MBC content.

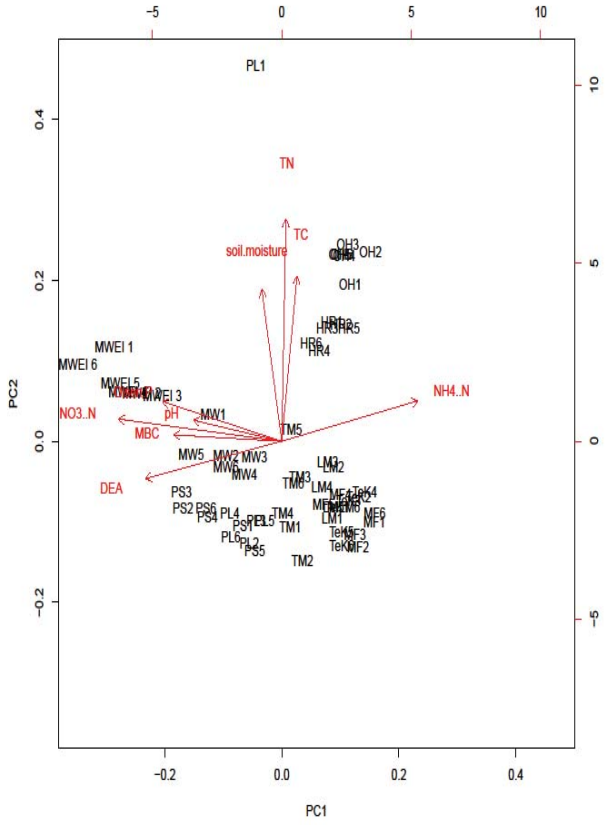


Figure 4.3 Projection of variables on 1st and 2nd factor planes. The arrows represent the relationship between soil parameters with PCs.

DEA = Denitrification enzyme activity; NO3.N = Nitrate –N; NH4-N = Ammonical-N; MBC = Microbial biomass carbon; TN = Total carbon; TN = Total nitrogen; MWEI = Manawatu fine sandy loam (effluent irrigated); MW = Manawatu fine sandy loam; TM = Tokomaru silt loam; TeK = Te Kowhai silt loam; OH = Otorohanga silt loam; HR = Horotiu silt loam; PS = Paparua silt loam (Springston); LM = Lismore stony silt loam; MF = Mayfield silt loam; PL = Paparua silt loam (Lincoln). Numbers adjacent to soil codes denote replicate number.

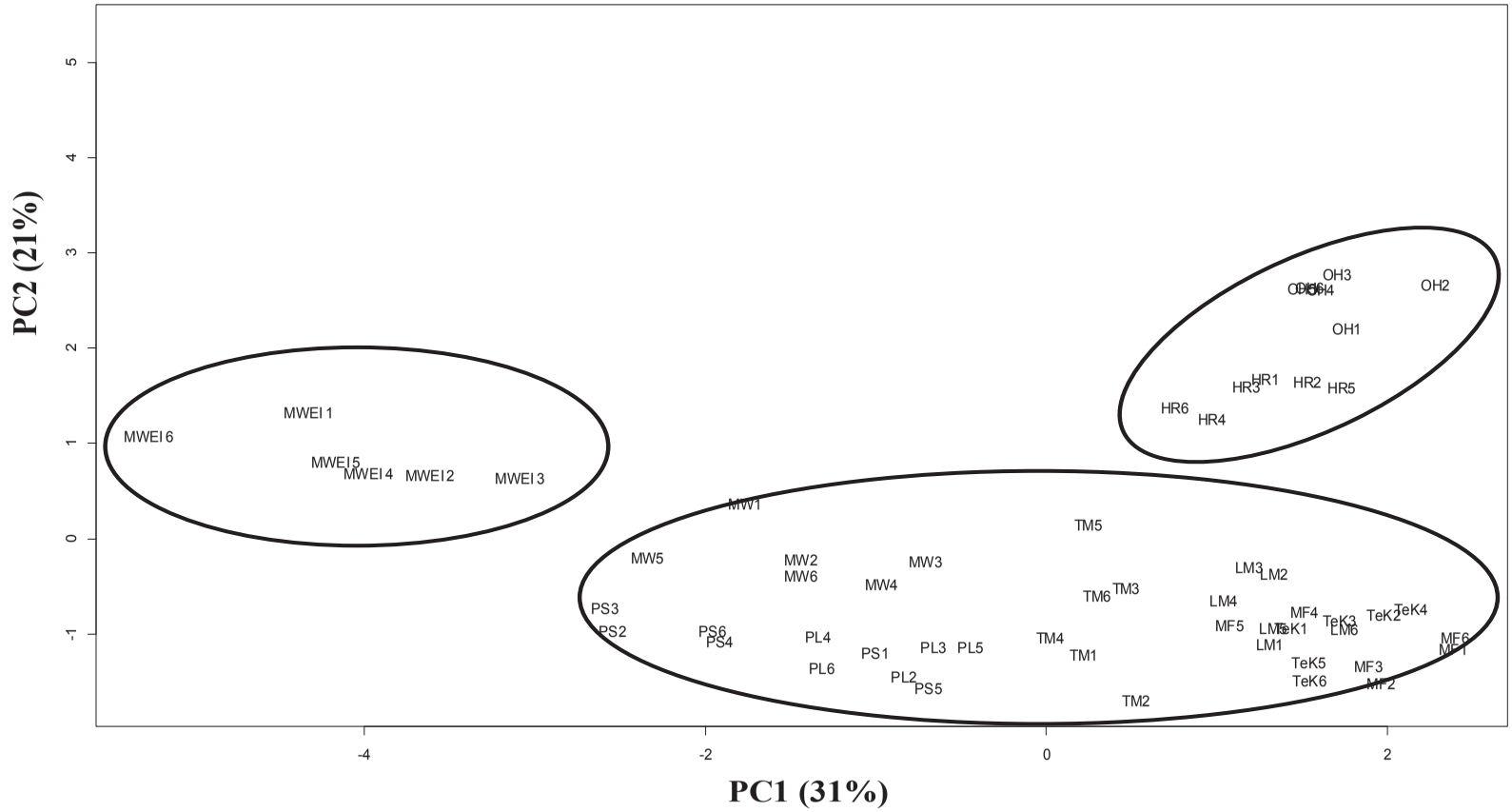


Figure 4.4 Principal components and cluster formation of various group based on their soil characteristics at 0–100 mm depth.

MWEI = Manawatu fine sandy loam (effluent irrigated); MW = Manawatu fine sandy loam; TM = Tokomaru silt loam; TeK = Te Kowhai silt loam; OH = Otorohanga silt loam; HR = Horotiu silt loam; PS = Paparua silt loam (Springston); LM = Lismore stony silt loam; MF = Mayfield silt loam; PL = Paparua silt loam (Lincoln). Numbers adjacent to soil codes denote replicate number.

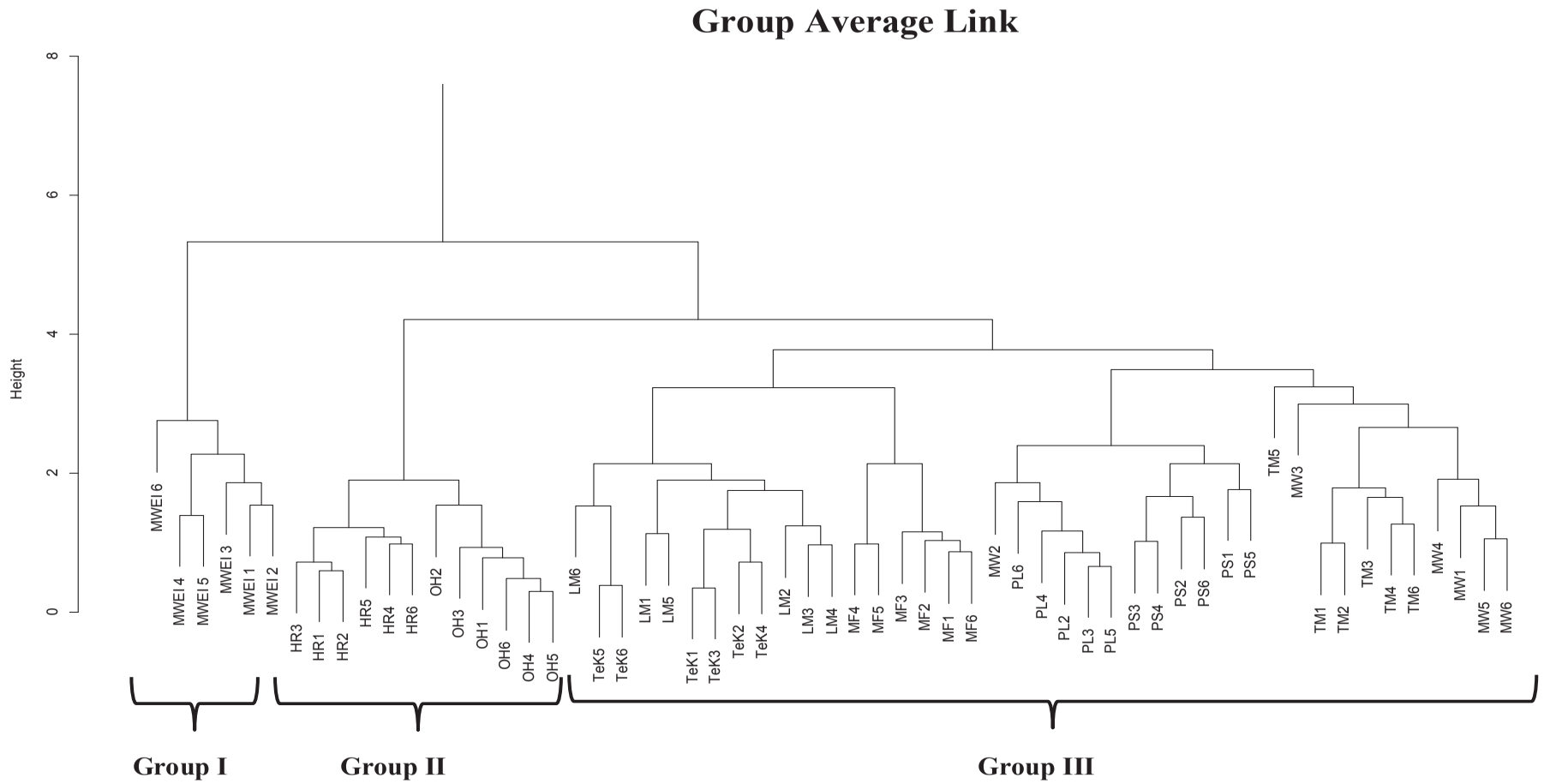


Figure 4.5 Cluster formation of various group based on their soil characteristics at 0–100 mm depth.

MWEI = Manawatu fine sandy loam (effluent irrigated); MW = Manawatu fine sandy loam; TM = Tokomaru silt loam; TeK = Te Kowhai silt loam; OH = Otorohanga silt loam; HR = Horotiu silt loam; PS = Paparua silt loam (Springston); LM = Lismore stony silt loam; MF = Mayfield silt loam; PL = Paparua silt loam (Lincoln). Numbers adjacent to soil codes denote replicate number.

Regression Analysis

The regression analysis (Table 4.11) showed that 46 % of total variation in DR in the studied soils (0–100 mm depth) at field-moist conditions could be explained by the measured physiochemical characteristics. Among all the measured soil characteristics soluble C, SWC, NO_3^--N, Olsen P, and pH significantly influenced DR in these soils. Despite denitrification being a microbial process the coefficients of Olsen P and MBC (non-significant) were negative in the model. The regression analysis for sub-surface samples (100–200 mm depth) suggested only 22 % variation in DR was explained by soil physicochemical parameters, and SWC and NO_3-N content significantly influenced DR.

Similarly, the regression analysis (Table 4.12) showed that 54 % of total variation in DEA in the studied soils (0–100 mm depth) at field-moist conditions could be explained by the measured physicochemical characteristics. Among all the measured soil characteristics TC, SWC, and NO_3-N contents significantly influenced DEA in these soils. In case of sub-surface samples physicochemical characteristics of soils only explained 15 % variation in their DEA with none of the parameters significantly influencing DEA.

In order to predict DEA, and DR, based on soil physicochemical characteristics and denitrifier community structure and abundance, regression analysis were performed. However, these analyses could not predict gaseous parameters based on denitrifier community structure and abundance, only part of the physiochemical characteristics of soils was significant in the models.

Table 4.11 Multiple linear regression analysis using backward elimination model of DR against soil factors

Variable	**Equation (Model)**			**R^2**	**R^2adj**	**S**
DR	DR = 33.7 – 4.13 pH, -0.246 NO_3-N - 0.027 NH_4-N-0.79 SolC+ 0.378 C/N ratio+0.193 Olsen P + 0.99 MBC +0.442 SWC			0.52	0.46	5.6
Significant Variables	Sol C (*P*=0.0001), SWC (*P*=0.000), NO_3-N (*P*=0.0001), pH (*P*=0.046), Olsen P (*P*=0.003)					
	SS	**df**	**MS**	**F**	***P***	
Regression	1743.85	8	217.98	6.94	0.0001	
Residual	1602.59	51	31.42			
Total	3346.44	59				

Table 4.12 Multiple linear regression analysis using backward elimination model of DEA against soil factors

Variable	**Equation (Model)**			**R^2**	**R^2adj**	**S**
DEA	DEA = 4.06 – 1.0 Olsen P-16.9 TC + 1.17 TN + 1.59 NO_3-N +48.7 SWC +1.27 MBC –3.93 SolC, -0.092 NH_4-N – 0.015 pH			0.61	0.54	1.7
Significant Variables	TC (*P*=0.0001), SWC (*P*=0.000), NO_3-N (*P*=0.0001)					
	SS	**Df**	**MS**	**F**	***p***	
Regression	228.921	9	25.44	8.58	0.0001	
Residual	148.208	50	2.96			
Total	377.129	59				

4.4 DISCUSSION

4.4.1 Physicochemical or biochemical characteristics of soils

The soil characteristics TC, TN, pH, Olsen P, MBC, soluble C, NO_3^--N, NH_4^+-N, SWC, DEA, and DR were significantly higher in surface samples than in sub-surface samples, which reflected the higher root mass, due to pasture growth and substrate availability in surface soils. The decomposing plant parts of pastures provide high organic matter content to soil, which in turn improves the fertility or nutrient status compared with sub-surface low organic matter soil. The results obtained in this study are in accordance with others (D'Haene *et al.*, 2003; Luo *et al.*, 1998; Murray *et al.*, 2004), who have also demonstrated lower DEA in soils with increasing depths. However, in LM and PL soils the magnitude of DR was very low in surface samples compared with other soils and thus was indistinguishable from the sub-surface DRs.

The physicochemical characteristics of the 10 dairy-grazed pasture soils were highly variable due to their origin, geographical location, and the management practices followed on the farms from which they were collected. The MWEI surface soil with continuous effluent application over last 4 years has the highest Olsen P, MBC, pH, NO_3^--N of the soils. The two well-drained allophanic soils OH and HR, with silt loam texture, are similar to each other and have higher TC, TN, soluble C, SWC, and lower Olsen P content than other soils. MF soil with the lowest pH, SWC, and high NH_4^+-N content, is different from other soils. Other soils (TeK, TM, MW, LM, PL, PS), with texture varying from fine sandy loam to stony silt loam and poorly drained soils share similar chemical characteristics.

4.4.2 Denitrifier community structure, total bacterial and denitrifier gene abundance in New Zealand dairy pasture soils

The abundance of denitrifier gene richness (numbers of gene T-RFs) in New Zealand pasture soils indicated that the denitrifier community in these soils is dominated by NO_2^- reducers rather than N_2O reducers. *Nir*S genes showed the highest richness of the 3 denitrifier genes studied. The PL soil had the fewest *nir*S, *nir*K and *nos*Z phylotypes of all soils tested in this study. The

Pielou's coefficient of evenness (J) for denitrifying bacteria illustrated that the number of T-RFs of N_2O reducing bacterial community is more equally present than NO_2^- reducing community in the pasture soils tested. This suggested that under stress or unfavourable soil conditions the N_2O reducing bacterial community will be more tolerant than the NO_2^- reducing community, which is dominated by only a few genotypes (Wittebolle *et al.*, 2009).

As expected, in all the 10 soils the universal bacterial genes were the most abundant genes, followed by *nir*S+*nir*K, *nir*S, and *nir*K; the least abundant was the *nos*Z gene, which was also confirmed in other studies (Chon *et al.*, 2011). The higher abundance of *nir*S+*nir*K genes than that of the *nos*Z gene is because the length of the base pair of *nir* genes is longer than the *nos*Z gene; also because some bacteria, like *Agrobacterium tumefaciens*, lack the *nos*Z gene. Recent studies have suggested that a large group of environmental *nos*Z genes is still unrecognised by commonly used primes also included in the current study (Jones *et al.*, 2011). Using qPCR, it was demonstrated that the unaccounted *nos*Z gene was most often as abundant as the other conventional *nos*Z gene, which might indicate that we are not accounting for the complete *nos*Z community in these soils. It could be possible that some of the soils with high denitrification potential might possess more N_2O reducers than NO_2^- reducers.

The relative abundance of *nos*Z, *nir*S, *nir*K, and *nir*S+*nir*K genes compared with *rpo*B genes varied from 0.28 to 6.5 %, 3.22 to 55.3 %, 17 to 74 %, and 46 to 91 %, respectively. This shows that a very large proportion of the total bacterial population in these pasture soils have the ability to denitrify, however, a very small proportion of total bacteria is capable of reducing N_2O to N_2. The studies by Chen *et al.* (2012b), Henry *et al.* (2006), and Kandeler *et al.* (2006) have reported lower (< 10 %) proportions of abundances of denitrifier genes to total bacterial genes than reported in the current study. In their measurements on arable croplands and permanent grasslands, Miller *et al.* (2009b) have also found higher relative abundance of denitrifiers; the proportion of the *nos*Z/16SrRNA gene was > 90 %. The results of the current study suggest that New Zealand pasture soils have great potential to emit N_2O through denitrification. It is already established that denitrification is the primary process of the emitted N_2O emission in New Zealand pasture soils (Luo *et al.*, 1999a; 1999b; Saggar, 2004; 2004a; 2007; 2009). One of the reasons for this could be that these soils are rich in N_2O-producing bacterial denitrifier communities.

4.4.3 Influence of soil characteristics on DR and DEA

The correlation analysis, PCA, and regression analysis were performed to relate soil characteristics to, denitrification rates and DEA measured in New Zealand pasture soils. The key variables suggested by correlation and PC analysis influencing DR, and DEA are SWC, NO_3^--N, NH_4^+-N, Olsen P, MBC, TC, TN.

The DEA of a soil is its maximum potential to denitrify under ideal condition. The soil characteristics such as pH, MBC, and Olsen P that might influence the total microbial activity related very well with DEA of the studied soils. The phosphorus availability in soil indicates its fertility status. Studies have demonstrated that soil phosphorus availability regulates microbial activity in soils (Cleveland *et al.*, 2002; Ilstedt *et al.*, 2006). Positive correlations have been observed between P content in soil solutions and other biochemical processes such as decomposition and mineralization of soil organic matter (Cleveland *et al.*, 2002; Saggar *et al.*, 1998). The PS and the MWEI soils with higher MBC and Olsen P similarly have higher DEA than other soils.

The water content of soils is one of the major limiting factor for denitrification (Barton *et al.*, 1999) and as a result minimal denitrification is observed at soil moisture contents below critical levels, i.e. field capacity soil water content (Aulakh *et al.*, 1991a; Linn & Doran, 1984). Most of the soils we studied had lower SWC than their field capacity and consequently we observed smaller DRs in these soils compared with their denitrification potential.

On a dairy-grazed pasture, N in active form (NO_3^-, NH_4^+) is added through fertilization, fixation by leguminous plants, and excretal deposition by grazing animals (Galloway *et al.*, 2003). In these soils DR negatively correlated with NO_3^--N content and positively with NH_4^+-N content. Despite high gravimetric SWC, due to higher NO_3^--N content than other soils, DR was lower in the MWEI soil compared with the soils with similar SWC. Luo *et al.* (1996) have shown lower denitrification in soils incubated with high NO_3^--N contents more than 50 mg NO_3^--N kg^{-1} soil, the reason being that higher NO_3^--N content in soils inhibits the reductase enzymes activity (Gaskell *et al.*, 1981; Terry & Tate, 1980) and thus affects denitrification rate.

The results of regression analysis have advised that DR and DEA could be predicted based on their SWC, soluble C, NO_3^--N, pH, Olsen P, and TC. Despite lower DEA in soils such as TeK, OH, and TM, these soils displayed higher DRs due to their higher SWC and the poor

drainage condition of soils compared with the rest of the soils. Therefore, the physical properties of the soils played a key role in affecting denitrification rate at lower SWC in these soils.

There are contradicting results in the literature relating soil and environmental factors to denitrifier community structure and the influence of denitrifier community structure and abundance on denitrification activity and N_2O emissions. The chemical characteristics, especially NO_3^--N, NH_4^+-N, Olsen P, and MBC contents are found to be closely related to denitrifier richness and denitrifier abundance in the studied soils. Previous studies have shown that soil chemical characteristics such as organic carbon, NO_3^- availability (Tiedje, 1988), and soil pH (Parkin *et al.*, 1985) influence the composition of the denitrifier community in a soil.

With higher MBC and Olsen P, MWEI soil has more abundant denitrifier bacterial community than other soils. On the other hand, with lower MBC and Olsen P contents, PL soil had lower abundances of denitrifier genes. Also, the higher NH_4^+-N content of this soil likely favoured nitrifying or ammonia oxidising bacteria over denitrifying bacteria. This soil also had lower *rpo*B copies, suggesting chemical conditions in PL support a small bacterial population, which results in a smaller denitrifier population. There was a significant negative correlation (r = –0.372, P = 0.043) between the individual abundances of *nir*S and *nir*K genes in these soils. It was also observed that *nir*S and *nir*K gene abundances were related to different chemical characteristics of the soils, illustrating that the denitrifiers carrying these genes prefer different niches. The ratio of *nir*K/*nir*S gene copy numbers is variable in studied soils. The higher *nir*K/*nir*S gene ratio in MWEI soil than in TeK, OH, and HR suggests this soil has more *nir*K-bearing bacterial community than *nir*S. Also the MWEI soil has less SWC than TeK, OH, and HR soils, which supports the fact that denitrifying bacteria with *nir*K genes prefer drier soil condition than bacteria with *nir*S genes (Petersen *et al.*, 2012).

4.4.4 Influence of denitrifier gene richness and denitrifier gene abundance on denitrification in NZ dairy pasture soils

The relationship between denitrifier gene richness and its abundance with DR is not very strong in the studied pasture soils. Liu *et al.* (2013) have also revealed denitrifier gene abundances to be driven by soil characteristics (pH, EOC, TN, TC, NO_3^-, MBC, and MBN); however, denitrifier gene abundance (*nar*G, *nir*K, *nir*S, *nos*Z) could not be related to N_2O emissions.

Soils of similar pH and textures to those used in our study have yielded conflicting results with regard to the relationship among denitrifier community structure and denitrification activity or N_2O emissions (Enwall *et al.*, 2005; Peralta *et al.*, 2010; Rich & Myrold, 2004). When observed together, *nir*S and *nir*K gene abundances relate significantly with DR measured in these soils. This finding is similar to some other studies that have shown an influence of denitrifier abundance on denitrification activity (Chroňáková *et al.*, 2009; Čuhel *et al.*, 2010; Dong *et al.*, 2009).

No correlation was observed between any of the denitrifier gene abundance with DEA. This suggests gene copy numbers might not likely provide the information about the real time processes occurring in soils. As a result, studies reporting similar results (Henderson *et al.*, 2010; Miller *et al.*, 2009a; 2012) could not find any link between N_2O emission or DEA with denitrifier gene abundance. Several studies report that abundances of denitrifier genes are not correlated to measured denitrification activities (Attard *et al.*, 2011; Dandie *et al.*, 2008; Miller *et al.*, 2008). Also the variations in the denitrifier gene abundances in the studied soils could be due to differences in their chemical characteristics and could not be related to their activity.

In this experiment it was observed that N_2O production was related positively with denitrifier gene abundance (*nir*K, *nir*S+K*, nos*Z) in these soils. Since there is more abundance of *nir*K genes in the soils used in current experiment, the abundance of this gene is correlated more to N_2O emission than *nir*S gene abundance. Higher abundance of the *nir*K gene might have facilitated more N_2O emission during denitrification. It is a known fact that drier soils or less aerobic soils are dominated by *nir*K genes (Petersen *et al.*, 2012) and correspond to higher N_2O emissions.

The relationship between the denitrification rate of a soil and its denitrifier community structure might depend on the prevalence of anaerobic condition in that soil. In the current experiment, most of the soils have lower SWC than required for denitrification, thus no robust relationship could be produced between denitrification rates and denitrifier community structure and abundance. The exact amount of anoxia required for denitrification gene expression can differ substantially among organisms (Ka *et al.*, 1997). The soils used in the current experiment exhibit wide range of biochemical characteristics, collected from sites representing variable geographical and climatic conditions, and consist of a wide range of denitrifier community structure and abundance. Cavigelli & Robertson (2001) demonstrated a diverse range of

sensitivity of N_2O reductase activity to oxygen over a range of taxonomically diverse denitrifying isolates from soil results in variable N_2O emission from soil.

The key soil properties influencing denitrification and leading to variabilities in different soils were identified using PCA. The influence of chemical characteristics and denitrifier population on denitrification arranged the tested soils in 3 groups. While soils with exclusive characteristics and denitrification were grouped away from other soils, soils with similar characteristics clustered together. The regression analysis helped explain the amount of variability explained by key soil variables in denitrification and DEA. The physicochemical characteristics of soils might either directly affect the DR and DEA or indirectly affect the denitrifier community carrying the denitrification activity. Cavigelli & Robertson (2000), (2001), and Holtan-Hartwig *et al.* (2000) have suggested that the N_2O emissions during denitrification are regulated by the denitrifier community structure, which could be due to physiological differences in the soil. They have emphasised that the ability of denitrifiers to either produce or reduce N_2O under certain soil or climatic conditions should be emphasised when making models to develop mitigation techniques for N_2O emissions from soil.

4.5 SUMMARY AND CONCLUSIONS

The soils used in the current study were selected from various geographical locations to provide variation in physicochemical characteristics. The results of this study substantiated the available information that the DR and DEA of a soil vary with sampling depth, SWC, NO_3^--N, NH_4^+-N, Olsen P, and MBC contents. Denitrifying bacteria with *nir*S and *nir*K genes were more abundant in these soils than in those with *nos*Z genes. These soils differed in their denitrification potentials, denitrification rates, denitrifier gene richness and abundance due to their variation in physicochemical characteristics, management condition, variable textural class, their mineralogy, geographical location, and climatic condition of collection site.

Based on their physicochemical characteristics, molecular parameters and measured DEA and DR the soils used in the current experiment could be divided into three groups. The MWEI soil with rich biochemical and microbial characteristics is distinct from other soils with lower MBC, Olsen P and less abundant denitrifier gene. The two allophanic soils (OH, & HR) clustered together representing those soils with high DR and NH_4^+-N contents. The PL soil with the least number of denitrifier gene T-RFs, gene copy numbers, and least Olsen P and MBC was

distinct from other soils. The rest of the soils used in this experiment, due to their common soil properties, formed a separate group. This suggested that the physicochemical characteristics of these soils strongly influenced their denitrifier gene abundance and denitrification under incubated conditions.

The results implied that soils collected from different sites with similar biochemical characteristics had more similar denitrifier gene richness/abundance and showed similar DRs. The limited relationship between denitrifier gene abundance and DR, DEA, and N_2O emission suggested a lack of connection between denitrifier activity and denitrifier community size in these soils. There are unaccounted *nos*Z genes, which might help better explain denitrification processes in the studied soils.

The results described in this chapter are based on soil incubations carried out at variable soil water contents. Since soil water content is the key factor influencing DR in soils, the next chapter discusses the variation in DR in selected soils incubated at the same soil water content.

Chapter 5

Effect of varying soil water content on denitrification in a range of New Zealand dairy-grazed pasture soils

5.1 INTRODUCTION

Various soil conditions and properties such as soil water content (SWC), temperature, the concentration of mineral N, soil pH, the availability of soil C, and denitrifier population will affect the rate and products of denitrification in pastoral agricultural systems (Bolan *et al.*, 2004b; Drury *et al.*, 2008; Saggar *et al.*, 2013; Wallenstein *et al.*, 2006). Several studies have documented these effects on the denitrification rate (DR) in grazed pastures (de Klein & Van Logtestijn, 1994a, b; Jarvis *et al.*, 1991a; Luo *et al.*, 1994a, b; 1998; Zaman *et al.*, 2008c). Studies suggest that SWC is a major factor controlling DR in pasture soils. Pilot & Patrick (1972) and Klemedtsson *et al.* (1991) have suggested DR remains independent of SWC until the latter exceeds a threshold level and thereafter DR increases sharply with increasing SWC. This threshold SWC varies with soil type, and is close to field capacity (FC) or 60 % WFPS in many soils (de Klein & van Logtestijn, 1996; Saggar *et al.*, 2004b).

The SWC has multiple effects on N oxide emissions. Increasing SWC determines the biogeochemical environment for the activity of microorganisms (Weitz *et al.*, 2001) by enhancing the solubility and movement of substrates such as organic C, ammonium (NH_4^+) and nitrate (NO_3^-). SWC also controls both soil aeration and the movement of gases in the soil profile. When soil is dry, nitrification is the dominant process and N_2O produced during the process diffuses out of soil profile, before being consumed further. With the onset of anaerobic conditions, reductase enzymes involved in denitrification get activated and initiate the reduction process, which leads to N_2O and N_2 emissions (Richardson *et al.*, 2009). In wet soils, denitrification dominates the consumption of N-oxides and much of the intermediate gases retained in the soil profile are finally reduced to N_2. A number of studies describe the impact of changes in SWC on the DR and denitrification products (Fellows *et al.*, 2011; Hernandez &

Mitsch, 2006, 2007b; Song *et al.*, 2010) but to date the mechanism behind this is still not completely understood.

As pointed out above, changes in soil condition with increasing SWC influence the end products of denitrification and may lead to variable N_2O production during denitrification in soils, varying in physical, and biochemical properties. How SWC and the biochemical properties of soils affect denitrification end products is not well understood in New Zealand pasture soils. Factors regulating denitrification may differ with soil types. An understanding of how soil characteristics influence denitrification is essential in developing technologies to mitigate the harmful environmental impacts of gaseous losses of N.

It is hypothesized that differences in the soil properties, especially denitrification enzyme activity (DEA) of soils, will be reflected in their denitrification activity at common SWC (either FC or saturation).

To test the above hypothesis, five soils contrasting in DEA were selected from the 10 soils studied in chapter 4 of this thesis. These soils represented groups I, II, and III indicated in the previous chapter (Figure 4.5). The soils were: Effluent irrigated Manawatu fine sandy loam (MWEI); Otorohanga silt loam (OH); Paparua silt loam (Springston) (PS); Mayfield silt loam (MF); and Lismore stony silt loam (LM). The physicochemical characteristics and description of these soils is given in chapter 4 (Tables, 4.1, 4.4, & 4.5) of this thesis. These soils were incubated at FC and saturation SWCs to determine the changes in N_2O production (N_2O production with acetylene and without acetylene in jars respectively). The objective of the current experiment was to enhance the understanding of denitrification with increasing SWC in soils with different physicochemical properties, denitrifier gene abundances and varying denitrification potentials.

5.2 MATERIALS AND METHODS

5.2.1 Collection of soil samples, chemical and biochemical analysis

The methods used for the collection, and chemical and molecular analyses of the soil samples are described in chapter 3 of this thesis and a brief outline is given in chapter 4 (sections 4.2.2 to 4.2.3). Field fresh soil cores were taken to the laboratory, sieved to 2 mm, and stored at 4 ºC in plastic bags. For incubation during this experiment, stored samples (used in previous experiment) were used within 6 months of collection.

5.2.2 Incubation of soils at field capacity (FC) and saturation

For gas sampling : Six field replicate soil samples (50 g dry weight equivalent each) from the 0–100 and 100–200 mm depths were packed to known bulk density into plastic containers (r = 2.25 cm, h = 7.4 cm, vol = 117.63 cm^3) having 1-mm holes (15 holes for exchange of gases) for incubation. A total of 120 samples (5 soils × 6 field replicates × 2 depths × 2 C_2H_2 treatments =120) were incubated for measurement of DR at FC and saturation. Correct volumes of deionized water were added gradually from the top with the help of a hand sprayer to increase the soil water content to FC for 24 hours. Samples were kept at constant temperature (25 ^oC). The DR was measured at FC as described below. After the DR measurements at FC, additional deionised water was added to the same samples to increase the SWC to saturation. Soils were incubated at saturation for 24 hours and then DR was measured again.

For mineral N measurement: Six replicated soil samples (20 g dry weight equivalent each) from the 0–100 and 100–200 mm depths were taken in plastic containers (r = 2.25 cm, h = 7.4 cm, vol = 117.63 cm^3). Correct volumes of deionised water were added to the soil as described above to increase the SWC to FC. After 24 hours of addition of water, sub-samples for mineral N measurement at FC were taken; thereafter, additional deionised water was added to the same samples to increase the SWC to saturation. Soils were incubated for another 24 hours and then sub-samples for mineral N measurement were taken. The subsamples for mineral N measurements were taken after final gas sampling for DR measurement both at FC and saturation.

5.2.2.1 Estimation of FC SWC: The amount of water needed to wet the soils to FC was determined using the suction plate method (Loveday, 1974). Sieved soils samples (20 g each), packed to the known bulk density, were arranged in PVC rings (diameter of 4.3 and 1.8 cm height) on a suction plate and were saturated with water for 24 hours. The suction plate was then fitted to the bubble tower, which was connected to a suction pump set at a pressure of 0.1 bars. The assembly was kept undisturbed for 24 hours to pump out the excess water absorbed by soils. After 24 hours, when there was no water dripping out of the suction plate, gravimetric SWC was determined by drying the wet soil at 105 ^oC for 24 hours and reweighing until a constant weight was obtained. This SWC corresponded to the amount of water in the soils at FC. With the

amount of water present in the soil known, the additional amount of water needed to increase the water content of 50 g soil to FC was calculated.

5.2.2.2 Estimation of saturation SWC: The amount of water needed to increase the SWC to saturation was calculated from the bulk density and porosity of repacked soil in plastic container determined using the method outlined in section 3.5.6 of this thesis. The porosity of a soil corresponds to volume of water required to saturate the soil as fraction of the total volume. The total soil volume (with pore space) was calculated from the bulk densities and dry masses of soils, and the volume of water already contained in the soil was determined. The volume of already present water was subtracted from the pore space volume to calculate the total volume of water needed to saturate the soil. Table 5.1 shows the gravimetric SWCs in soils during the incubation. The water contents at FC were variable in the soils with the highest water content in the OH soil and the lowest in the PS soil ($P < 0.05$). At saturation there was no significant difference in the water content in the soils.

Table 5.1 Gravimetric soil water content (SWC) (%) before and after addition of water during the incubation of soils; OH = Otorohanga silt loam, MW = Manawatu fine sandy loam, MF = Mayfield silt loam, PS = Paparua silt loam, and LM = Lismore stony silt loam collected from 0 to 100 and from100 to 200 mm depths. Data are mean (n = 6) ± Standard error of mean (S.E.M.)

Soil Abbreviation	Sampling Depth (mm)	SWC in field fresh soil	SWC at FC	SWC at saturation
OH	**0–100**	54.5 ± 0.7	78.3 ± 1.0	105.7 ± 2.3
MWEI		52.8 ± 1.0	58.0 ± 1.3	96.2 ± 3.1
MF		24.3 ± 0.6	46.8 ± 0.5	98.3 ± 1.2
PS		33.5 ± 1.3	42.1 ± 1.0	95.2 ± 4.2
LM		31.7 ± 1.6	44.4 ± 0.5	97.4 ± 2.4
OH	**100–200**	54.6 ± 0.8	78.3 ± 0.8	94.3 ± 4.2
MWEI		38.5 ± 0.7	52.2 ± 0.3	95.2 ± 2.5
MF		22.9 ± 0.5	46.4 ± 0.7	96.5 ± 3.1
PS		29.3 ± 0.6	32.3 ± 0.3	95.4 ± 4.2
LM		23.9 ± 0.9	40.9 ± 0.8	94.1 ± 2.8

5.2.3 Measurement of N_2O production and DR during incubation

DR in the original field-moist samples was determined as described in section 3.3. After incubating the soils at FC or saturation for 24 hours, undisturbed soil samples were transferred to 1-L glass jars, lids were tightened, and the samples were left to incubate for the next 24 hours to determine DR as described in section 3.3. The N_2O production in soils incubated with and without C_2H_2 was calculated using Eqs 3.4 & 3.5. The addition of acytelene to the soil incubation jars inhibits nitrification and reduction of N_2O to dinitrogen. Therefore, N_2O produced in non-acetylene treated jars (N_2O-NA) corresponds to N_2O emitted both from nitrification and denitrification processes occurring in incubated soil sample. And N_2O produced in acetylene treated jars (N_2O-A) corresponded to denitrification rate (DR).

5.2.4 Statistical Analysis

The detailed statistical analysis is described in section 4.2.4 of this thesis; therefore, only a brief outline is given here. The dataset was normalised using Box Cox transformation and all the statistical tests were performed on normalised data. Repeated measures analysis of variance (ANOVA) was used to assess differences among means of N_2O-NA, N_2O-A, NO_3^--N, and NH_4^+-N contents in the five soils at two soil water contents and at two sampling depths. Tukey's Studentized Range Test at $\alpha = 0.05$ significance level was used *post hoc* to reveal significant differences among means. The relationships among the soil characteristics mineral N, DEA, N_2O-NA, and N_2O-A measured at the three water contents were determined using Pearson's correlation. Relationships between the number of denitrifier gene T-RFs and abundance analysis measured and reported in sections 4.3 of this thesis with the DR, N_2O-NA, and N_2O-A at FC and saturation for surface samples was also assessed.

5.3 RESULTS

5.3.1 Soil mineral N content at variable SWCs

The overall comparison between the mineral N content in original field-moist and amended soils (FC & S) at each sampling depth using 2-way ANOVA (Tables 5.2 & 5.3) indicated the significant effects of SWC, soil type and the interaction term. In the original field-moist unamended soils the NO_3^--N & NH_4^+-N contents varied from 8.1 to 58.7 mg kg^{-1} soil and 0.9 to 12.9 mg kg^{-1} soil respectively in the surface samples (Table 5.4). These values in the sub-surface samples ranged from 2.0 to 26.2 mg kg^{-1} soil for NO_3^--N content and 0.7 to 9.6 mg kg^{-1} soil for NH_4-N content. At FC, NO_3^--N and NH_4^+-N contents in surface soils ranged from 17.2 to 68.4 mg kg^{-1} soil and from 6.4 to 42.5 mg kg^{-1} soil respectively (Table 5.4). After incubation to saturation the NO_3^--N and NH_4^+-N contents in surface soils changed and ranged from 6.0 to 117.6 mg kg^{-1} soil and from 3.0 to 103.9 mg kg^{-1} soil, respectively. In sub-surface soils incubated at FC, the NO_3^--N and NH_4^+-N contents ranged from 8.4 to 67.5 mg kg^{-1} soil and from

4.2 to 12.6 mg kg^{-1} soil respectively. At saturation in sub-surface soils, NO_3^--N and NH_4^+-N contents ranged from 1.8 to 46.8 mg kg^{-1} soil and from 2.1 to 7.4 mg kg^{-1} soil respectively.

In the surface and subsurface samples, NO_3^--N content was the highest in the MF soil and the lowest in the OH soil (Table 5.4). The NO_3^--N contents in the incubated soils varied with their water contents, in the surface samples soils had more NO_3^--N contents at saturation than at field capacity or original field-moist condition. In the subsurface samples the NO_3^--N contents at saturation and field capacity were not significantly different, but higher than the NO_3^--N contents at the original field-moist condition. With the change in water contents in soils from the original to field capacity and then to saturation water content there was significant increase in the NO_3^--N contents in the MF and the LM soils. In the other three soils this change in NO_3^--N contents with moisture level was not significant at both the sampling depths.

Table 5.2 Repeated measures analysis of variance table to test the effect of soils; OH = Otorohanga silt loam, MW = Manawatu fine sandy loam, MF = Mayfield silt loam, PS = Paparua silt loam, and LM = Lismore stony silt loam, sampling depths (0–100 and 100–200 mm) and soil water content (original, field capacity & saturation) on the nitrate –N (NO_3^-–N) content in soils

Source	**SS**	**F**	***p***
Soil (0–100 mm)	28428.0	43.11	0.0001
Soil water content (SWC)	9252.1	28.06	0.0001
Soil × SWC	39920.8	30.27	0.0001
Error	12364.5		
Soil (100–200 mm)	10774.9	57.31	0.0001
Soil water content (SWC)	3252.6	34.60	0.0001
Soil × SWC	13052.3	34.71	0.0001
Error	3525.2		

Table 5.3 Repeated measures analysis of variance table to test the effect of soils; OH = Otorohanga silt loam, MW = Manawatu fine sandy loam, MF = Mayfield silt loam, PS = Paparua silt loam, and LM = Lismore stony silt loam, sampling depths (0–100 and 100–200 mm) and soil water content (original, field capacity & saturation) on the ammonical–N (NH_4^+–N) content in soils

Source	**SS**	**F**	***p***
Soil (0–100 mm)	29501.9	22.03	0.0001
Soil water content (SWC)	3866.1	5.77	0.0001
Soil × SWC	25113.7	9.37	0.0001
Error	25102.5		
Soil (100–200 mm)	365.03	20.77	0.0001
Soil water content (SWC)	135.9	15.47	0.0001
Soil × SWC	325.8	9.27	0.0001
Error	329.5		

Table 5.4 Mineral N contents (mg kg^{-1} soil) measured at field-moist condition, field capacity and saturation soil water content in soils; OH = Otorohanga silt loam, MW = Manawatu fine sandy loam, MF = Mayfield silt loam, PS = Paparua silt loam, and LM = Lismore stony silt loam collected from 0-100 and 100-200 mm depths. Data are mean (n = 6) ± standard error of mean

Soil Depth (mm)	Soil Abbreviation	NO_3^-–N (mg kg^{-1} soil)			NH_4^+–N (mg kg^{-1} soil)		
		FM	FC	S	FM	FC	S
0–100	OH	12.6 ± 0.9^{ef}	30.9 ± 3.3^{def}	6.0 ± 0.7^{f}	11.6 ± 0.3^{bc}	7.4 ± 0.4^{bc}	4.1 ± 0.4^{c}
	MWEI	58.7 ± 3.5^{bc}	50.4 ± 6.1^{bcd}	47.3 ± 3.1^{cd}	0.9 ± 0.1^{d}	7.9 ± 0.9^{bc}	3.0 ± 0.3^{c}
	MF	8.1 ± 0.8^{ef}	68.4 ± 1.0^{b}	117.6 ± 9.6^{a}	8.1 ± 0.1^{bc}	42.5 ± 0.6^{b}	103.9 ± 4.5^{a}
	PS	32.8 ± 3.1^{cde}	32.6 ± 3.5^{de}	13.4 ± 3.2^{ef}	4.5 ± 0.4^{c}	6.4 ± 0.2^{bc}	3.5 ± 0.4^{c}
	LM	10.6 ± 1.5^{ef}	17.2 ± 2.9^{ef}	61.5 ± 8.8^{b}	12.9 ± 0.9^{bc}	12.6 ± 0.3^{bc}	4.4 ± 0.3^{c}
100–200	OH	7.9 ± 0.8^{f}	8.7 ± 0.3^{ef}	1.8 ± 0.2^{f}	8.0 ± 0.8^{bc}	5.2 ± 0.1^{cde}	3.3 ± 0.4^{def}
	MWEI	26.2 ± 2.3^{cd}	28.3 ± 0.9^{cd}	25.1 ± 2.3^{cd}	0.7 ± 0.0^{f}	6.0 ± 0.5^{bcde}	2.1 ± 0.3^{ef}
	MF	2.0 ± 0.3^{f}	67.5 ± 2.0^{a}	46.8 ± 6.7^{b}	7.9 ± 1.1^{bc}	4.5 ± 1.0^{cde}	7.4 ± 2.3^{bcd}
	PS	23.9 ± 4.1^{cd}	22.0 ± 1.3^{cde}	15.4 ± 1.8^{def}	4.0 ± 0.4^{cdef}	4.2 ± 0.2^{cdef}	2.9 ± 0.4^{ef}
	LM	5.0 ± 0.4^{f}	8.4 ± 1.1^{ef}	33.2 ± 8.8^{c}	9.6 ± 1.4^{ab}	12.6 ± 0.2^{a}	3.6 ± 0.7^{def}

Values sharing same letter are not significantly different. The letters indicate the differences in the means tested with two way ANOVA for each sampling depth. The letters with same section of table denote one test. FM= field-moist condition, FC= field capacity & S= saturation

5.3.2 N_2O production rates (non-acetylene jars) at variable SWCs and incubation times

The results of the 2-way ANOVA (Table 5.5) at each sampling depth indicated the significant effect at $\alpha = 0.05$ of soil type, SWC, and the interaction of soil type, and SWC on the measured N_2O-NA in five different soils. In the original field-moist unamended soils, the N_2O-NA varied from 0.01 to 7.00 µg N_2O-N kg^{-1} soil hr^{-1} in the surface samples; these values in the sub-surface samples ranged from 0.01 to 5.31 µg N_2O-N kg^{-1} soil hr^{-1} (Table 5.6). At FC, the N_2O-NA in the surface samples ranged from 0.66 to 12.99 and from 0.59 to 1.47 µg N_2O-N kg^{-1} soil hr^{-1}in the sub-surface samples (Table 5.6). After incubation to saturation the N_2O-NA in surface and sub-surface samples ranged from 2.36 to 22.89µg N_2O-N kg^{-1} soil $hr^{-1}$and from 0.45 to 7.57 µg N_2O-N kg^{-1}soil hr^{-1} respectively.

Table 5.5 Repeated measures analysis of variance to test the effect of soils; OH = Otorohanga silt loam, MW = Manawatu fine sandy loam, MF = Mayfield silt loam, PS = Paparua silt loam, and LM = Lismore stony silt loam, sampling depths (0–100 and 100–200 mm) and soil water content (field capacity & saturation) on the N_2O-NA (N_2O production rates in soils with no acetylene)

Source	SS	F	*P*
Soil (0–100 mm)	882.6	54.72	0.0001
Soil water content (SWC)	927.5	115.0	0.0001
Soil × SWC	1094.8	33.94	0.0001
Error	302.45		0.0001
Soil (100–200 mm)	163.9	35.18	0.0001
Soil water content (SWC)	75.3	32.31	0.0001
Soil × SWC	133.4	14.31	0.0001
Error	87.4		

Table 5.6 N_2O-NA [N_2O production rates (µg N_2O-N kg^{-1} soil hr^{-1}) in jars without acetylene] measured at field-moist condition, field capacity and saturation soil water content in soils; OH = Otorohanga silt loam, MW = Manawatu fine sandy loam, MF = Mayfield silt loam, PS = Paparua silt loam, and LM = Lismore stony silt loam collected from 0–100 and 100–200 mm depths. Data are mean ± standard error of mean (*n* = 6)

		N_2O-NA (µg N_2O-N kg^{-1} soil hr^{-1})		
Sampling depth (mm)	**Soil Abbreviation**	**FM**	**FC**	**S**
0–100	OH	2.84 ± 0.47^{d}	1.79 ± 0.08^{d}	22.89 ± 2.50^{a}
	MWEI	7.00 ± 0.17^{c}	12.99 ± 0.40^{b}	9.56 ± 3.59^{bc}
	MF	2.13 ± 0.49^{d}	1.95 ± 0.12^{d}	2.36 ± 0.26^{d}
	PS	0.01 ± 0.001^{d}	1.56 ± 0.08^{d}	9.82 ± 0.95^{bc}
	LM	1.47 ± 0.29^{d}	0.66 ± 0.03^{d}	7.06 ± 0.10^{c}
100–200	OH	1.01 ± 0.38^{c}	0.96 ± 0.06^{c}	1.52 ± 0.04^{c}
	MWEI	5.31 ± 0.06^{b}	1.27 ± 0.02^{c}	7.57 ± 0.04^{a}
	MF	1.85 ± 0.12^{c}	1.47 ± 0.12^{c}	0.45 ± 0.01^{c}
	PS	0.01 ± 0.001^{c}	0.95 ± 0.07^{c}	4.68 ± 1.31^{b}
	LM	1.18 ± 0.3^{c}	0.59 ± 0.001^{c}	2.17 ± 0.11^{c}

Values sharing same letter are not significantly different. The letters indicate the differences in the means tested with two way analysis of variance at each sampling depth., FM= field-moist condition, FC= field capacity & S= saturation.

In the surface samples of MWEI soil the N_2O-NA was significantly higher than the other four soils both at original field-moist and field capacity water contents. On the other hand, at saturation the N_2O production rate was highest in the OH soil, followed by the MWEI, PS, and the LM soils, and the lowest in the MF soil. The change in N_2O-NA with the increase in SWC from original field-moist condition to FC in the surface samples was observed only in the MWEI soil. In the other four soils, significantly, there was no change in N_2O-NA between field moist and FC SWC. With increase in the SWC from FC to saturation there was significant increase in the N_2O-NA only in the PS, OH, and LM soils. In the MWEI and MF soils there was no further increase in N_2O-NA with the increase in SWC. There was least increase in N_2O-NA in the MF and the highest in the well-drained volcanic OH soil with the increase in SWC.

In the sub-surface samples the N_2O-NA was the highest in the MWEI soil and similar in the other four soils. The N_2O-NA in the original field-moist samples was significantly higher in the MW soil than in the other soils. At FC the N_2O-NA was not significantly different in any of the five soils. At saturation N_2O-NA was highest in the MWEI and the PS soil compared with the rest of the three soils (Table 5.6) Increase in N_2O-NA with the change in SWC was observed only in the MWEI and the PS soils, in rest of the soil there was no significant difference in the N_2O-NA with the increase in SWC.

The increase in N_2O-NA from field-moist samples to samples incubated at FC ranged from 1.1 to 2.0 times that of N_2O-NA at field moisture. The changes in N_2O-NA from FC to saturation ranged from 0.6 to 1.9 times that of N_2O-NA at FC. There was higher N_2O-NA in soils incubated at saturation than at field-moist condition. However, there was no significant difference in the N_2O-NA in the soils incubated at FC and at field-moist condition. These increases in N_2O production in soils with increase in SWCs to saturation could also be due to the combined effect of SWC and sampling duration, since the samples incubated at saturation were incubated twice, once at FC and then at saturation.

5.3.3 Denitrification rate (DR) or nitrous oxide produced in acetylene added jars (N_2O-A) at variable SWCs

The results of the 2-way ANOVA (Table 5.7) at each sampling depth indicated the significant effect at $\alpha = 0.05$ of soil type, SWC and the interaction of soil type, and SWC on the measured DR in five different soils.

In the original field-moist unamended soils (as received), the N_2O-A varied from 2.9 to 7.3μg N_2O-N kg^{-1} soil hr^{-1} in the surface samples; these values in the sub-surface samples ranged from 1.7 to 5.5μg N_2O-N kg^{-1} soil hr^{-1} (Fig. 5.1). At FC, the N_2O-A in the surface samples ranged from 0.8 to 50.4μg N_2O-N kg^{-1} soil hr^{-1} and from 0.7 to 2.5μg N_2O-N kg^{-1} soil hr^{-1} in the sub-surface samples. After incubation to saturation the N_2O-A in surface and sub-surface samples ranged from 21.5 to 73.9μg N_2O-N kg^{-1} soil hr^{-1} and from 0.7 to 16.9μg N_2O-N kg^{-1} soil hr^{-1} respectively.

In the surface samples, N_2O-A was greater in the MW soil and the least in the LM soil. With the increase in SWC, the N_2O-A varied in the incubated soils. In the MWEI soil the N_2O-A was the lowest in the original field-moist soil, increased when this soil was incubated to FC, and continued to increase when the soil was further saturated. In the PS, OH, and LM soils there was no significant difference in the N_2O-A between the original field-moist samples and the samples incubated to field capacity. However, there were significantly higher N_2O-A in these soils when incubated at saturation than at FC or original field-moist condition. Only in the MF soil was there no significant difference in the N_2O-A between the field-moist and FC or between the FC and saturation. There was slightly higher N_2O-A in the saturated sample than the original field-moist sample.

In the sub-surface samples, N_2O-A was significantly higher in the PS and MWEI soils than in the other three soils in which N_2O-A was not significantly different. N_2O-A increased in PS, MW, and LM soils with the change in SWC from field moist to saturation. However, there was no difference in the N_2O-A in the MF and the OH soils with increase in SWC.

Overall, the increase in the N_2O-A from FC to saturation ranged from 1.8 to 30 times the N_2O-A at FC and varied with soil type. There was least increase in the N_2O-A in the MWEI soil, and the highest in the LM soil, with an increase in SWC from FC to saturation.

Table 5.7 Repeated measures analysis of variance to test the effects of soils: OH = Otorohanga silt loam, MW = Manawatu fine sandy loam, MF = Mayfield silt loam, PS = Paparua silt loam, and LM = Lismore stony silt loam sampling depths (0–100 and 100–200 mm), and soil water content (field capacity & saturation) on the denitrification rates (DR) or nitrous oxide produced in acetylene treated jars (N_2O-A) in soils

Source	**SS**	**F**	**P**
Soil (0–100 mm)	14033.0	33.15	0.0001
Soil water content (SWC)	27960.7	132.12	0.0001
Soil × SWC	9139.1	10.8	0.0001
Error	7936.4		0.0001
Soil (100–200 mm)	508.3	81.02	0.0001
Soil water content (SWC)	583.25	185.91	0.0001
Soil × SWC	708.52	56.46	0.0001
Error	117.64		

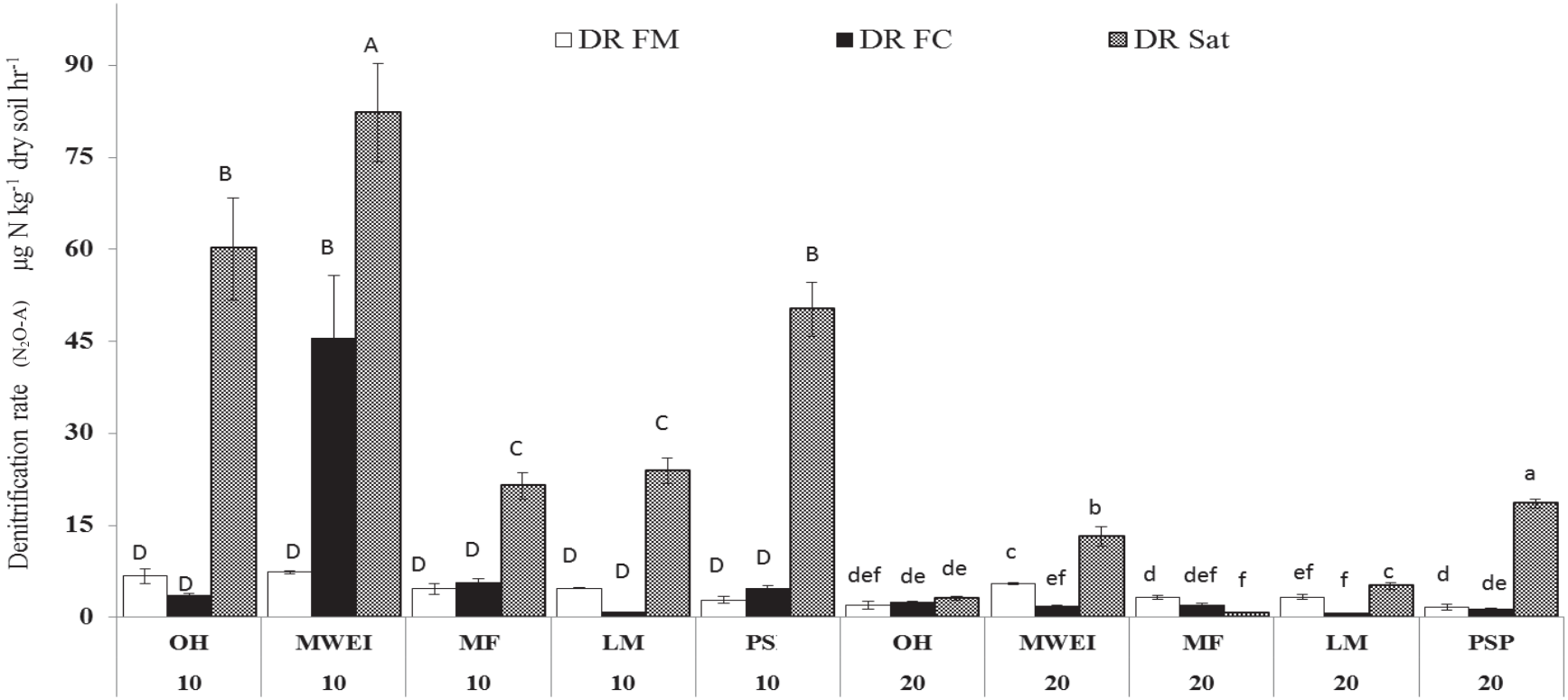


Figure 5.1 DR (µg N kg^{-1} dry soil hr^{-1}) measured in soils as nitrous oxide produced in acetylene treated jars; OH = Otorohanga silt loam, MW = Manawatu fine sandy loam, MF = Mayfield silt loam, PS = Paparua silt loam, and LM = Lismore stony silt loam collected from 0–100 and 100–200 mm depths at field-moist condition, field capacity and saturation. Bars denote mean ± S.E.M. Bars with same letter values are not significantly different. The letter values apply to means of N_2O-A. Numbers adjacent to soil abbreviation denote sampling depth, 10 = 0–100 mm and 20 = 100–200 mm depth. DR FM = denitrification rate at original field-moist condition, DR FC = denitrification rate at field capacity, and DR Sat = denitrification rate at saturation water content in soils.

Table 5.8 Pearson's correlation coefficients ($P < 0.05$) between DR and N_2O emission rate and soil mineral N contents and DEA

	Variable	R
DR (FM)	NO_3-N (FM)	0.535
	NH_4-N(FM)	Ns
	DEA	Ns
DR(FC)	NO_3-N (FC)	–0.610
	NH_4-N(FC)	–0.352
	DEA	Ns
DR(Sat)	NO_3-N (Sat)	–0.521
	NH_4-N(Sat)	–0.430
	DEA	0.342
N_2O-NA (FM)	NO_3-N (FM)	0.641
N_2O-NA (FC)	NO_3-N (FC)	0.624
N_2O-NA (Sat)	NO_3-N (Sat)	–0.485

DR = denitrification rate, N_2O = nitrous oxide, DEA = denitrification enzyme activity, FM = field moist (as received), FC = field capacity, sat = saturation, NA = non-acetylene treated jars.

Table 5.9 Pearson's correlation coefficients ($P < 0.05$) between DR, N_2O emission, and molecular parameters

Variables	DR (N_2O-A)			N_2O-NA		
	FM	**FC**	**S**	**FM**	**FC**	**S**
***nir*K T-RF**	ns	0.809	0.567	0.759	Ns	0.582
***nosZ* T-RF**	ns	0.661	ns	ns	Ns	ns
***Nir*S gene copies**	ns	ns	ns	ns	Ns	ns
***Nir*K gene copies**	0.515	0.853	0.582	0.874	0.568	0.701
***Nir*S+K gene copies**	ns	ns	ns	0.534	Ns	0.641
***NosZ* gene copies**	ns	0.763	0.516	ns	0.655	ns

DR = denitrification rate, N_2O= nitrous oxide, N_2O-A = nitrous oxide produced in acetylene added jars, N_2O-NA = nitrous oxide produced in non-acetylene added jars, FM = field moist, FC = field capacity, S = saturation soil water content, ns= not significant, *nir*K and *nir*S = nitrite reductase genes, *nosZ* = nitrous oxide reductase gene.

5.3.4 Relationship of mineral N contents and DEA with N_2O-NA, and N_2O-A in soils incubated at variable SWC

The results of correlation analysis between soil characteristics and gaseous emissions illustrated that at field-moist condition the N_2O production rates in these soils were positively correlated with NO_3^--N content measured at field-moist condition. The DRs at FC and saturation were negatively correlated to the NO_3^--N & NH_4^+-N contents of the soils measured at respective SWCs. The DR at saturation was also positively correlated to DEA of soils. The N_2O production rate was positively correlated to NO_3-N content at FC and negatively at saturation (Table 5.8).

In an attempt to look into the possible relationship between gaseous emissions during denitrification with the denitrifier community structure and abundance, even though these characteristics were not measured during the incubation, a correlation analysis was conducted based on the measurements in the same soil in the previous experiment. The correlation analysis (Table 5.9) suggested that DR in the original field-moist soil was significantly correlated only with *nirK* gene copy numbers. At FC and saturation the DR was positively correlated to the numbers of *nir*K and *nos*Z gene T-RFs and *nir*K and *nos*Z gene copy numbers. The N_2O production rate was positively correlated to numbers of *nir*K gene T-RFs and *nir*S+*nir*K gene copy numbers.

The regression analysis (Table 5.10) showed that 61 % of total variation in DR in the studied soils at FC water content could be explained by the measured physiochemical characteristics. Among all the measured soil characteristics sampling depth, Olsen P, soluble C, and NO_3-N contents significantly influenced DR at FC in these soils. Similarly, the regression analysis (Table 5.11) showed that 76 % of total variation in DR at saturation soil water content in the studied soils could be explained by the measured physiochemical characteristics. Among all the measured soil characteristics sampling depth, soluble C, and NO_3^--N contents significantly influenced DR at saturation. The visual examination of the residual plots of the regression models appeared satisfactory.

Table 5.10 Multiple linear regression analysis using backward elimination model of DR at field capacity soil water content against soil factors

Variable	**Equation (Model)**			**R^2**	**R^2adj**	**S**
DR (FC)	DR (FC) = –60.9 + 0.236 Olsen P + 3.01 pH+ 3.39 TN + 0.259 TC + 0.281 NO_3-N -0.101 NH_4-N- 0.126 Sol C – 0.30 MBC – 13.3 Sampling depth			0.61	0.55	10.05
Significant Variables	Olsen P (*P*=0.0001), NO_3-N (*P*=0.003), soluble C (*P*=0.014), sampling depth (*P*=0.012)					
	SS	**Df**	**MS**		**F**	***p***
Regression	8180.1	9	908.9		9.00	0.0001
Residual	5049.5	50	101.0			
Total	13229.6	59				

Table 5.11 Multiple linear regression analysis using backward elimination model of DR at saturation soil water content against soil factors

Variable	**Equation (Model)**			**R^2**	**R^2adj**	**S**
DR (Sat)	DR (Sat) = 104 + 0.116 Olsen P –8.07 pH + 3.66 TN + 0.666 TC –0.24 Soluble C – 0.20 MBC –24.7 sampling depth –0.361 NO_3-N + 0.193 NH_4-N			0.76	0.72	14.64
Significant Variables	Soluble C (*P*=0.002), sampling depth (*P*=0.002), NO_3-N(Sat) (*P*=0.025)					
	SS	**df**	**MS**		**F**	***p***
Regression	34646.9	9	3849.7		17.95	0.0001
Residual	10724.7	50	214.5			
Total	45371.5	59				

5.4 DISCUSSION

5.4.1 Changes in soil mineral N with changing SWC

The five soils displayed a large variation in NO_3^--N and NH_4^+-N contents, reflecting the historical variable N inputs either through periodic fertilizer application and/or effluent irrigation, or excretal-N deposition. The changes in NO_3^--N content with increasing SWC were not consistent in all five soils. While it was expected that increasing SWC would lead to reductions of soil NO_3^--N through denitrification (Gillam *et al.*, 2008), a significant decrease in NO_3^--N with increasing SWC was observed only in the OH soil. Though there were decreases in NO_3^--N contents in the MWEI and PS soils, with the increase in SWC these decreases were not significant. There was a significant increase in the NO_3^--N contents in the LM and MF soils at higher SWC, which in contrast to expectation, are due to higher nitrification and lower denitrification taking place in incubated soils. The decrease in NO_3^--N content (although insignificant) in selected soils was not comparable to the N contained in N_2O-A at saturation. The loss of N as N_2O-A during denitrification was lower than the decrease in NO_3^--N content during incubation, suggesting there could be other N transformation processes apart from denitrification taking place in the incubated soils.

In this study, increasing SWC had no effect on NH_4^+-N in either surface or sub-surface samples. One exception was the MF soil, where there was a significant increase in NH_4^+-N with increasing SWC at both soil depths (suggesting mineralization of biomass). There could be various reasons for the increase in NH_4^+-N content in the MF soil. The MF soil has the least pH of all the soils, and inhibition of nitrification at lower soil pH (Li *et al.*, 2009) must have resulted in higher NH_4^+-N content than other soils at saturation. Also N mineralisation could have led to higher NH_4^+-N contents at higher SWC (Aciego Pietri & Brookes, 2008). The increase in NH_4^+-N content in MF soil at saturation could also be due to dissimilatory NO_3^- reduction to NH_4^+ (DNRA) as NO_3^- is reduced to NH_4^+ via (NO_2^-) by fermentative microorganisms (Tiedje *et al.*, 1982). The subsequent nitrification of part of the available NH_4^+-N could have resulted in the increased NO_3^--N content that was observed in this soil. There might have been limited opportunity for the available NO_3^--N to be denitrified at low pH because of the inhibition of nitrate reductase enzymes at low pH (Čuhel & Šimek, 2011; Nogales *et al.*, 2002).

Even at similar SWC, the N transformations can be highly variable between soils with different inherent physicochemical properties, such as higher TC, TN in the OH soil, higher Olsen P, MBC, and DEA in the MWEI and PS soils. The variation may also arise due to different N fertilizer application on farms, generating different microbial populations capable of carrying different N transformations at a given time. The higher numbers of *nir*S+*nir*K gene copies in the MWEI and OH soils than in the MF and LM soils might have led to higher reduction of NO_3^--N in these soils than in others. The higher NH_4^+-N content in the MF field-moist soil may be capable of sustaining ammonium oxidising organisms producing higher NO_3^--N in the soil during incubation. The lowest pH content of the MF soil might not be supporting the bacterial denitrifier population, and the pathways mediated by fungal population could be contributing to N transformations.

5.4.2 N_2O production rates (N_2O-NA and N_2O-A) at variable SWCs

The DRs observed in this experiment at variable SWCs are comparable to the DRs and within the range reported in grassland soils under various incubation conditions (Saggar *et al.*, 2013). The increase in N_2O-A was more than the increase in N_2O-NA with increasing SWC. This suggested that with the advance of favourable conditions, denitrification activity increased in soils, resulting in an increase in the total denitrification product. With an overall increase in DR, N_2O production increased, but at the same time the reduction of N_2O to N_2 was also enhanced, which did not allow N_2O-NA to increase at same rate as the N_2O-A. The increase in N_2O with increasing SWC is in agreement with the results reported in earlier studies (Bolan *et al.*, 2004b; de Klein & Ledgard, 2005; Luo *et al.*, 2000).

5.4.3 Soil properties influencing N_2O-NA and N_2O-A in the incubated soils at variable SWCs.

The response pattern of N_2O production rates with increasing SWC were variable among the five soils in the current study and support the hypothesis of this study, which proposes the increase in the N_2O-NA and N_2O-A with increasing SWC will be variable among soils. The inherent physicochemical characteristics of these soils might have resulted in differences in denitrification at same water content. Mathieu *et al.* (2006) have also shown that at higher SWC the relative contribution of denitrification to N_2O emissions in various soils differs with soil type. The variability in the N_2O production rates in the five soils incubated at common

SWCs (either FC or S) is typical of grazed grassland soils, reflecting the natural heterogeneity of such soils due to uneven urine and dung patches over months to year (Baily *et al.*, 2012; Hutchings *et al.*, 2007). This natural variability has led to differences in their NO_3^--N content, DEA, TC, TN, MBC, Olsen P or denitrifier population acknowledged in section 4.3. The other reason for variability in the N_2O production rate, especially at FC, could be variable moisture content in soils.

In the current study, the largest increase in N_2O-NA with the increase in SWC was in the OH and the MWEI soils; it was similar in the rest of the soils. The OH soil also displayed the highest decrease in the NO_3^--N content with an increase in SWC from FC to saturation. This indicated the NO_3^- reduction in this soil might be generating high N_2O-A. In all the soils used in this study, except the MWEI soil, it is significant that the N_2O-NA production rates measured at field-moist conditions (Table 5.6) are not different from the N_2O-NA at FC as a consequence of lower denitrification activity in soils at and below FC (de Klein & van Logtestijn, 1996).

The highest N_2O-NA and N_2O-A measured in the MWEI soils at FC could be due to the higher denitrification activity at relatively aerobic condition in this soil. The MWEI soil comparable NO_3^--N content to the PS and OH soils, and had higher Olsen P, MBC, and DEA than the OH soil, which might have facilitated the higher microbial activity in the MWEI soil and helped increased denitrification activity, even at comparably aerobic condition to the OH soil. Since the OH soil had the highest SWC at FC compared with the other four soils, this soil was expected to have the least increase in DR with the change in SWC from FC to saturation. In contrast to this, the OH soil, along with the PS soil, resulted in the highest increase in the DR with the increase in SWC from FC to saturation.

Compared with the OH and PS soils, there was low/no significant increase in N_2O-A in the MWEI, LM, and MF soils. The small increase in DR from FC to saturation in the MWEI soil could be due to the already very high DR in the MWEI soil compared with the other four soils at FC, suggesting the soil reached almost its maximum potential at FC and exhausted available resources, which could be due to prolonged incubation of the same set of samples, first at FC, and then at saturation, and might not have allowed further increase in the denitrification activity in the MWEI soil.

On the other hand, less increase in the DR with the increase in SWC in the LM soil compared with the other soils suggested the conditions in the LM soil, lower NO_3^--N, and TC content were not optimum for denitrification with increasing SWC. In the case of the MF soil

there was no significant increase in DR with the increase in SWC during incubation. The lower denitrification activity in the MF soil could be due to its chemical or biological condition not facilitating denitrification even at higher SWC. The MF soil has the lowest pH, the highest NO_3^--N, and NH_4-N contents at both saturation and FC compared with the other four soils. The low pH inhibits the N_2O reductase activity, suggesting this soil might lack the active denitrifier population to carry out denitrification, even under complete anaerobic conditions (Hansen *et al.*, 2014; Šimek & Cooper, 2002). The denitrifier gene abundance might only provide the overall picture of the denitrifier community, and the activities of these denitrifiers depend mainly on the environmental conditions and physicochemical properties of soil. The observed increase in NO_3^--N content with increasing SWC in the MF and LM soils (with intermediate DEA), suggested the contribution of N transformations other than denitrification during the transition. The variability in the inherent microbial population driving denitrification, directly or indirectly due to physicochemical characteristics of soils, was reflected in the current incubation study.

Inherent soil property and management condition, such as effluent application, proper irrigation, maintenance of higher soil pH, and organic matter content will help overall microbial population and denitrifiers to contribute to the transformation of available N to N_2O.

5.5 SUMMARY AND CONCLUSIONS

This study highlights the influence of changing SWC on the N oxide transformations and emissions of N_2O during nitrification and denitrification. The results of this experiment support the proposed hypothesis that the increase in N_2O production (with and without C_2H_2) with increasing soil water content will vary in soils due to differences in their denitrification enzyme activities. The differences in DR with respect to changes in the SWC, variations in soils, and their physicochemical conditions, were reflected in this experiment. The DR at saturation revealed variability in the DEA of the 5 soils. There was a sharp increase in DR in the MWEI soil at FC compared with the DR at field-moist condition. In the other four soils the increase in DR was observed at saturation. Even at saturation of the water content, the OH produced more N_2O from nitrification and denitrification than from the denitrification alone. The presence of high allophane in the OH soil compared with the other soils might have restricted the enzyme activity and thus complete denitrification.

The differences in N_2O production (with and without C_2H_2) in the five studied soils at similar SWC depended on their NO_3^--N, TC, TN, MBC, Olsen P, DEA, denitrifier gene richness, and abundance. The relationship between the molecular parameters and the gaseous measurements at elevated SWC was stronger in the five studied soils in comparison to when measured at field-moist condition. The inherent variability in the presence of the denitrifier community and their abundance and variations in the physicochemical conditions of soils might have led to the variations in DRs observed under similar incubation conditions. This study supports the view that increased SWC, due to rainfall or irrigation, and better management of soil, higher pH, availability of organic matter, and phosphorus generates optimum conditions in fertilized and grazed farms for denitrification. Under most favourable soil conditions for denitrification, the soils abundant with NO_2^- reducing denitrifiers will lead to higher N_2O production.

The analysis using molecular results reported in this chapter has highlighted the general relationships between denitrifier communities, DEA, and DR among the five soils contrasting in DEA. These relationships have been drawn from the molecular measurements performed on field-moist soil reported in the previous chapter. As the denitrifier communities are subject to changes with changing SWC conditions, these data representing general relationships must be interpreted with caution. How will the denitrifier communities respond to changes in SWC between field-moist conditions to saturation need to be covered for understanding the effects of soil manipulations to encourage the reduction of N_2O to N_2 during denitrification? In this study DR responded to increased SWC beyond FC. Since the soils were first wetted to FC, SWC, and subsequently to saturation, this made it difficult to separate the effects of FC and saturation on denitrification. Studies addressing these issues are the focus of the following two chapters. The next chapter discusses variations in N_2O production (with and without C_2H_2) and changes in denitrifier gene structure and abundance measured *in situ* at field-moist condition and after bringing the soil to saturation and incubation for 28 days.

Chapter 6

Effects of saturation and length of incubation on denitrifier gene richness, denitrifier gene abundance, and denitrification rate in incubated dairy-grazed pasture soils

6.1 INTRODUCTION

The results obtained in the previous chapter confirmed the already established fact that with increasing soil water content (SWC) (field capacity & saturation) nitrous oxide (N_2O) production from both nitrification and denitrification increases (Weier *et al.*, 1993a). Due to methodological constraints, the results obtained in the previous study from saturated soils may have been confounded with both additional incubation time and SWC. Apart from the physiochemical characteristics of soils, the denitrifier community structure and its abundance critically control the denitrification (DR) from soils (Braker *et al.*, 2001). The relationships between N_2O production, (from nitrification and denitrification), denitrification enzyme activity (DEA), and denitrifier population were drawn from the molecular measurements performed on field-moist soil.

The denitrifier communities are subject to changes with changing SWC conditions (Zhou *et al.*, 2002). Denitrifier bacterial community structure, abundance, and activities determine the DR and N_2O production, especially under anaerobic conditions (Cavigelli & Robertson, 2000; 2001; Philippot & Hallin, 2005; Rich *et al.*, 2003). Wetting of a paddy soil, for example, significantly altered its bacterial denitrifier community structure and abundance (Somenahally *et al.*, 2011). Hence it is expected that saturation of studied soils with water will influence the denitrifier community structure and abundance, and hence the denitrification activities in the incubated soils. The response of denitrifier communities to changes in SWC between field-moist conditions to saturation needs to be determined to further understand the effects of irrigation, rainfall or urination events from grazing animals and to develop strategies to reduce N_2O production during denitrification.

Despite a number of recent studies of denitrifier genes based on molecular techniques, understanding of the effect of various soil and environmental factors on denitrifier

community structure, size and function is limited (Dandie *et al.*, 2011; Enwall *et al.*, 2010; García-Lledó *et al.*, 2011; Philippot *et al.*, 2009). While the effect of SWC on DR and N_2O production has been explored in New Zealand pasture soils (Ledgard *et al.*, 1999; Luo *et al.*, 2000; Ruz- Jerez *et al.*, 1994), its reaction on denitrifier community structure and abundance is unclear. Understanding the ecology of denitrifying bacteria is extremely important in order to make strategies for N_2O mitigation in pasture systems. *Nir*S, *nir*K, and *nos*Z genes encode for key denitrification steps and thus in this study were used as molecular markers to target both complete and incomplete denitrifiers by measuring their richness (numbers of gene T-RFs) and abundance (gene copy numbers) in contrasting soils.

The current experiment was therefore conducted to study the denitrification activity and denitrifier community structure and abundance in soils under 4-week incubation at saturation. The objective was to understand the changes in denitrification activity, denitrifier community structure and abundance in soils with contrasting physicochemical and molecular characteristics incubated at saturation for an extended period.

For the current study three soils were used: Manawatu fine sandy loam effluent irrigated (MWEI) and Otorohanga silt loam (OH) having the highest (2533 ug N_2O-N $kgsoil^{-1}hr^{-1}$) and the lowest denitrification enzyme activity (DEA) (173 ug N_2O-N $kgsoil^{-1}hr^{-1}$), respectively of the five soils used in chapter 5; the third soil was Tokomaru silt loam (TM) soil with an intermediate DEA (608 ug N_2O-N $kgsoil^{-1}hr^{-1}$). The physicochemical characteristics and description of these soils is given in Tables 4.1, 4.3, & 4.4. These soils belong to one of each group described in chapter 4.

6.2 MATERIALS AND METHODS

6.2.1 Collection of soil samples

The three soils that were used for this study were: MWEI, TM. and OH. Four field-replicate samples for each soil from 0–100 mm depth were collected in October 2013 from the same sites as described in section 4.2.1 by following the procedure detailed in section 4.2.2. The collected soil samples were sieved to 2 mm and stored at 4 ^{o}C for biochemical analysis and –20 ^{o}C for molecular analysis. The denitrification measurements for Day 0 and preparation of extracts for chemical and molecular analysis were performed within 1 week of collection of

samples. The extracts were stored in –20 ^{o}C until used for chemical or molecular analysis within 3 months of extraction.

6.2.2 Incubation of soils

For gas sampling: Fifty g (dry weight equivalent) of each soil subsample was placed in a plastic container (r = 2.25 cm, h = 7.4 cm, vol = 117.63 cm^3) with 1-mm holes (15 in number) on the walls of the container to allow the acetylene (C_2H_2) gas to penetrate the soil and the N_2O to be released from the soil and incubated for denitrification measurement under field-moist conditions (original unamended as received soil), similarl to the method described in the previous chapter (Figure 6.1). Correct volumes of deionised water were gradually added to increase soil water content (SWC) to saturation.

For chemical and molecular analysis: Four field replicate soil samples (250 g each dry weight equivalent) for each treatment were taken in glass jars. Correct volumes of deionised water were added gradually to increase soil water content (SWC) to saturation.

The samples for both the sets were incubated for 28 days. Deionised water was added daily to keep the soil samples saturated during the entire incubation duration. The lids of the jars in which soils were incubated were closed only during the measurement of DR.

Figure 6.1 Incubation of soil samples in jars for gas analysis.

6.2.3 Measurements of soil chemical characteristics and denitrification

The methods used for gaseous, chemical, and molecular analysis of soil samples are described in sections 3.2 to 3.5 of this thesis and a brief outline is given below.

Fresh soil samples were collected from the same sites as described in Table 4.1. DEA, TC, TN, Olsen P, MBC, soluble C (extract from non-fumigated soil), soil pH, NO_3^-, NH_4^+ content, N_2O-NA, N_2O-A, numbers of denitrifier gene T-RFs, and gene copies were measured in soil samples before incubation (original unamended as received field-moist soil). The gas samples during the study for N_2O-NA (soils in non-acetylene jars), and N_2O-A (soils in acetylene jars) measurements were taken at Day 0 (in unamended original as received field-moist soil) 1, 3, 15, and 28 days after saturation. Cumulative N_2O-NA and N_2O-A were calculated (for Day 0 to Day 28) using the trapezoidal rule of under the curve method (Purves, 1992). N_2O-NA corresponded to nitrous oxide produced by nitrification and denitrification and N_2O-A corresponded to nitrous oxide produced by complete denitrification.

6.2.4 Statistical Analysis

The differences in the means of soil characteristics such as gravimetric soil water content TC, TN, Olsen P, MBC, pH, NO_3^--N, and NH_4^+-N, were assessed using one-way analysis of variance The effects of soil type, sampling duration, and saturation on the means of soil characteristics (pH, NO_3^--N, NH_4^+-N, N_2O-NA, and N_2O-A) and molecular parameters (numbers of T-RFs and gene copies) were assessed using repeated measures general linear model ANOVA. Tukey's Studentized Range Test, at $\alpha = 0.05$ significance level, was used *post hoc* to reveal significant differences among the means. The differences in the means of N_2O-NA and N_2O-A reported for the same soils in chapter 5 and this chapter were assessed using two samples T-test. The relationship between soil chemical characteristics, gaseous emissions and molecular parameters during incubation was assessed using Pearson's correlation coefficient at $\alpha = 0.05$ significance.

6.3 RESULTS

6.3.1 Soil properties in the samples collected for this experiment

DEA measured in the original field-moist soils were highest for the MWEI, followed by the TM, and lowest in the OH soil. The MWEI with the highest DEA also had the highest Olsen P, MBC, and NO_3^--N content among the three soils. The TM soil with moderate DEA also had moderate Olsen P, MBC, and lowest TC, TN, and NO_3-N content. The OH soil with least DEA had the highest TC and TN contents, the lowest Olsen P and MBC, and moderate NO_3-N content. The soil pH and NH_4^+-N contents were not significantly different in the three soils used in this study (Table 6.1).

6.3.2 Changes in soil pH, mineral N, soluble C, microbial biomass C (MBC), denitrifier gene T-RFs and denitrifier gene copy numbers during incubations after saturation of soil with only water and no other amendment

F and *P* values from repeated measures ANOVA presented in Table 6.2 shows that for NH_4^+–N, NO_3^-–N, soluble C, and *nir*S+*nir*K gene copy numbers, the effect of soil type and incubation time, and the interaction of soils and incubation period were significant. For numbers of *nos*Z gene T-RFs and *nos*Z gene copy numbers, only the effect of incubation period was significant. Numbers of denitrifier gene T-RFs were unaffected by applied treatments. For MBC, numbers of denitrifier gene T-RFs and *nos*Z gene copy numbers, the effect of soil type was significant. For soil pH, neither the interaction of factors nor the effect of individual factors was significant.

6.3.2.1 Soil pH and mineral N contents in the field-moist and saturated soils

Measured pH of the three field-moist soils did not differ significantly among each other. Saturation of these field-moist soils and incubation resulted in small but insignificant changes in pH (Table 6.2, Fig. 6.2). Since there was no other amendment apart from water added to the soil, there was no significant change in soil pH in any of the three soils.

NH_4^+-N content varied from 15.9 to 85.0 mg kg^{-1} soil during the incubation (Day 0 to Day 28). NH_4^+-N content decreased following saturation until Day 3 or Day 15 and then stabilized in all the soils. NH_4^+-N content was higher in the TM soil than the OH and the

MWEI soils. The interaction term for soil and incubation period was significant suggesting NH_4^+-N content varied with incubation time differently in three soils (Fig. 6.3). NH_4^+-N was significantly the highest on Day 0 in all the three soils and started to decrease from Day 1 onwards. However, the extent of this decrease was variable among three soils. In the MWEI and the OH soil NH_4^+-N content decreased faster than in the TM soil and the least NH_4-N was from Day 1 onwards in the OH soil, Day 3 in the MWEI soil, and Days 15 and 28 in the TM soil

The NO_3^--N content of all the soils varied from 0.7 to 124.9 mg kg^{-1} soil during incubation: the highest NO_3^--N content was in the OH soil, followed by the MWEI and the TM soil. The interaction term for soil and incubation time was significant, suggesting NO_3^--N content varied with sampling time differently in three soils. The NO_3^--N content in all the three soils initially declined (Day 1) following saturation. This was followed by a significant increase in the MWEI and the OH soil between Days 3 and 15 (Fig. 6.4), and no change in the TM soil.

6.3.2.3 Soluble C and MBC contents in the field-moist and saturated soils

The soluble C content (measured as C in the un-fumigated soil samples during measurement of MBC) changed in incubated samples both with saturation and with the increase in incubation time (Fig. 6.5). The increase in soluble C contents in the MWEI and the OH soil was gradual compared with the rapid change in the TM soil. In all three soils the soluble C content increased for the first 15 days of incubation and stabilised thereafter. The pattern of change in the MBC content with increasing incubation time was similar in all three: initially slightly decreasing, thereafter increasing and remaining constant in the soils. However, MBC was significantly the highest in the MWEI soil, followed by the TM and the OH soils (Fig. 6.6).

Table 6.1 Soil characteristics of the collected soils (original field-moist samples) for the experiment. Data = mean ± standard error of mean (*n* = 4)

Soil abbreviation	DEA	Total C (g kg^{-1} soil)	Total N (g kg^{-1} soil)	Olsen P (mg kg^{-1} soil)	MBC (mg kg^{-1} soil)	Soil pH (1:2.5,soil: water ratio)	Nitrate-N	Ammo-N	Gravimetric SWC
MWEI	938.8 ± 183.0^{a}	46.7 ± 2.3^{b}	5.3 ± 0.2^{b}	67.2 ± 12.0^{a}	1175 ± 62^{a}	6.3 ± 0.07^{a}	55.3 ± 2.2^{a}	70.8 ± 2.9^{a}	26.6±1.2^{b}
TM	471.4 ± 139.9^{b}	28.02 ± 0.8^{c}	2.7 ± 0.1^{c}	54.8 ± 2.0^{b}	721 ± 54^{b}	5.9 ± 0.05^{a}	12.0 ± 1.0^{c}	85.0 ± 6.8^{a}	30.3±0.5^{b}
OH	149.9 ± 39.8^{c}	80.62 ± 3.0^{a}	8.7 ± 0.3^{a}	22.2 ± 5.4^{c}	528 ± 42^{c}	6.0 ± 0.23^{a}	43.1 ± 3.9^{b}	74.5 ± 5.3^{a}	58.2±2.7^{a}

Values sharing same letter are not significantly different. The letters indicate the differences in the values only within the column they are presented in MWEI = Manawatu fine sandy loam, TM = Tokomaru silt loam, OH = Otorohanga silt loam, DEA = denitrification enzyme activity, C = carbon, N = nitrogen, P = phosphorus, MBC = microbial biomass C, Ammo-N = ammonical N, SWC = soil water content.

Table 6.2 F and *P* values from repeated measures analysis of variance (ANOVA) to test the effect of soil (S; Manawatu fine sandy loam, Tokomaru silt loam & Otorohanga silt loam), and incubation period (I; 0, 1, 3, 15, or 28 days) on soil characteristics after addition of only water and no other amendments

Source		Soil pH *(1:2.5)*	NH_4-N *(mg kg^{-1} soil)*	NO_3^-N *(mg kg^{-1} soil)*	Soluble C *(mg kg^{-1} soil)*	MBC *(mg kg^{-1} soil)*	*Number of nir*S+*nir*K gene T-RFs	*Number of nos*Z gene T-RFs	*Nir*S+*nir*K gene copies kg^{-1} *soil*	*Nos*Z gene copies kg^{-1} *soil*
I	F	2.11	132.26	44.89	14.03	0.49	0.14	2.67	3.87	3.39
	p	0.0950	0.0001	0.0001	0.0001	0.7440	0.967	0.0440	0.0090	0.0160
S	F	0.37	15.39	84.57	167.70	35.60	69.42	43.30	6.20	4.44
	p	0.692	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0040	0.0170
I × S	F	1.40	2.77	18.88	7.72	1.00	1.17	0.94	3.73	1.08
	p	0.2230	0.0140	0.0001	0.0001	0.4500	0.3360	0.4960	0.0020	0.397

MBC = microbial biomass C, *nir*S and *nir*K = nitrite reductase gene, *nos*Z = nitrous oxide reductase gene, T-RFs = terminal restriction fragments.

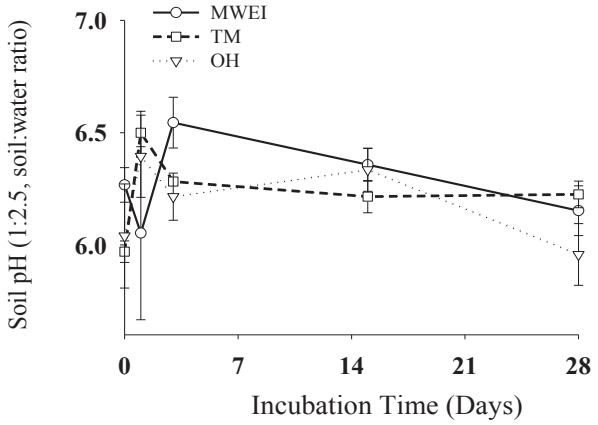


Figure 6.2 Soil pH at various incubation times in Manawatu fine sandy loam (MWEI), Tokomaru silt loam (TM) and Otorohanga silt loam (OH) incubated at saturation with only water and no other amendments for 28 days. Data are means ± standard error of mean (n = 4).

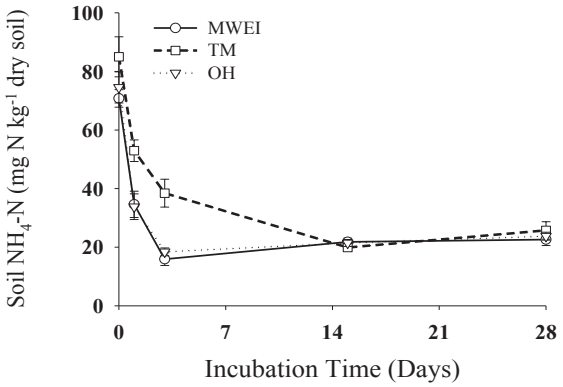


Figure 6.3 Ammonical –N (**(**NH_4^+-N) content (mg-N kg^{-1} dry soil) at various incubation times in Manawatu fine sandy loam (MWEI), Tokomaru silt loam (TM) and Otorohanga silt loam (OH) incubated at saturation with only water and no other amendments for 28 days. Data are means ± standard error of mean. (n = 4).

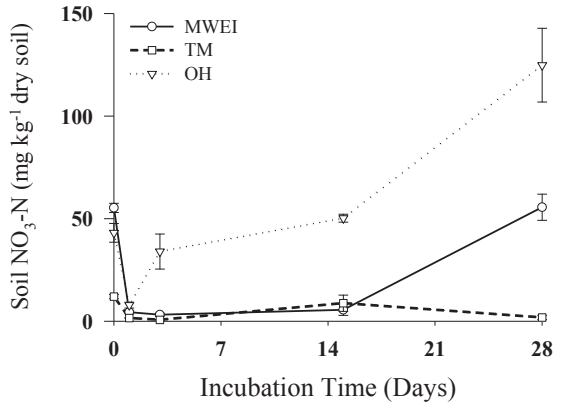


Figure 6.4 Nitrate-N (NO_3-N) content (mg-N kg^{-1} dry soil) at various incubation times in Manawatu fine sandy loam (MWEI), Tokomaru silt loam (TM) and Otorohanga silt loam (OH) incubated at saturation with only water and no other amendments for 28 days. Data are means ± standard error of mean. (n = 4).

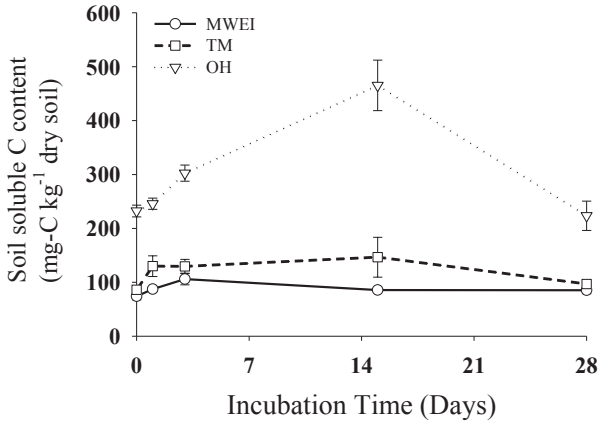


Figure 6.5 Soluble C content (mg-C kg^{-1} dry soil) at various incubation times in Manawatu fine sandy loam (MWEI), Tokomaru silt loam (TM) and Otorohanga silt loam (OH) incubated at saturation with only water and no other amendments for 28 days. Data are means ± standard error of mean (n = 4).

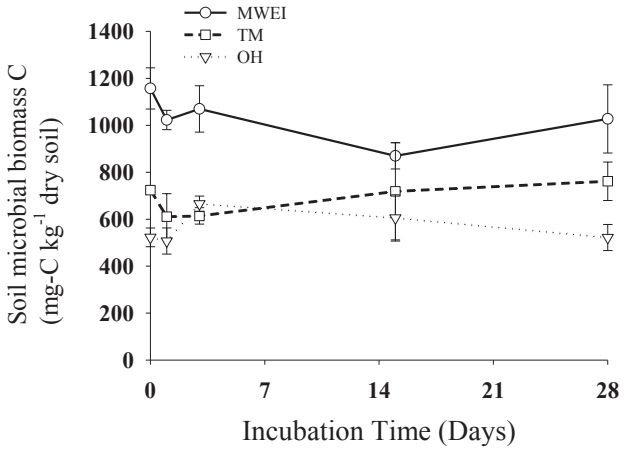


Figure 6.6 Microbial biomass C content (mg-C kg^{-1} dry soil) at various incubation times in Manawatu fine sandy loam (MWEI), Tokomaru silt loam (TM) and Otorohanga silt loam (OH) incubated at saturation with only water and no other amendments for 28 days. Data are means ± standard error of mean (n = 4).

6.3.2.4 Denitrifier gene richness in the incubated soils

In the incubated samples numbers of *nir*S+*nir*K, gene T-RFs varied from 17 to 47 and *nos*Z T-RFs from 12 to 27. The numbers of denitrifier gene T-RFs were higher in the MWEI and TM soil than in the OH soil, especially until Day 20 of incubation of samples. There were large, significant differences in the number of gene T-RFs in field-moist soils and samples measured at Day 15 and Day 28. The trend in the changes in the numbers of *nir*S+*nir*K and *nos*Z gene T-RFs with saturation and period of incubation were similar in three soils (Fig. 6.7). After an initial drop on Day 3 there was not much change in gene copy numbers except for *nir*S+K gene in the TM soil, which increased continuously till the end of incubation. However, the numbers of *nir*S+*nir*K and *nos*Z gene T-RFs varied significantly among the three soils. The numbers of denitrifier gene T-RFs were higher in the MWEI and TM soil than in the OH soil. While there was no change in the numbers of *nir*S+*nir*K gene T-RFs in the incubated soils, with the increase in incubation time, the numbers of *nos*Z gene T-RF varied in soils. Numbers of *nos*Z gene T-RFs slightly but significantly decreased on Day 3 after the addition of water, increased again, and reached the original value by Day 15.

6.3.2.5 Denitrifier gene abundances in the incubated soils

The *nir*S+*nir*K gene copy numbers were higher in the MWEI and TM soils than in the OH soil ($F = 6.15$, $P < 0.05$). There was a small but significant effect from the duration of incubation on the numbers of *nir*S+*nir*K gene copies ($F = 3.91$, $P < 0.05$). There were significantly higher *nir*S+*nir*K gene copy numbers at Day 1 and 15. There was no significant change in the *nir*S+*nir*K gene copy numbers in the MWEI and the OH soils; however, in the TM soil these gene copy numbers increased with incubation period and were measured highest on Day 28. The *nir*S+*nir*K gene copy numbers (Figure 6.8) increased on Day 1 but decreased on Day 3 in all three soils. Subsequently, the *nir*S+*nir*K gene copy numbers started to increase. This increase continued in the TM soil until the end of incubation (Day 28), and until Day 15 in the MWEI and the OH soils. The *nir*S+*nir*K gene copy numbers were higher in the MWEI and TM soils than in the OH soil.

The *nos*Z gene copy numbers were higher in the MWEI soil than in the TM and OH soils ($F = 4.52$, $P < 0.05$). Also the *nos*Z gene copy numbers were higher on Day 28. The changes in *nos*Z gene copy numbers followed the same trend as had been observed with

*nir*S+*nir*K gene copy numbers. The *nos*Z gene copy numbers also increased on Day 1 and decreased on Day 3, with subsequent increases in all the three soils. However, the increase in *nos*Z gene copy numbers continued till Day 28 in the TM and MWEI soils and till Day 15 in the OH soil (Fig. 6.8). At the end of the incubation (Day 28) significantly higher denitrifier gene copy numbers were measured in the MWEI and TM soils than in the OH soil. Apart from a decrease in gene copies on Day 3, the changes in the numbers of denitrifier gene copies were non-significant with change in time in all the three soils.

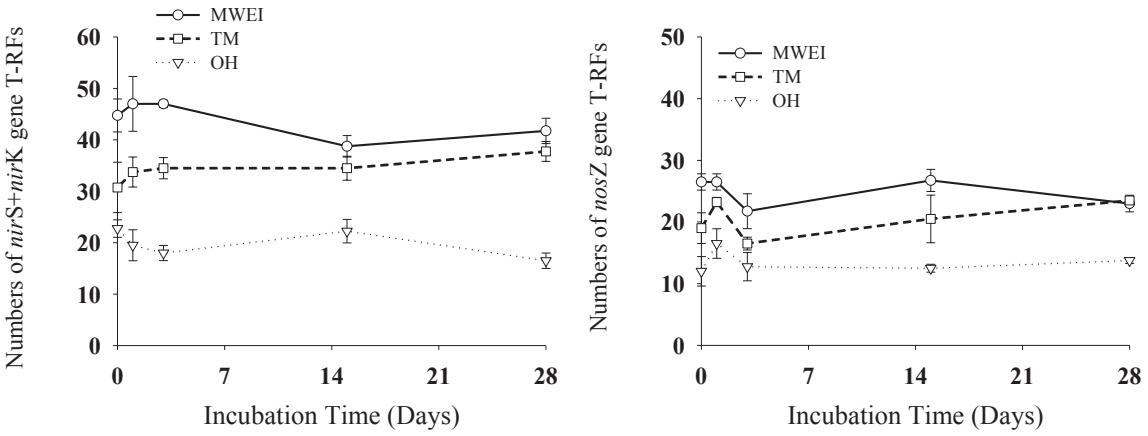


Figure 6.7 Numbers of denitrifier gene terminal restriction fragments (T-RFs) at various incubation times in Manawatu fine sandy loam (MWEI), Tokomaru silt loam (TM), and Otorohanga silt loam (OH) incubated at saturation with only water and no other amendments for 28 days. Data are means ± standard error of mean (n = 4). *nir*S and *nir*K = nitrite reductase, *nos*Z = nitrous oxide reductase gene.

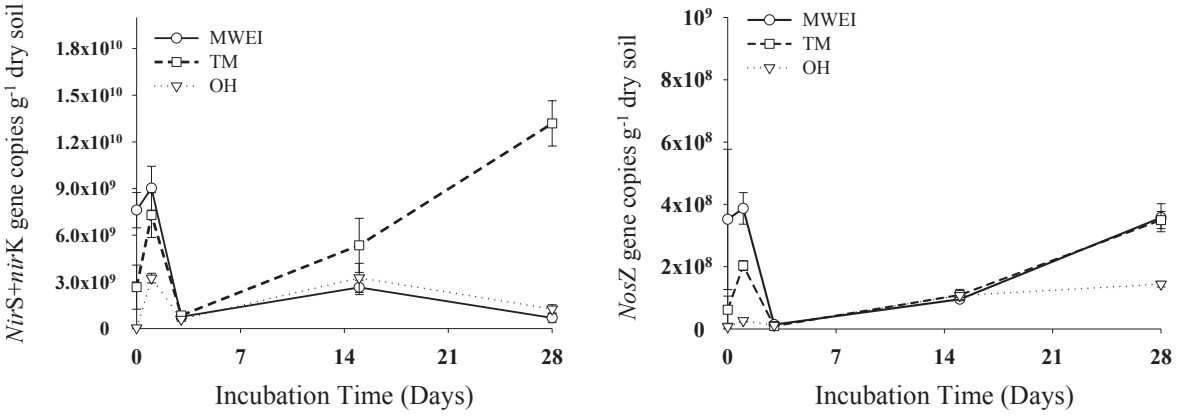


Figure 6.8 Denitrifier gene copy numbers at various incubation times in Manawatu fine sandy loam (MWEI), Tokomaru silt loam (TM), and Otorohanga silt loam (OH) incubated at saturation with only water and no other amendments for 28 days. Data are means standard error of mean (n = 4). *nir*S and *nir*K = nitrite reductase, *nos*Z = nitrous oxide reductase gene.

6.3.3 Changes in the N_2O production in the incubated soils

F and *P* values from the repeated measures ANOVA for N_2O production (from nitrification and denitrification) and DR (Table 6.3) indicate a significant 2-way interaction between soils and incubation period.

6.3.3.1 N_2O production rates in soils from non-acetylene treated jars (N_2O-NA)

The N_2O-NA during incubation was highest in the MWEI soil and similar in the TM and the OH soils ($F = 7.10$, $P < 0.05$). the effect of incubation showed that overall in all three soils N_2O-NA were the least in the original field-moist samples and were measured highest on the Day 28 after saturation ($F = 13.49$, $P < 0.05$). The interaction term for soil and the incubation period was significant, suggesting the differences in N_2O-NA with time differed among the three soils. N_2O-NA significantly increased on Day 1 of saturation of field-moist samples in all the three soils (Fig. 6.9). On Day 1 the magnitude of N_2O-NA was higher in the TM and MWEI soils than in the OH soil compared with that of Day 0 (Figure 6.9). In this experiment in the OH soil, the N_2O-NA remained constant till Day 15 of incubation, and increased again thereafter, suggesting a delayed response to saturation on denitrification in the OH soil. In the MWEI soil, the rate of N_2O-NA decreased on Day 15 of saturation, and increased again until the end of incubation. In the TM soil, after an initial increase the N_2O production rate decreased on Day 3, increased again till Day 15, and remained constant thereafter. In the previous experiment the N_2O-NA increased in all the soils after saturation; however, the N_2O-NA was higher in the OH soil than in the MWEI soil. The cumulative N_2O-NA between Day 0 and Day 28 of incubation in this experiment was not significantly different among the three soils and was measured at 18.0 mg N_2O-N kg^{-1} soil in the MWEI soil, 16.6 mg N_2O-N kg^{-1} soil in the TM soil, and 15.5 mg N_2O-N kg^{-1} soil in the OH soil.

6.3.3.2 Denitrification Rates or N_2O production rates in soils from acetylene treated jars (N_2O-A)

The changes in N_2O-A (either increase or decrease) with the increase in incubation time after saturation was variable among the three soils (Table 6.3). During the 28 days of incubation N_2O-A at saturation varied among the three soil types ($F = 18.98$, $P < 0.05$), with tN_2O-A highest in the MWEI soil and least in the OH soil. With the addition of water to the field-

moist samples on Day 0 there was increase in N_2O-A in all three soils on Day 1 (Fig. 6.9). This higher N_2O-A continued until the end of incubation on Day 28 in the MWEI soil. In the TM and OH soils, after an initial increase N_2O-A subsequently decreased from Day 15 in the TM soil, and from Day 3 in the OH soil. The N_2O-A was significantly higher only in the MWEI soil in the saturated samples rather than the in the field-moist samples at all time-points during the incubation. The highest cumulative denitrification during the entire incubation period was observed in the MWEI soil (102.4 mg N_2O-N kg^{-1} soil), followed by the TM soil (77.2 mg N_2O-N kg^{-1} soil), and the OH soil (54.3 mg N_2O-N kg^{-1} soil).

The fluctuations in the N_2O productions in the incubated soils with sampling time are, to some extent, in agreement with the decrease or increase in NO_3^--N and NH_4^+-N contents in these soils during the incubation. However, these changes were variable in three soils. There was increase in N_2O-A and N_2O-NA within 3 days of saturation when both NO_3^--N and NH_4^+-N are declining in soils. After Day 3 there was no increase or a slight decrease in N_2O-A and an increase in N_2O-NA when NO_3^--N content started to increase in the MWEI and the OH soil, suggesting there was more nitrification than denitrification in these two soils. In the TM soil NO_3^--N content either remained unchanged or slightly decreased after Day 3, and so the DR either remained unchanged or increased after Day 15, suggesting an increase in N_2O-A at a constant rate under saturated conditions in this soil.

Another factor contributing to the increase in denitrification with saturation and until Day 15 of incubation is the available soluble C in the soils after saturation, which gradually increased in soil with the increase in incubation duration and declined again to original levels after Day 15, as did the N_2O-A in the three soils.

The increases in the N_2O-NA and N_2O-A on Day 1 in the MWEI and OH soils are in agreement with the results reported in chapter 5 of this thesis. The differences in the means of the N_2O-NA and N_2O-A at saturation were not significantly different ($P < 0.005$) during the two incubation experiments in the OH soil. However, these values were significantly higher in the MWEI samples incubated at saturation in the current experiment than in the previous one.

Table 6.3 F and *P* values from repeated measures analysis of variances to test the effect of soil (S; Manawatu fine sandy loam, Tokomaru silt loam & Otorohanga silt loam), and incubation period (I; 0, 1, 3, 15, or 28 days) on nitrous oxide emission with acetylene (N_2O-A) and without acetylene (N_2O-NA) in soils after addition of only water and no other amendments

Source		N_2O-NA *(mg N kg^{-1} soil d^{-1})*	N_2O-A *(mg N kg^{-1} soil d^{-1})*
I	**F**	21.93	30.77
	p	0.0001	0.0001
S	**F**	3.37	16.54
	p	0.0430	0.0001
I × S	**F**	4.35	6.58
	p	0.0001	0.0001

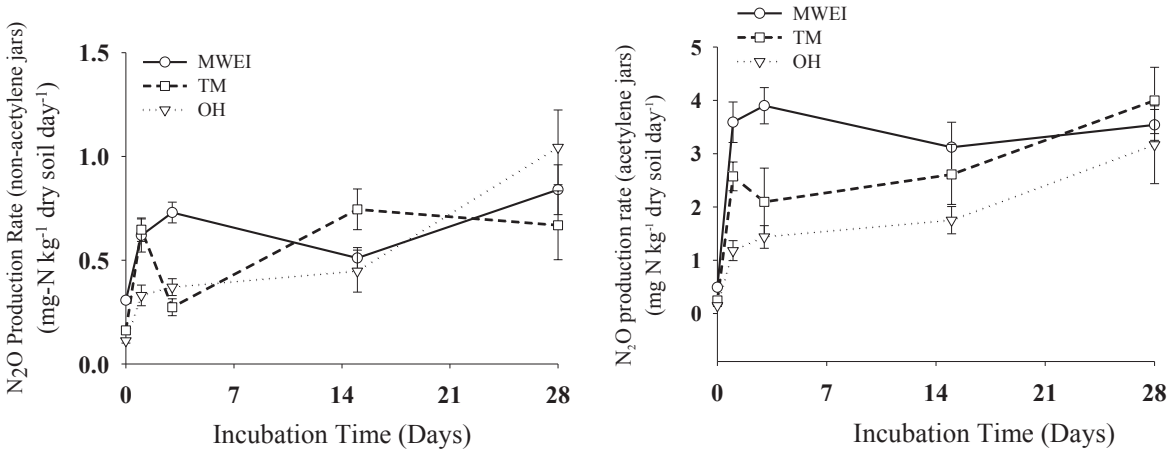


Figure 6.9 Nitrous oxide (N_2O) production (mg-N kg^{-1} dry soil day^{-1}) at various incubation times in Manawatu fine sandy loam (MWEI), Tokomaru silt loam (TM) and Otorohanga silt loam (OH) incubated at saturation with only water and no other amendments for 28 days. Data are means ± standard error of mean (n = 4).

6.3.4 Relationship of denitrifier gene richness and abundance with DR, N_2O, from soils incubated at saturation

The correlation analysis among the soil parameters and denitrification for the entire incubation duration suggested there was no correlation between soil pH and denitrification in these soils after the addition of only water and no other amendment (Table 6.4). Soil NH_4^+-N content was negatively correlated with the N_2O-NA, and DR. N_2O-NA and DRs in the incubated soils related positively to the numbers of *nir*S+*nir*K and *nos*Z gene T-RFs. The denitrifier gene copy numbers did not relate significantly to N_2O-NA and DRs in the studied soils. Cumulative DR was significantly correlated to DEA (r = 0.822), Olsen P (r = 0.714), *nir*S+K gene T-RFs (r = 0.820), and *nos*Z gene T-RFs (r = 0.674).

Table 6.4 Pearson's correlation coefficient (r) among soil properties and denitrification measurements significant at $P < 0.05$

	pH *(1:2.5 ratio)*	**NH_4^+-N** *(mg kg^{-1} soil)*	**NO_3^--N** *(mg kg^{-1} soil)*	**MBC** *(mg kg^{-1} soil)*	***nir*S+*nir*K gene T-RFs**	***nos*Z gene T-RFs**
N_2O-NA (*mg N_2O-N kg^{-1} soil d^{-1})*	ns	–0.500	0.365	0.334	0.289	0.473
DR (N_2O-A) (*mg N_2O-N kg^{-1} soil d^{-1})*	ns	–0.594	ns	0.333	0.417	0.538

Where N_2O-A = nitrous oxide production in soils with acetylene, N_2O-NA = nitrous oxide production in soils without acetylene, NH_4^+-N = ammonical N, NO_3^--N = nitrate N, MBC = microbial biomass carbon, *nir*S, K = nitrite reductase gene, *nos*Z = nitrous oxide reductase gene, T-RFs = terminal restriction fragments.

6.4 DISCUSSION

6.4.1 Effect of saturation and incubation on changes in soil pH, mineral N, soluble C and MBC contents

The incubated soils saturated with deionised water did not show significant pH change. The small pH fluctuations observed during incubation are in line with the literature (Zaman & Blennerhassett, 2010).

Increased SWC creates anaerobic conditions and stimulates denitrification by activation of various reductase enzymes involved in denitrification (Morley *et al.*, 2008). The decline in NO_3^--N content immediately after saturation of soils (Day 1) suggested the initiation of denitrification and reduction of NO_3^- present in soils. The differential capacities of the three incubated soils to mineralise organic N, nitrify NH_4^+, and reduce NO_3^- may have led to the observed differences in NO_3-N content in three soils at different time points during incubation. The fact that NO_3^--N contents continued lower in the MWEI and TM soils corresponded to the continuation of denitrification activity in those two soils. However, an increase in NO_3-N under saturated condition in the OH soil, suggested the possibility of some nitrification in the soil,

especially when the lids were opened in between the gas sampling. Few studies have reported nitrification contributing to N_2O emission even at higher SWC (Adams & Akhtar, 1994; Sánchez-Martín *et al.*, 2008). Comparatively lower DEA in the OH soil might have resulted in the lower reduction of NO_3^- in the incubated samples than the MWEI and TM soil.

In contrast to the NO_3^--N content, the NH_4^+-N content initially increased after the addition of water, and continuously decreased in the incubated samples. The increase in NO_3^--N and decrease in NH_4-N content in incubated samples is attributed to nitrification and anaerobic ammonium oxidation (Rice & Tiedje, 1989) occurring during the incubation in the soils, higher in case of the OH and MWEI soils than the TM. Due to the opening of the lids of the jars in between the gas samplings for denitrification measurements, although the soil was saturated, the exposed surface of soils might be contributing to nitrification of the available NH_4^+-N contents in soils. Nitrification might have resulted in a decrease in the NH_4^+-N contents in the incubated soils. In the non-acetylene-treated jars nitrification was not inhibited, which may have contributed to N_2O production in soils during incubation The fluctuations in the NO_3^--N and NH_4^+-N contents in the incubated soils to some extent were also reflected in their patterns of N_2O-NA and N_2O-A during the incubation, and are discussed in subsequent sections.

The changes in soluble C and MBC content in the three soils were similar, with an initial increase with saturation of soil and followed by a decline with increasing incubation time. Similar trends have been observed in previous studies (Orwin *et al.*, 2010; Uchida *et al.*, 2011), suggesting solubilisation of organic C and then its utilization by active microbial population in soils.

6.4.2 Numbers of denitrifier gene T-RFs and denitrifier gene copy numbers in the incubated soils

The numbers of denitrifier gene T-RFs in a sample reflects denitrifier richness in that soil. In the MWEI and TM soils with higher MBC, Olsen P, and DEA, greater denitrifier gene richness was observed than in the OH soil. Denitrifier communities with *nir*S+*nir*K genes were more richly populated in these soils than the denitrifiers with *nos*Z genes. Since incomplete denitrifiers are richer in these soils, they are more resistant to change (Wittebolle *et al.*, 2009) and remain stable during the incubation compared with the complete denitrifier community.

As with denitrifier gene richness, the denitrifier gene abundance is higher in the MWEI and TM soils than in the OH soil. The changes in gene copy numbers with saturation and increase in incubation time were similar for *nir*S+*nir*K genes and the *nos*Z gene. These gene copy numbers increased immediately after saturation. The initial increase in SWC and the saturated condition of the soil might have resulted in an initial decrease in the gene copy number; with time, the recovery in microbial population might have increased their number again, and the gene copies returned to their original level. The rate of this recovery was variable in the three soils. By measuring denitrifier gene abundance using a similar method, Dandie *et al.* (2008) also reported no significant change in denitrifier gene abundance with time.

The responses of numbers of denitrifier gene T-RFs and copy numbers with increasing SWC in the three soils explained their N_2O-NA and N_2O-A during the incubation. Due to the originally rich denitrifier population in the MWEI and TM soils, compared with the OH soil, for the first 15 days of saturation the numbers of denitrifier gene copies increased with time (except for a drop in gene copies on Day 3) in these two soils, as did the N_2O-NA and N_2O-A. Lower numbers of denitrifier gene T-RFs and a non-significant increase in gene copies were in synchronisation with lower N_2O-NA and N_2O-A for the first 15 days in the OH soil compared with the TM and MWEI soils.

6.4.3 N_2O production rates (N_2O-NA) and DR (N_2O-A) in the incubated samples

High SWC creates anaerobic conditions and stimulates denitrification, which results in th increased production of N_2O and N_2 (Bollmann & Conrad, 1998). Higher DRs and N_2O production rates (from nitrification and denitrification) in selected saturated soils are also presented in the previous chapter of this thesis. The anaerobic conditions activate the reductase enzymes, facilitating the production of N_2O and its reduction to N_2, thus increasing both N_2O production and its total denitrification in saturated soils (Petersen & Andersen, 1996; Weier *et al.*, 1993a).

In the current study the magnitude of the production of N_2O (from nitrification and denitrification) and DR at saturation varied among the three soils and the incubation period. Among the three soils used in this study, the MWEI soil has the highest DEA, MBC, Olsen P content, numbers of *nos*Z gene T-RFs, and *nir*S+*nir*K gene copy numbers, ensuring overall

higher microbial activity. Under high SWC favourable for denitrification this soil produced the highest N_2O (from nitrification and denitrification) during the 28-day incubation. Since the richness of the denitrifier community is highest in the MWEI soil compared with the other two soils, the high Olsen P (corresponding to high ATP and phosphatase contents) of the MWEI soil and the high MBC levels may have increased the activity of the rich denitrifying population, causing higher denitrification and generating higher N_2O (from nitrification and denitrification) than did the other two soils. The allophanic OH soil, with the least DEA, Olsen P, MBC, and denitrifier abundance, produced the least N_2O (from nitrification and denitrification) and had lower DR than the MWEI and TM soils during the entire incubation. Compared with other two soils, the OH soil is allophanic, and the fraction of soluble C out of the total C content is smaller.

Similar increases in N_2O (from nitrification and denitrification) production and DR with saturation were observed in our earlier experiment (chapter 5); the direction of increase in both N_2O (from nitrification and denitrification) production and DR were similar in both the incubation studies, and the results of this study corroborate the findings discussed in chapter 5. However, the denitrification activity in both the incubations was similar only in the OH soil. The soils used for this experiment were collected 2 years after the soils used in previous experiment (chapter 5). Therefore, the MWEI soil would have had 2 additional years of continuous effluent irrigation, which might have initiated a more active microbial population, probably resulting in higher denitrifier activity and, subsequently, higher N_2O (from nitrification and denitrification) production than the previous experiment.

This increase in N_2O production at saturation together with an increase in the duration of incubation, was also observed by Mathieu *et al.* (2006) and Zaman *et al.* (2008c). The increased reductase enzyme activity with time under anaerobic conditions in incubated soils might have influenced higher denitrification activity and N_2O production with increase in incubation time in soils. This might relate to the higher DR or N_2O production in the saturated soils in the previous experiment, which were incubated under elevated SWC for longer duration than the samples incubated at FC. In the MWEI soil, the increase in DR was faster compared with the other two soils (also observed in the previous experiment reported in chapter 5) and this increase was more during the first half of the incubation; however, in the TM and OH soils DR increased gradually with the increase in incubation time.

The soil temperature in field conditions are much lower in winter than the incubation conditions used in the current experiment (25 ^{o}C), suggesting that, despite similar patterns, the amount of N_2O production in the field might be a couple of magnitudes lower than is reported in this experiment, since the Q_{10} value for denitrification is approximately 2 between a temperature range of 15 and 35 ^{o}C (Pelletier *et al.*, 1999). Alternatively, the prolonged anaerobic condition and deposition of animal excreta might generate hot spots for denitrification, and increased denitrifier activity under favourable conditions might override the temperature effect on denitrification activity. Hence higher denitrification could be observed even under cold moist conditions. Prolonged lower temperature in temperate grasslands like New Zealand might produce denitrifier populations that are either cold-tolerant or cold-adapted and can continue denitrification under anaerobic conditions even at low soil temperature (Dorland & Beauchamp, 1991).

6.4.4 Inter-relationships between soil characteristics and denitrification

There were differences among the three soil types in the response to saturation and incubation time in terms of N_2O production and DR. These differences were attributed to the differences in their TC, TN, DEA, Olsen P, MBC, and numbers of denitrifier gene T-RFs in the three soils (Tables 6.4 & 6.5). The DR was higher in the MWEI and TM soils than in the OH soil. The higher DEA, MBC, and Olsen P contents, along with higher numbers of denitrifier gene T-RFs in the MWEI and TM soils compared with the OH soil resulted in a higher denitrification activity overall in these two soils. There was also a strong positive correlation between denitrification and numbers of denitrifier gene T-RFs in the incubated soils. Rich denitrifier communities in soils, actively participating in denitrification at saturation water condition, led to higher denitrification in incubated soils. The higher numbers of denitrifier genes might have facilitated higher denitrification activity in the MWEI and TM soils than in the allophanic free-draining OH soil (Chen *et al.*, 2012b; Deslippe *et al.*, 2014).

The increase in N_2O-NA and N_2O-A was correlated with the numbers of denitrifier gene T-RFs in the tested soils, suggesting saturated condition in soils over the entire incubation period might have influenced the soil environment, facilitating an increase in denitrifier activity in soils

(Dandie *et al.*, 2008; Philippot, 2002; Wallenstein *et al.*, 2006; Zumft & Kroneck, 2006). The results previously reported in chapter 5 also showed the positive relationship of N_2O-NA and N_2O-A at saturation only with the numbers of *nir*K gene T-RFs, but not with the *nir*S or *nos*Z gene. In this chapter the simultaneous measurements of DR and *nir*S+*nir*K and *nos*Z gene T-RFs correlated positively with each other. The positive relationship between *nos*Z-TRFs and N_2O-NA suggests that although these soils have rich denitrifying population with *nos*Z gene, these denitrifiers are involved more in the reduction of NO_3^- to N_2O than in the reduction of N_2O to N_2. The anaerobic conditions during incubation might have influenced both the denitrifiers and their activities in incubated soils.

Even though there was initial synchronisation in the increase in denitrifier bacterial gene copy numbers and DR in soils within 24 hours of saturation, there was no correlation to denitrifier gene copy numbers and denitrification activities in the saturated soils during the entire incubation duration in the current experiment in any of the three soils. The response of the abundance of denitrifying communities to the changes in soil condition or the response of denitrification to the changes in the soil conditions are not necessarily in accordance with each other. Several studies report that abundances of denitrifier genes are not correlated to measured denitrification activities (Attard *et al.*, 2011; Dandie *et al.*, 2008; Miller *et al.*, 2008). While the prolonged incubation condition might have had a major impact on the abundance and activity of a few dominant denitrifiers, and consequently produced higher denitrification activity, they did not influence the total gene copy numbers.

Although NO_3^--N content is one of the important substrates for denitrification, no significant relationship was observed between NO_3^--N content and denitrification or the abundance of denitrifier genes in the current study; this has also been reported in several other studies (Enwall *et al.*, 2010; Kandeler *et al.*, 2006; Ligi *et al.*, 2013). On the other hand, the N_2O-NA and N_2O-A and *nir*S+*nir*K gene T-RFs were negatively correlated to the NH_4-N content in the soils, suggesting higher NH_4-N contents in soils stimulate nitrification and the populations of ammonia oxidizing bacteria and not the denitrifiers. The relationships between N_2O production and DR with denitrifier community structure and abundance suggest that, under favourable conditions, an increase in selected dominant denitrifier population results in higher DR.

6.5 SUMMARY AND CONCLUSIONS

Bringing the soils to saturation and incubating for 4 weeks increased overall N_2O-NA and N_2O-A during first few days of incubation.

These increases in N_2O-NA and N_2O-A were variable in three soils and depended on their inherent soil characteristics such as DEA, Olsen P, MBC, TN, TC, and denitrifier richness and abundance. The patterns of increase and decrease in N_2O-NA in the incubated soils were in accordance with the changes in their NO_3^--N NH_4^+-N and soluble C contents. The numbers of denitrifier gene T-RFs remained constant or decreased with time. Overall, the denitrifier gene copy numbers decreased initially and increased later to their original values with the increase in incubation time under saturation (apart from the increase in *nir*S+*nir*K gene copy number in the TM soil).

The higher denitrification in those saturated soils incubated for a longer period confirmed the combined effect of both saturation and incubation period on the denitrification activity in the incubated soils considered in the previous study reported in chapter 5. The results of the two incubation studies also showed an increase in denitrification with saturation. Denitrification under saturated condition in these soils is related more to the numbers of denitrifier T-RFs than to denitrifier gene copy numbers, suggesting denitrifier abundance in soils remained constant throughout the incubation.

This study only reports the changes measured in soils after the application of water. Since year-round grazing is common practice in New Zealand pasture soils, how denitrification activity, denitrifier richness, and denitrifier abundance change with urine from grazing animals in the field needs to be determined. The results of the incubation describing the effect of cattle urine with and without DCD on denitrification and denitrifier community structure and abundance are discussed in next chapter.

Chapter 7

Effects of cattle urine and DCD on denitrifier gene richness, denitrifier gene abundance, and denitrification rates in incubated dairy-grazed pasture soils

7.1 INTRODUCTION

Because urine patches provide readily available N and C, they are among the highest sources of nitrous oxide (N_2O) emissions from agricultural soils (Van Groenigen *et al.*, 2005a; Yamulki *et al.*, 2000). In New Zealand, 80 % of agricultural N_2O emissions arise from excreta deposition by grazing animals on farms (de Klein & Ledgard, 2005). About 60 % of N_2O emitted from a urine patch is attributed to denitrification (Di & Cameron, 2008). Based on a review of 241 datasets of denitrification rates (DR) and the percentage of N denitrified, Saggar *et al.* (2013) estimated an annual loss of 5.6 Tg of N through denitrification in temperate grasslands.

Nitrous oxide emissions resulting from denitrification could be mitigated by improved drainage of soils, optimum mineral N fertilizer application, use of nitrification inhibitors, and improved animal and pasture management (Luo *et al.*, 2008b; Saggar *et al.*, 2009; 2011). Commercially formulated nitrification inhibitors such as dicyandiamide (DCD), are generally used to reduce N_2O emissions in New Zealand and around the world. The average reduction of N_2O emissions with DCD addition (10 kg ha^{-1}) (standard recommendation is 2 applications of DCD) has been reported to be 57 % from soils affected by urine (de Klein *et al.*, 2011).

The available NH_4^+ in soil is transformed via nitrification by ammonia-oxidising bacteria (AOB) to NO_3^- during which N_2O is released. This NO_3^- is subsequently denitrified either to N_2O or N_2 by denitrifying bacteria. DCD reduces N_2O emissions by inhibiting the active site of ammonia mono-oxygenase (AMO) (Amberger, 1989; Subbarao *et al.*, 2006) and a subsequent decrease in the abundance of AOB (O'Callaghan *et al.*, 2010). Lower abundance or activity of AOB will result in lesser availability of NO_3^- for denitrifiers to either reduce to N_2O or N_2.While

considerable information is available on the influence of N fertilization or manure application on the changes in denitrifier gene abundance (Chen *et al.*, 2012b; Chon *et al.*, 2011; Miller *et al.*, 2009a), less is known about the effect of urine and urine + DCD application on the abundance of denitrifiers in soils. Di *et al.* (2014) reported lower *nir*K gene abundance in soils treated with urine with added DCD than without DCD. Since most AOB also code for the *nir*K gene (Lund *et al.*, 2012), reduction in its abundance was more likely an indication of reduction of AOB population than of denitrifiers. Similarly, Wakelin *et al.* (2013) reported small but significantly lower *nir*S gene abundances in DCD applied urine treatment compared with urine only; however, this difference was not observed after subsequent DCD applications to soil.

High N_2O emissions following rainfall events have been measured in urine patches deposited by grazing animals. A previous chapter addressed denitrifier gene richness, denitrifier gene abundance, and N_2O production (with and without C_2H_2) in soils saturated with water. The richness and abundance of the denitrifiers remained stable during the incubation; however, the inherent variability in denitrifier population was reflected in N_2O production (with and without C_2H_2) during the incubation. An animal urination event discharges very high concentrations of nutrients in a smaller volume of soils and might impact inherent denitrifier population and their activity differently in soils with varying physicochemical characteristics. Similarly, application of DCD with urine by controlling NO_3^- concentration indirectly might affect the denitrifier population and their activity.

Therefore in order to understand the impact of urine and urine + DCD application on existing denitrifier population and their involvement in denitrification in three soils with contrasting denitrification, enzyme activities (DEA) and physicochemical characteristics were selected for this experiment. *Nir*S, *nir*K, and *nos*Z genes encode for key denitrification steps and thus were used as molecular markers to target both incomplete and complete denitrifiers by measuring their richness (numbers of gene T-RFs) and abundance (gene copy numbers) in contrasting soils with application of urine and urine + DCD. How abundance of incomplete and complete denitrifiers change in soils with application of urine and urine + DCD and thus overall N_2O production needs to be tested. The overall biochemical and molecular changes in soils with the addition of urine and urine + DCD would help us understand the course of denitrification and the denitrifier population involved in the process under urination and rainfall events on farms.

This information is vital to develop mitigation strategies to enhance the reduction of N_2O during denitrification from dairy pasture soils.

The objectives of this experiment were: (i) to measure total denitrification with urine and urine + DCD applications on dairy pasture soils with contrasting DEA; (ii) to observe denitrifier gene richness with the application of urine and urine + DCD; and (iii) to determine changes in the denitrifier gene copy numbers with the application of urine and urine + DCD

7.2 MATERIALS AND METHODS

7.2.1 Collection of soil samples

The collection of soil samples, Manawatu fine sandy loam effluent irrigated (MWEI), Otorohanga silt loam (OH), and Tokomaru silt loam (TM), has already been described in detail in section 6.2.1.

7.2.2 Application of treatments and incubation of soils

Fresh cattle urine was collected from cows during milking, (avoiding contamination from dung), and stored in tightly sealed plastic bottles at 4 ^{o}C to avoid urea hydrolysis. The chemical composition of the collected urine was determined using the protocols described in section 3.5 and presented in Table 7.1.

Table 7.1 Characteristics of collected bovine urine used in the experiment. Data are mean ± standard error of mean (n = 4)

Total N (g- N L^{-1})	7.6 ± 0.4
Total C (g- C L^{-1})	15.6 ± 0.2
NH_4-N (mg- N L^{-1})	32.5 ± 4.2
NO_3-N (mg- N L^{-1})	2.3 ± 0.8
pH (1:2.5, urine: water ratio)	7.6 ± 0.3

The treatments were: Control (CT) (deionised water); Urine (700 mg N kg^{-1} dry soil) (U); and Urine + DCD (10 mg DCD kg^{-1} dry soil) (UI). The total amount of pre-calculated deionised water in the C treatments and the amounts minus urine or urine +DCD solution were added to the U and UI treatments to saturate the soils. After application of the required amount of water 4.6 ml of cattle urine in the U and urine + 1 ml of DCD solution (10 mg DCD dissolved in 20 ml deionised water) were added to the UI treatments.

Incubation: The detailed procedure for incubation of soil samples has been described in section 6.2.2. Samples for chemical, gaseous and molecular analysis were taken on Day 1, 3, 15 and 28 after addition of treatments (CT, U and UI).

7.2.3 Measurements of soil chemical characteristics and denitrification

Soils with applied treatments were incubated for 28 days and during the incubation gas samples were taken to measure N_2O and N_2O+N_2 production in the incubated soils. Soil sub-samples were also taken to analyse soil pH, nitrate-N, ammonical-N, microbial biomass C, and soluble C denitrifier (*nir* and *nos*) gene richness and abundance. The methods for collection and analysis of gases for denitrification measurement, chemical and molecular analysis of soil samples are described in chapters 3 and 6. In this chapter measurements from all the treatments starting from Day 1 to Day 28 are described. The preceding chapter contains measurements taken from water only applied treatment from Day 0 to Day 28.

7.2.4 Calculation of changes in applied N

Since there was no trace element added with cattle urine in this experiment, measurements of the remained N in the soil, the proportion of added N denitrified or the reduction in N_2O emission with the use of DCD with urine may not be exact but are the closest estimation.

The percentage of that added N (as cattle urine) which remained in soil samples as mineral N (NH_4^+-N or NO_3^--N) at the end of incubation was calculated as:

$$\text{Percent N remained} = \left[\frac{N_U - N_{CT}}{\text{Urine-N applied}}\right] \times 100 \qquad \text{Eq 7.1}$$

where N_U and N_{CT} correspond to the mineral N contents in the U and control treatments respectively.

The proportion of applied urine N denitrified (DR_N) in the U and UI treatments were calculated using following equations:

$$DR_N = \left(\frac{Cum_U - Cum_{CT}}{\text{Urine-N applied}}\right) \times 100 \qquad \text{Eq 7.2}$$

where Cum_U corresponds to cumulative N_2O-A produced in the urine or urine +DCD treatment and Cum_{CT} corresponds to respective measurement in the control treatment.

The percentage reduction in N_2O-NA with addition of DCD to urine was calculated using following equation

$$\text{Percent reduction in } N_2O\text{-NA} = \left(\frac{Cum_U - Cum_{UI}}{Cum_U - Cum_{CT}}\right) \times 100 \qquad \text{Eq 7.3}$$

where Cum_U, Cum_{UI} and Cum_{CT} correspond to the cumulative N_2O-NA in the urine, urine + DCD and control treatments respectively.

7.2.5 Statistical Analysis

The data for soil chemical characteristics, gaseous emissions, denitrifier gene richness, and denitrifier gene abundance were analysed as described previously in Section 6.2.4.

7.3 RESULTS

7.3.1 Changes in soil pH, mineral N, soluble C, microbial biomass C (MBC), denitrifier gene T-RFs and denitrifier gene copy numbers during incubations with application of urine and urine + DCD

F and *P* values from repeated measures ANOVA presented in Table 7.2 show that for NH_4^+-N, NO_3^--N, soluble C, and *nir*S+*nir*K gene copy numbers effects of individual factors, soil type, incubation duration, and applied treatments, and their the three-way interaction were significant. For numbers of denitrifier gene T-RFs and *nos*Z gene copy numbers in addition to individual factors, the effect of 2-way interactions of incubation duration and soil were significant. Numbers of denitrifier gene T-RFs were unaffected by applied treatments. For numbers of *nos*Z gene T-RFs, 2-way interactions of incubation duration with treatments, duration with soil, and soils with treatments were significant. For MBC and *nos*Z gene copy numbers only the individual effects of these factors were significant.

7.3.1.1 Soil pH

The effect of applied urine and urine + DCD on changes in soil pH with increasing incubation time differed in the three soils. The application of urine immediately increased soil pH in the MWEI soil (Fig. 7.1). In the MWEI soil on Day 1 after application of treatments (CT, U, &,UI), soil pH was higher in the UI treatment than the U treatment and least in the CT treatment. In the TM soil on Day 1 soil pH in the U & UI treatments were significantly different from CT. In the OH soil there was no significant difference in the soil pH in any of the three applied treatments (CT, U &UI) on Day 1. The variable response of the three soils in pH change with the application of urine and urine + DCD on Day 1 could be due both to differential urea hydrolysis in the three soils and to the variable capacities of the soils to buffer pH changes with application of urine.

With the increase in incubation time, soil pH was not significantly different in any of the three treatments in the MWEI and OH soils. However, in the TM soil higher soil pH in the two urine treatments continued until Day 3, when soil pH was significantly highest in the UI

treatment. The rates of increase in soil pH in the U & UI treatments were significantly higher than the rate of change in soil pH in the CT treatment. After an initial increase, pH started to decrease from Day 3 onwards in the MWEI and TM soils, and only after Day 1 in the OH soil. This decrease in pH indicated cessation of urea hydrolysis, which lasts for 24 to 72 hours after addition. The pH continued to decrease, and by the Day 15 it was not significantly different in the three treatments in all three soils.

7.3.1.2 Ammonical-N (NH_4^+-N) Content

Although peak NH_4^+-N contents were observed in the U & UI treatments on Day 1, it subsequently decreased in these treatments in all three soils (Fig. 7.1). This decrease could be attributed to the nitrification and ammonia volatilisation of available NH_4^+ after urea hydrolysis. However, the pattern of changes in NH_4^+-N content with increasing incubation duration varied among the three soils used here. Only in the OH soil was there significantly higher NH_4^+-N content in the UI treatment than the U treatment on Day 1 after application of treatments. This suggested DCD addition with urine immediately inhibited nitrification in this soil. In the OH soil there was very sharp decrease in the NH_4^+-N content in the U treatment between Days 1 and 3, which could be due either to nitrification or to ammonia volatilisation. In the MWEI and TM soils this decrease was significant between Days 3 and 15, which showed the gradual effect of DCD in these soils. In the TM soil decrease in the NH_4^+-N content was gradual and NH_4^+-N content was higher in the U treatment than CT during the entire incubation. In the TM soil there was a significant increase in the NH_4^+-N content in the U & UI treatments between Days 15 and 28. At the end of the 28-days of incubation the TM soil still had 31 % of the added N as NH_4-N in the U and 41 % in the UI treatment (using Eq. 7.1). In the MWEI soil this value was 16 % for U, and 12 % for UI. In the OH soil a very small amount of urine N was left as NH_4^+-N by the end of incubation – only 3 % in the U and 2 % in the UI treatment.

7.3.1.3 Nitrate-N (NO_3^--N) Content

The increases in the NO_3^--N contents with the application of urine and urine + DCD were more immediate and sharper in the OH soil than in the MWEI and TM soils, in which the increases in NO_3^--N contents were gradual (Fig. 7.2). Higher NO_3^--N content in the OH soil signals higher nitrification than denitrification in the OH soil compared with the TM and MWEI soils. In the OH soil the NO_3^--N content in the U & UI treatments was significantly higher than in the CT treatment on Day 3. In the MWEI and TM soils the increases in NO_3^--N content in the U & UI treatments started from Day 3 and continued. However, NO_3^--N content remained higher in the U & UI treatments than in the C treatment until Day 28 in all three soils. In the TM soil the NO_3^--N content was significantly lower in the UI treatment than in the U treatment on Day 15. At the end of the 28 days incubation, the MWEI soil had 33 % of the added N as NO_3-N in the U and 31 % in the UI treatment (using Eq. 7.1). In the TM soil these values were 24 % for U and 31 % for UI. In the OH soil the amount of NO_3^--N left at the end of incubation was 31 % in U and 29 % in UI of the total N applied in urine.

7.3.1.4 Soluble C and MBC content in the incubated soils

Soluble C content changed in the incubated samples both with application of treatments (U &UI) and increase in incubation time (Fig. 7.3). The increases in soluble C contents in the MWEI and OH soils were gradual compared with the rapid change in the TM soil. In all three soils the soluble C content increased for the first 15 days of incubation. There was higher soluble C content in the U and UI treatments than in the control in all the three soils, especially on the Day 15.

Similar to the soluble C content in the incubated soils, the MBC content also changed with the application of treatments and incubation duration (Fig. 7.3). The changes in MBC content with urine and urine + DCD application during incubation were similar in the three soils. MBC contents in each soil increased for the first 15 days and were significantly highest on Day 15 than at other time-points. There were higher MBC contents in the U & UI treatments than in the control but the MBC content was not significantly different among the UI & CT treatments. The MBC content was significantly highest in the MWEI soil and least in the OH soil. Urine

application increased the MBC in the soils; however, significant increases as compared with the control were only observed in the MWEI and TM soils, and that too only on Day 15.

Table 7.2 F and *P* values from repeated measures analysis of variance to test the effect of soil type (S; Manawatu fine sandy loam, Tokomaru silt loam & Otorohanga silt loam), treatment (T; control, urine, urine + DCD), and incubation period (I; 1, 3, 15, or 28 days) on soil characteristics

Source		Soil pH *(1:2.5)*	NH_4-N *(mg kg^{-1} soil)*	NO_3^-N *(mg kg^{-1} soil)*	Soluble C *(mg kg^{-1} soil)*	MBC *(mg kg^{-1} soil)*	*Number of nir*S+*nir*K gene T-RFs	*Number of nos*Z gene T-RFs	*Nir*S+*nir*K gene copies *kg^{-1} soil*	*Nos*Z gene copies *Kg^{-1} soil*
I	**F**	76.42	85.76	491.06	22.47	4.06	2.67	2.55	12.60	10.43
	p	0.0001	0.0001	0.0001	0.0001	0.0090	0.0510	0.0060	0.0001	0.0001
S	**F**	11.23	16.41	280.10	223.60	75.24	206.03	116.04	42.06	40.80
	p	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
T	**F**	15.91	147.72	498.82	28.26	10.32	1.01	0.51	11.47	4.78
	p	0.0001	0.0001	0.0001	0.0001	0.0001	0.3070	0.6010	0.0001	0.0100
I × S	**F**	3.06	3.12	40.91	9.31	0.98	2.83	5.44	4.13	3.08
	p	0.0080	0.0070	0.0001	0.0001	0.4400	0.0130	0.0001	0.0001	0.008
I × T	**F**	12.24	19.63	66.19	3.86	1.68	1.29	3.21	0.02	1.68
	p	0.0001	0.0001	0.0001	0.0020	0.1330	0.2660	0.0060	1.000	0.1330
S × T	**F**	1.48	3.78	18.00	14.54	1.80	0.71	2.81	13.65	1.68
	p	0.2120	0.0060	0.0001	0.0001	0.1330	0.5860	0.0290	0.0001	0.1330
I× S × T	**F**	2.12	2.57	11.15	3.56	0.68	0.69	0.41	1.95	0.77
	p	0.0210	0.0050	0.0001	0.0001	0.7710	0.757	0.958	0.0360	0.6840

NH_4^+-N = ammonical N, NO_3^--N = nitrate N, MBC = microbial biomass carbon, *nir*S and *nir*K = nitrite reductase gene, *nos*Z =nitrous oxide reductase gene.

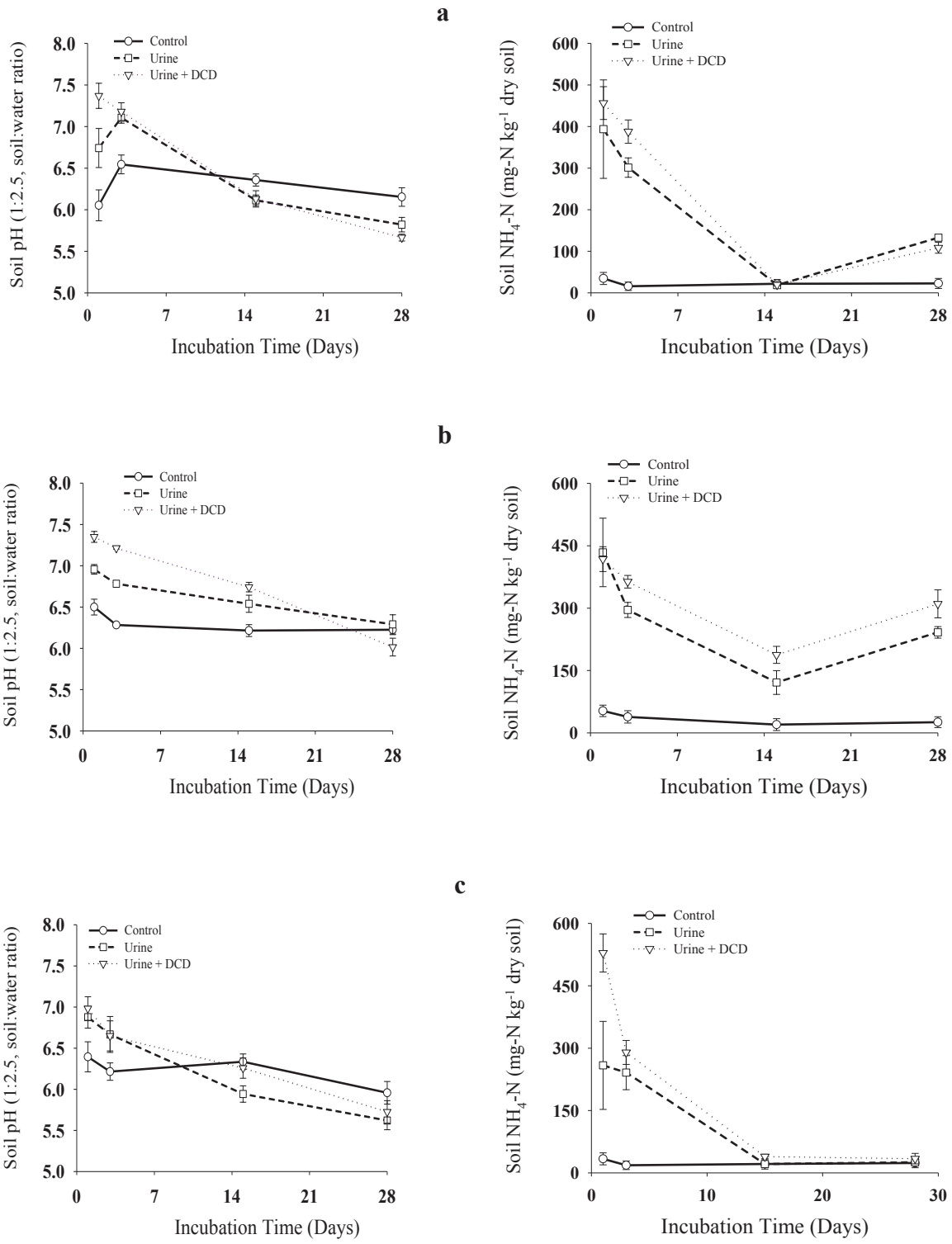


Figure 7.1 Soil pH and ammonical-N (NH_4^+-N) content (mg-N kg^{-1} dry soil) in (a) Manawatu fine sandy loam, (b) Tokomaru silt loam, and (c) Otorohanga silt loam incubated at saturation with three applied treatments (control, urine & urine + DCD) at various incubation times. Data are means ± standard error of mean (n = 4).

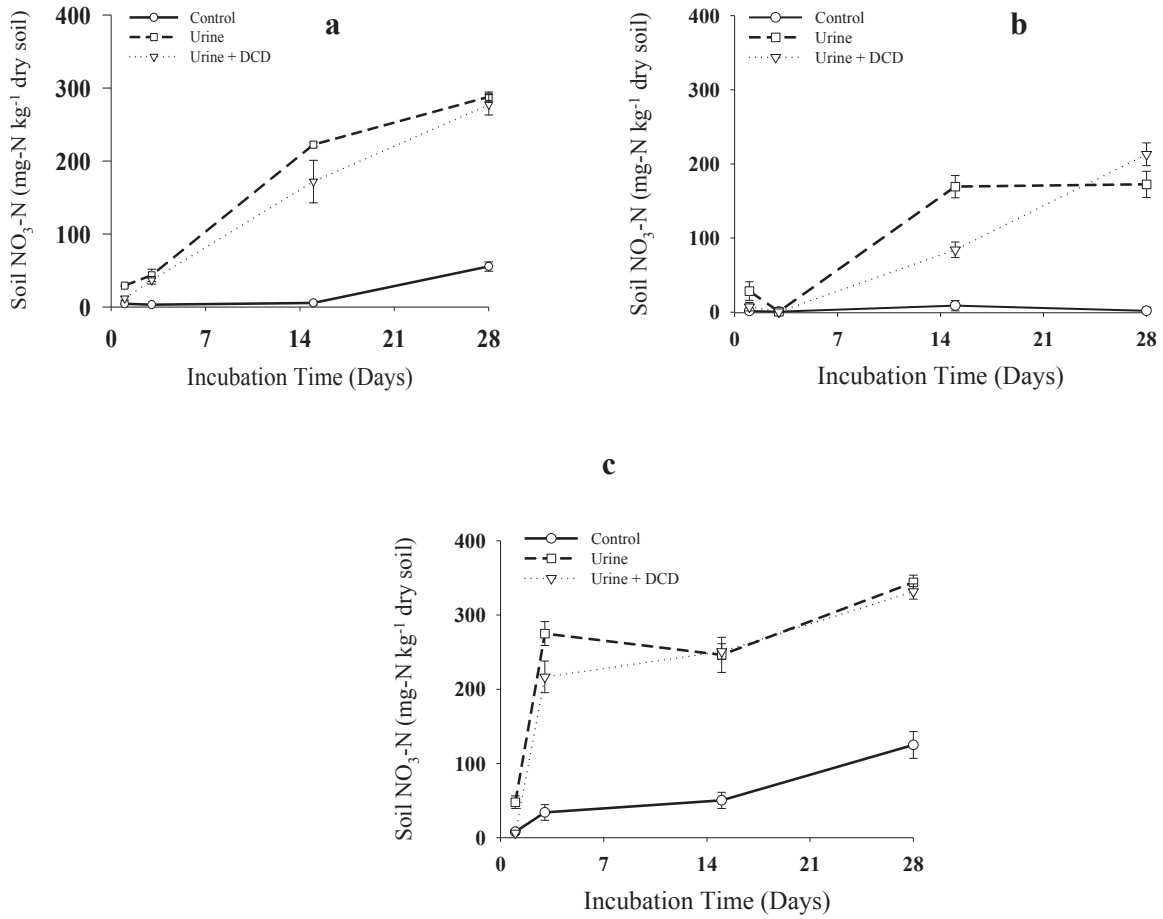


Figure 7.2 Soil nitrate-N (NO_3^--N) (mg-N kg^{-1} soil) content in (a) Manawatu fine sandy loam, (b) Tokomaru silt loam, and (c) Otorohanga silt loam incubated at saturation with three applied treatments (control, urine & urine + DCD) at various incubation times. Data are means ± Standard error of mean (n = 4).

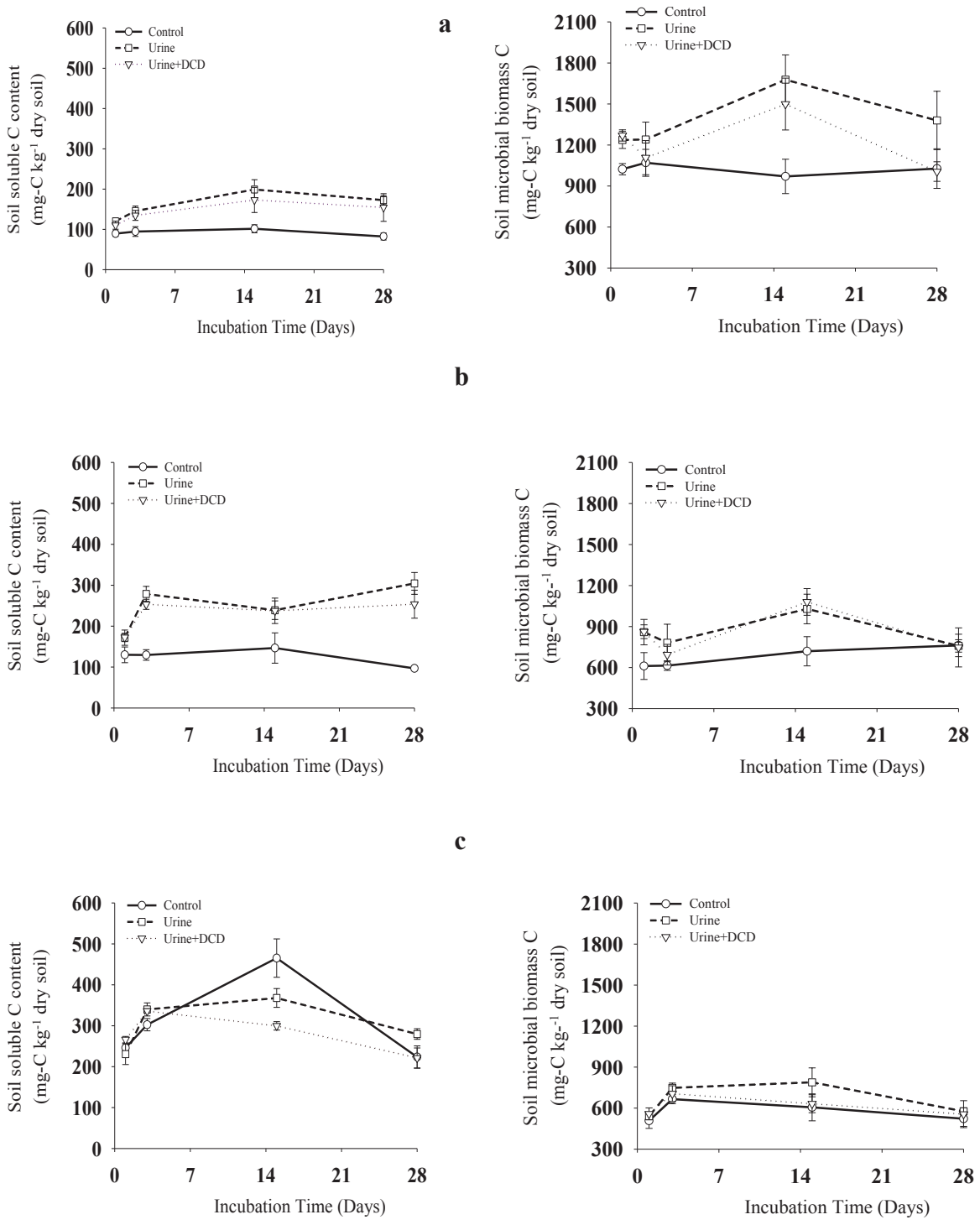


Figure 7.3 Soluble C and microbial biomass carbon contents (mg-N kg^{-1} dry soil) in (a) Manawatu fine sandy loam, (b) Tokomaru silt loam, and (c) Otorohanga silt loam incubated at saturation with three applied treatments (control, urine & urine + DCD) at various incubation times. Data are means ± standard error of mean (n = 4).

7.3.2 Denitrifier gene richness in the incubated soils

Comparatively longer base pair lengths of *nir*S and *nir*K genes than *nos*Z genes resulted in higher richness (numbers of gene T-RFs) of the former in all three soils. The effect of incubation period (Days 1, 3, 15, and 28) on changes in numbers of *nir*S+*nir*K gene T-RFs was variable in the three soils (Table 7.2). The decrease in numbers of *nir*S+*nir*K gene T-RFs was significant only in the MWEI soil, and these numbers were significantly higher on Day 3 than on Days 15 and 28 (Figure 7.4). In the TM and OH soils there was no significant difference in the numbers of *nir*S+*nir*K gene T-RFs at any time-point during the 28-days incubation. Overall, the numbers of *nir*S+*nir*K gene T-RFs were significantly higher in the MWEI soil than in the OH soil.

The effects of both sampling duration and applied treatments on numbers of *nos*Z gene T-RFs were variable in the three soils. The effect of sampling time-points on numbers of *nos*Z gene T-RFs were also variable with applied treatments. The increase in the numbers of *nos*Z gene T-RFs with respect to incubation duration was significant both in the MWEI and the TM soils. In the MWEI soil the numbers of *nos*Z gene T-RFs increased after Day 1 and continued until Day 15. In the TM soil numbers of *nos*Z gene T-RFs increased between Days 3 and 15. There was a greater increase in the numbers of *nos*Z gene T-RFs in the CT treatment in the MWEI and TM soils than in the U and UI treatments. Numbers of *nos*Z gene T-RFs in the CT and U treatments in the MWEI and TM soils were significantly higher than *nos*Z T-RFs in the OH soil. In the UI treatment, the numbers of *nos*Z gene T-RFs were significantly higher in the MWEI soil, followed by the TM soil, and the least in the OH soil.

7.3.3 Denitrifier gene abundances in the incubated soils

The richness of denitrifier genes was positively correlated with the abundance of *nir*S+*nir*K (r = 0.404, $P < 0.0001$) and *nos*Z gene (r = 0.422, $P < 0.0001$). MWEI and TM soils, which had greater numbers of denitrifier gene T-RFs also, had more of these genes relative to the OH soil. The effect of treatments over the duration of incubation on *nir*S+*nir*K gene copy numbers differed among the three soils, however, soils did not differ with respect to *nos*Z gene copy numbers. After an initial decrease in the *nir*S+*nir*K gene copy numbers on Day 3, *nir* gene numbers increased in all the three soils (Figure 7.5). However, in the MWEI soil *nir* gene copies were significantly higher in the U & UI treatments than CT at all sampling time-points. While there was increase in the *nir*S+*nir*K gene copy numbers in the TM and OH soil with increase in incubation length, there was no significant difference in the gene copy numbers among the three treatments (CT, U & UI).

Similar to *nir*S+*nir*K gene copy numbers, the *nos*Z gene copy numbers also decreased on Day 3; however, only in the MWEI and TM soils. Gene copy numbers increased again in these two soils and, *nos*Z gene copy numbers on Days 15 and 28 were not significantly different than on Day 1. Overall, with the increase in the *nos*Z gene copy numbers during the latter part of incubation these gene copies were significantly higher on Days 1 and 28 than on Days 3 and 15. Also the *nos*Z gene copy numbers were significantly higher in the MWEI soil than in the other two soils, with the OH soil having the least *nos*Z gene copies (Fig. 7.5). *Nos*Z gene copy numbers were significantly higher in the U treatment than in the C treatment; however, there was no significant difference between the *nos*Z gene copy numbers in the U and UI treatments and in the UI and CT treatments. There were slight fluctuations in the numbers of denitrifier gene T-RFs during the incubation, but overall the numbers were quite stable and not significantly different on Day 28 than on Day 1 of incubation.

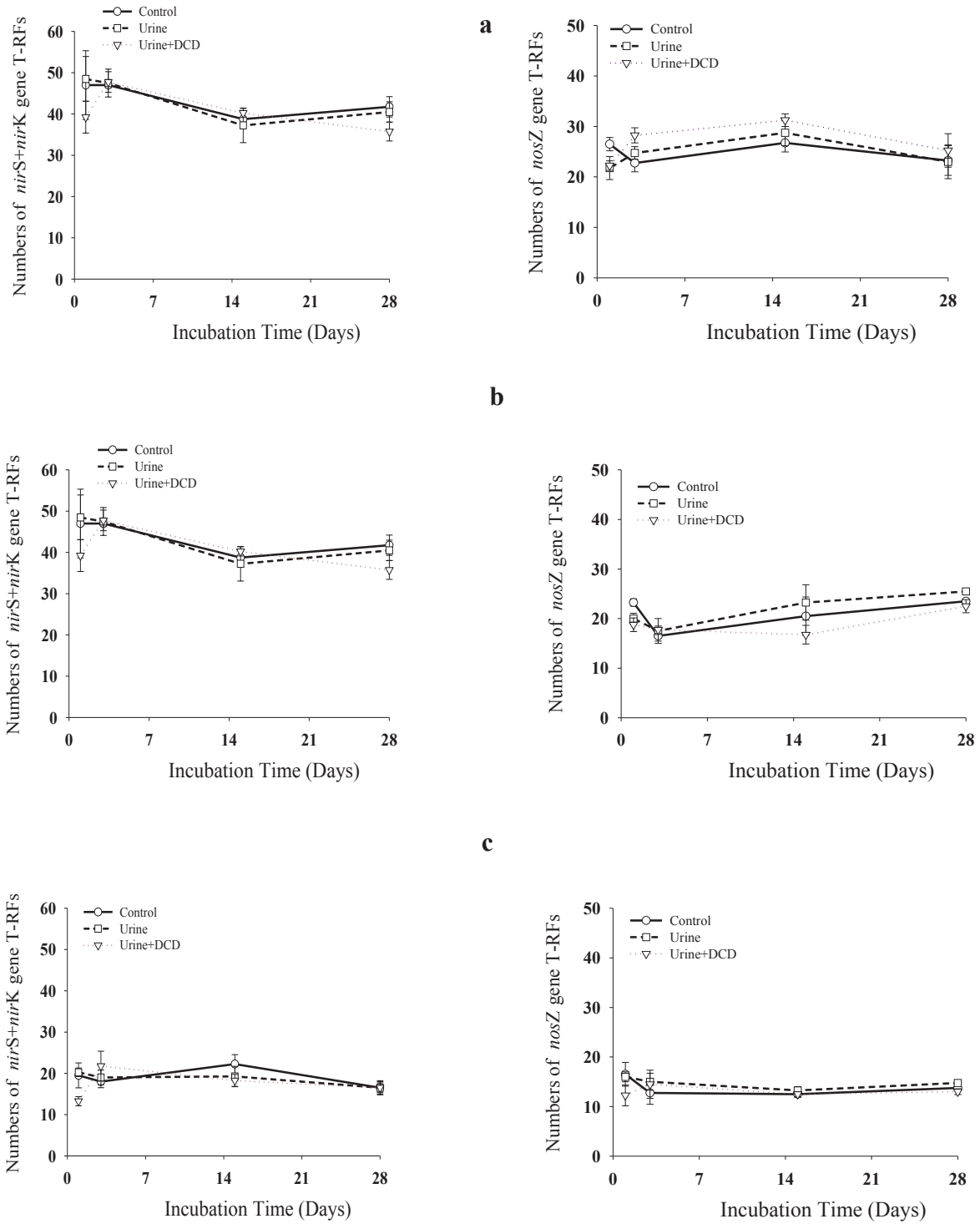


Figure 7.4 Numbers of denitrifier gene terminal restriction fragments (T-RFs) in (a) Manawatu fine sandy loam, (b) Tokomaru silt loam, and (c) Otorohanga silt loam incubated at saturation with three applied treatments (control, urine & urine + DCD) at various incubation times. Data are means ± standard error of mean (n = 4), *nir*S and *nir*K = nitrite reductase gene, *nos*Z = nitrous oxide reductase gene.

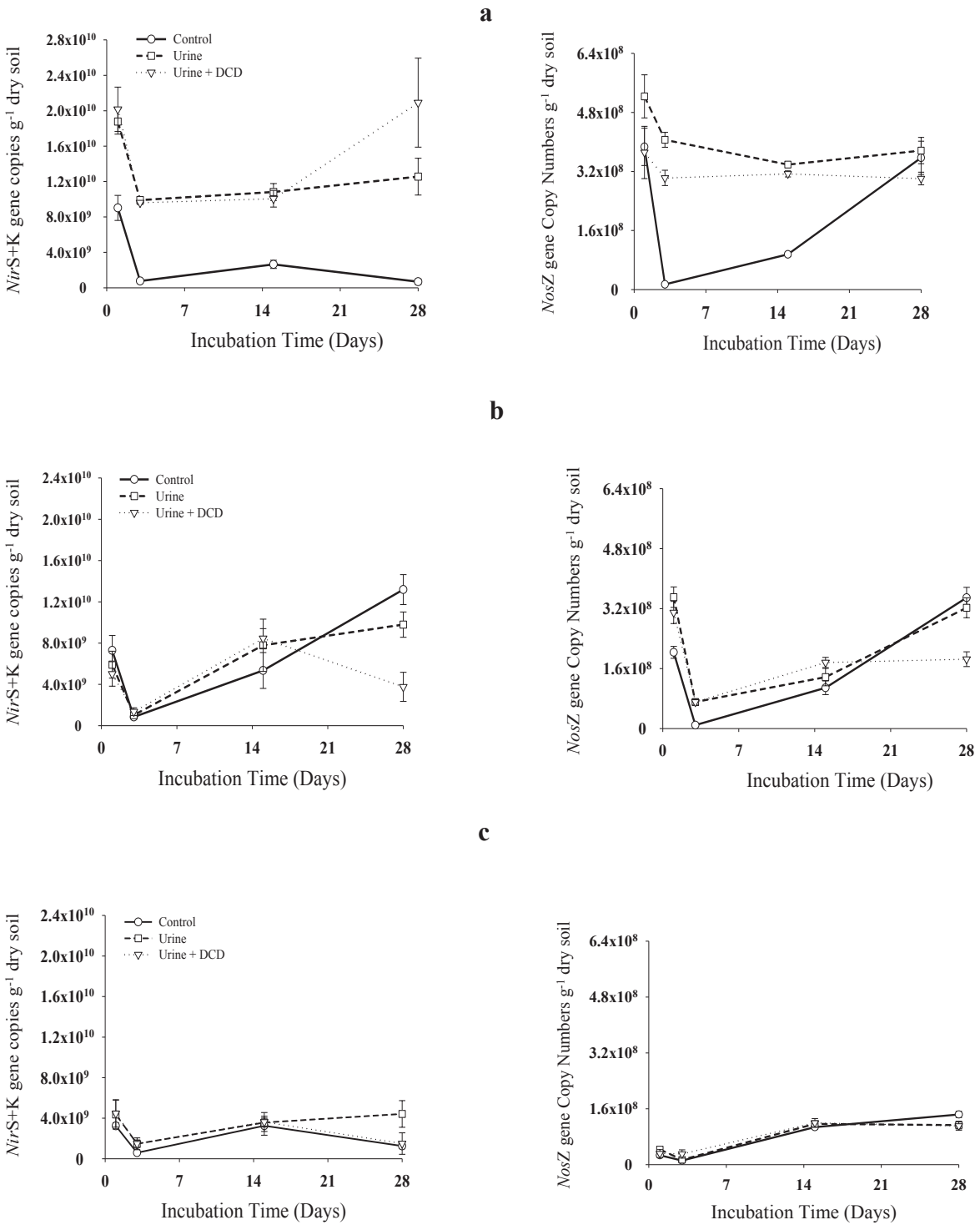


Figure 7.5 Denitrifier gene copy numbers in (a) Manawatu fine sandy loam, (b) Tokomaru silt loam, and (c) Otorohanga silt loam incubated at saturation with three applied treatments (control, urine & urine + DCD) at various incubation times. Data are means standard error of mean (n = 4), *nir*S and *nir*K = nitrite reductase gene, *nos*Z = nitrous oxide reductase gene.

7.3.4 Changes in denitrification during incubation with application of urine and urine +DCD

F and *P* values from the repeated measures ANOVA for N_2O-NA (from nitrification and denitrification) and N_2O-A (DR) (Table 7.3) indicate a significant 3-way interaction between soils, incubation duration, and treatment.

In the MWEI soil on Day 1 the N_2O-NA was significantly higher in the U treatment than the CT treatment (Fig. 7.6). The increase in N_2O production was observed in all the three soils; however, it initiated more quickly in both the MWEI and OH soils than in the TM soil. Although an increase in the N_2O-NA was delayed in the TM soil, it remained higher in the U treatment than in the control until the end of incubation on the Day 28. A significantly higher increase in N_2O-NA in the U treatment than in the other two treatments in the TM soil led to a higher N_2O-NA rate in the U treatment on Day 15 than in the UI treatment.

N_2O-A increased nearly 2–6 fold within 24 hours of application of urine compared with the water only addition in the control treatments. N_2O-A was significantly highest on Day 1 after application of treatments due to very high N_2O-A in the MWEI soil with the application of urine compared with the other two soils. In the MWEI soil, after an initially very high N_2O-A on Day 1 in the U treatment, the denitrification sharply decreased on Day 3 and then gradually increased again until the end of incubation on Day 28 (Fig. 7.6). In the TM soil there was a gradual increase in N_2O-A in the U treatment. In the OH soil there was no significant increase in N_2O-A during the increase in incubation after the application of treatments (CT, U, and UI).

7.3.5 Cumulative denitrification and amount of added N lost during denitrification

The influence of each treatment on the cumulative N_2O-NA (from nitrification and denitrification) and N_2O-A (from complete denitrification) differed among the three soils. In the MWEI and OH soils cumulative N_2O-NA was significantly higher in the U treatment than in the CT treatment (Table 7.4). But there was no significant difference in the cumulative N_2O-NA between the U and UI and UI and CT treatments. In the TM soil, N_2O-NA was significantly highest in the U treatment, followed by the UI treatment, and least in the CT treatment. During the 28-days incubation, the addition of DCD with urine application (UI)

reduced the cumulative N_2O-NA rates by 54 % , 67 %, and 23 % in the MWEI soil, the TM soil and the OH soil respectively (using Eq 7.2).

There was significantly higher N_2O-A in the U treatment than in the CT treatment in all three soils. Compared with the U treatment, the application of DCD with urine was more effective in restricting N_2O-A in the MWEI (77 %) and TM soils (73 %) than in the OH soil (28 %).

The proportion of added N completely denitrified (DR_N) was calculated using Eq 7.3 and ranged from 6.3 to 22.4 % (Table 7.4) in the incubated soils. DR_N in the U treatments was not significantly different in the MWEI and TM soils but was significantly higher in these two soils than in the OH soil. There were higher reductions in the DR_N through the application of DCD to the urine treatments in the MWEI and TM soils than in the OH soil.

Table 7.3 F and *P*-values for repeated measures analysis of variance to test the effects of soils (S: Manawatu fine sandy loam, Tokomaru silt loam & Otorohanga silt loam), treatments (T: control, urine, urine + DCD), and incubation period (I: 1, 3, 15, or 28 days) on nitrous oxide emissions with (N_2O-A) and without acetylene (N_2O-NA) addition to soils in jars

Source		N_2O-NA *(mg N kg^{-1} soil d^{-1})*	N_2O-A *(mg N kg^{-1} soil d^{-1})*
I	**F**	19.94	10.37
	p	0.0001	0.0001
S	**F**	8.61	84.11
	p	0.0001	0.0001
T	**F**	31.75	156.45
	p	0.0001	0.0001
I × S	**F**	15.12	21.60
	p	0.0001	0.0001
I × T	**F**	0.93	2.96
	p	0.480	0.0001
S × T	**F**	1.58	19.35
	p	0.1850	0.0001
I× S × T	**F**	2.79	12.85
	p	0.0020	0.0001

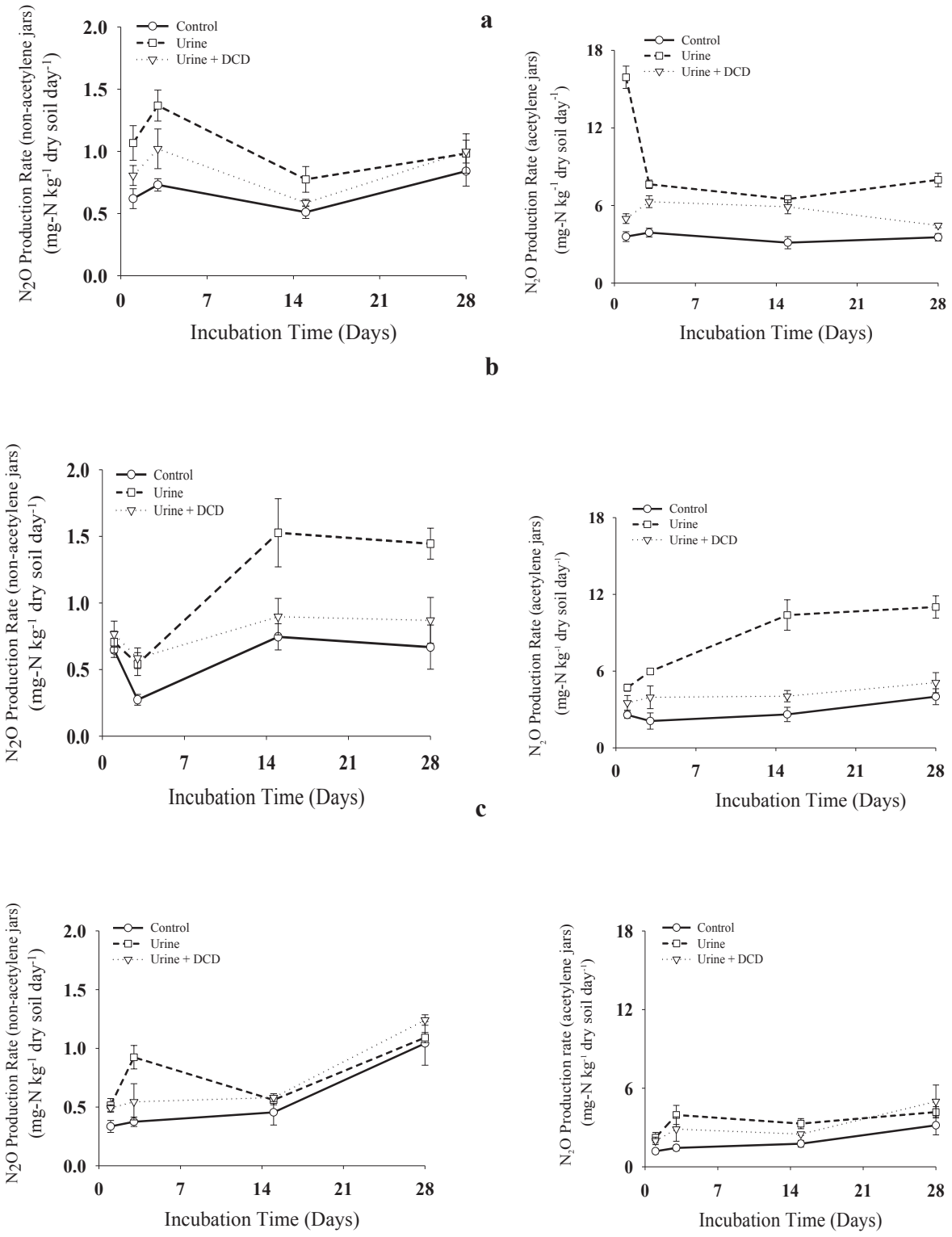


Figure 7.6 Nitrous oxide (N_2O) production (mg-N kg^{-1} dry soil day^{-1}) in (a) Manawatu fine sandy loam, (b) Tokomaru silt loam, and (c) Otorohanga silt loam incubated at saturation with three applied treatments (control, urine & urine + DCD) at various incubation times. Data are means ± standard error of mean (n = 4).

Table 7.4 Cumulative nitrous oxide with addition of acetylene (N_2O-A) and without acetylene (N_2O-NA) in soils after 28 days incubation at saturation with applied treatments water (CT), urine (U), and urine + DCD (UI) to Manawatu fine sandy loam (MWEI), Tokomaru silt loam (TM), and Otorohanga silt loam (OH), proportion of added urine N denitrified (DR_N) and reduction of DR_N by application of DCD to urine treated soils. Data are mean ± standard error of mean (n = 4)

Soil	Treatment	N_2O-A *(mg-N Kg^{-1} dry soil)*	N_2O-NA *(mg-N Kg^{-1} dry soil)*	DR_N (%)	Reduction of DR_N by DCD (%)
MWEI	CT	83.9 ± 4.6^{cd}	17.5 ± 0.6^{cde}	-	
	U	240.2 ± 9.4^{a}	26.7 ± 1.9^{b}	22.4 ± 1.1^{a}	77
	UI	119.4 ± 7.9^{b}	21.7 ± 0.7^{bc}	5.1 ± 2.3^{c}	
TM	CT	75.9 ± 6.5^{d}	16.2 ± 0.8^{de}	-	
	U	222.4 ± 7.2^{a}	33.0 ± 4.3^{a}	20.9 ± 0.9^{ab}	73
	UI	114.8 ± 8.3^{b}	21.7 ± 0.9^{bc}	5.6 ± 1.2^{c}	
OH	CT	53.7 ± 7.4^{e}	15.3 ± 2.2^{e}	-	
	U	98.1 ± 9.5^{bc}	20.9 ± 1.0^{cd}	6.3 ± 1.6^{c}	43
	UI	85.7 ± 5.8^{cd}	19.6 ± 1.4^{cde}	3.6 ± 1.3^{d}	

Values sharing same letter are not significantly different. The letters indicate the differences in the mean values tested by 2-way ANOVA only within the column in which they are presented.

7.3.6 Interrelations of gas emissions and soil properties

Correlation analysis suggested N_2O-NA was positively correlated with NO_3^--N, and MBC content, numbers *nir*S+*nir*K and *nos*Z gene T-RFs, and *nir*S+*nir*K and *nos*Z gene copy numbers in soils (Table 7.5). DR was positively correlated to NH_4^+-N, MBC content, numbers *nir*S+*nir*K and *nos*Z gene T-RFs, and *nir*S+*nir*K and *nos*Z gene copy numbers in soils.

The relationship between the cumulative N_2O-NA and N_2O-A in the control samples and soil characteristics was described in section 6.3.4. Cumulative N_2O-A production in both the urine treatments were positively correlated to DEA and Olsen P contents in soils, and negatively correlated to TN and TC contents in soils (Table 7.6). Cumulative N_2O-A production in the urine-only treatment positively correlates to denitrifier gene richness and *nos*Z gene abundance.

Regression analysis of cumulative N_2O-A in urine-only treatment with soil properties showed 74 % variance in DR ($P < 0.05$) was explained by DEA ($P = 0.152$), *nir*S+K ($P = 0.799$) and *nos*Z ($P = 0.046$) gene abundance and *nir*S+K ($P = 0.512$) and *nos*Z ($P = 0.792$) gene phylotypes.

Table 7.5 Pearson's correlation coefficients (r) among soil properties and gas measurements (n = 144) significant at $P < 0.05$

	pH *(1:2.5 ratio)*	NH_4-N *(mg kg^{-1} soil)*	NO_3-N *(mg kg^{-1} soil)*	MBC *(mg kg^{-1} soil)*	*nir*S+*nir*K gene T-RFs	*nos*Z gene T-RFs	*nir*S+*nir*K gene copy numbers	*nos*Z gene copy numbers
N_2O-NA *(mg N_2O-N kg^{-1} soil d^{-1})*	ns	ns	0.365	0.334	0.348	0.233	0.338	0.265
N_2O-A *(mg N_2O-N kg^{-1} soil d^{-1})*	ns	0.296	Ns	0.333	0.386	0.278	0.475	0.436

Table 7.6 Pearson's correlation coefficients (r) among soil properties and cumulative gas measurements (n = 12) significant at $P < 0.05$

	DEA *(μg N_2O-N kg^{-1} soil hr^{-1})*	Olsen P *(mg kg^{-1} soil)*	TN *(g kg^{-1} soil)*	TC *(g kg^{-1} soil)*	*nir*S+*nir*K gene T-RFs	*nos*Z gene T-RFs	*nir*S+*nir*K gene copy numbers	*nos*Z gene copy numbers
Cumulative (N_2O-A) (U) *(mg N_2O-N kg^{-1} soil)*	0.818	0.859	–0.822	-0.845	0.684	0.728	ns	0.519
Cumulative (N_2O-A) (UI) *(mg N_2O-N kg^{-1} soil)*	0.614	0.805	–0.604	-0.655	ns	0.506	ns	0.643

Where N_2O-A = nitrous oxide production in soils with acetylene, N_2O-NA = nitrous oxide production in soils without acetylene, NH_4-N = ammonical N, NO_3-N = nitrate N, MBC = microbial biomass carbon, *nir*S, K = nitrite reductase gene, *nos*Z = nitrous oxide reductase gene, T-RFs = terminal restriction fragments, DEA = denitrification enzyme activity, P = phosphorus, TN = total nitrogen, TC = total carbon

7.4 DISCUSSION

7.4.1 Changes in soil pH, mineral N, soluble C and MBC during incubation with application of urine and urine +DCD

The higher pH and NH_4-N contents of soils in the U and UI treatments were probably due to the hydrolysis of urea, which generally lasts for the first 3 days following urine application (Zaman *et al.*, 2008a). Later decreases in both the soil pH and NH_4-N contents were attributed to nitrification, ammonia volatilisation from the soil surface, or anaerobic ammonium oxidation over the length of the incubation (Rice & Tiedje, 1989; Zaman *et al.*, 1999). DCD reduced nitrification leading to higher pH and NH_4-N contents of soils in the UI than U treatment, which agrees with another study (Zaman & Nguyen, 2012). Temporal patterns in pH and NH_4-N were variable among the three soils. There was a rapid change in both soil pH and NH_4-N contents in the MWEI and OH soils; however, these changes were gradual in the TM soil.

The NO_3-N contents of incubated soils increased continuously during incubations, suggesting that nitrification continued in these saturated soils. One explanation for this observation is that nitrification continued on the exposed soil surface when lids were opened and O_2 might have been introduced in between the gas sampling. Lower NO_3-N contents in UI samples than in U suggest that DCD effectively inhibited nitrification in the incubated soils (Singh *et al.*, 2008). The decrease in NH_4^+-N content in urine treatments over the course of incubation period or the increase in NO_3^--N content were similar in magnitude, suggesting substantial nitrification of applied urine in soils.

The increase in soluble C observed in the urine-treated soils could also be due to the degradation of complex organic C to more soluble form dissolved by the addition of urine to the soil samples (Petersen *et al.*, 2004). Other reasons for an increase in soluble C could be increased solubility of dissolved organic carbon with initial increase in soil pH, and the need of anions to balance the NH_4^+ charge (Curtin *et al.*, 2011). After a gradual increase for the first 15 days, the soluble C contents of soils decreased. This could be due to the utilisation of available C by the growing microbial population in the incubated soils (negative correlation between SC and denitrifier gene copy numbers and MBC). Decline in soluble C could also be due to the increase in microbial respiration in the incubated soils (Singh *et al.*, 2008). Throughout the incubation,

although the soluble C content was significantly higher in the allophanic OH soil than in the other two soils, the MBC content was lower in the OH soil than in the other two soils. The allophanic OH soil might have a lower fraction of the microbial C in the total organic C in the soil, resulting in a lower MBC per unit of organic C than of other soils (Sparling, 1992). Comparatively lower decomposition and higher stabilization of the organic C due to the presence of allophane in the OH soil (Sparling, 1992) might also have resulted in very low MBC compared with non-allophanic MWEI and TM soils. The addition of urine increased the MBC with the increase in incubation time but the application of DCD did not have a significant effect on the MBC content during the incubation. Similar increases in biomass C have been reported by Orwin *et al.* (2010), especially with the urine addition to wet soil samples rather than to dry soil samples.

7.4.2 Denitrifier gene richness in the incubated soils

The numbers of denitrifier gene T-RFs in a sample reflect denitrifier richness in that soil. In the MWEI and TM soils with higher MBC, Olsen P, and DEA greater denitrifier gene richness was observed than in the OH soil. Small but significant changes in the *nir*S+*nir*K gene and *nos*Z gene T-RFs with respect to increasing incubation time were observed only in the MWEI and TM soils. The OH soil with originally less rich denitrifier genes did not respond to changes in incubation duration in soils. Although smaller population of denitrifiers are present in the OH soil, these denitrifiers could be more tolerant of chemical changes in soil than the MWEI and OH soils. Slight changes in the denitrifier communities in the OH soil with urine or DCD addition might be hidden in the overall stable bacterial community (Avrahami *et al.*, 2002). Since the denitrifier community is richer in the MWEI and TM soils, some of the sensitive denitrifier genotypes might have responded to the chemical changes in these soils during the incubation, thus the slight decrease in the numbers of gene T-RFs was observed.

7.4.3 Denitrifier gene abundances in the incubated soils

After the application of treatments there was an initial decrease in *nir*S+*nir*K and *nos*Z gene copy numbers in soils. With urine application, the ionic strength of soil solution increases with the addition of K^+, Ca^{2+}, Mg^{2+}, Na^+, and Cl^- (Haynes & Williams, 1992) and thus generates osmotic stress for microbial populations present in soil. High pH and high NH_4^+ contents in soil after urine addition are inhibitory for some bacteria (Monaghan & Barraclough, 1992). Thus initial decrease in denitrifier gene copy numbers with urine application could be due to the harsh environment created by urine application (e.g. osmotic stress, ammonia toxicity), which generated a stressful condition for the microbial populations (Uchida *et al.*, 2008), including denitrifiers in soils. After the initial reduction, denitrifier gene copies increased in all the treatments in the three soils, suggesting the incubations led to conditions that were favourable for the growth of denitrifiers. Continuous anaerobic conditions and the increased availability of NO_3^-, NH_4^+, and soluble C contents in the incubated soils might have resulted in increased denitrifier abundances in soils.

Inherently higher Olsen P, MBC and DEA in the MWEI soil and enhanced pH, MBC, and soluble C content with urine application may have resulted in higher *nir*S+*nir*K gene copy numbers in the U and UI treatments in the MWEI soil than in the control treatment. There was higher *nos*Z gene abundance in the MWEI soil than in the other two soils, and *nos*Z gene copy numbers increased with the application of urine in this soil. Similar increases in denitrifier gene copies have previously been reported by Wakelin *et al.* (2013) and Di *et al.* (2014), suggesting increases in the abundance of *nos*Z gene, *nir*K, and *nos*Z genes with urine application respectively.

The results of this experiment show that the impact of DCD on denitrifier communities in these soils is small. This suggests that the effect of DCD is organism specific and only restricted to nitrifiers. It also suggests that the available C, NO_3^-, and NH_4^+ pools in incubated soils were sufficient to maintain a denitrifier community of a constant size.

Similar to these results, both Di *et al.* (2014) and Wakelin *et al.* (2013) did not find any reduction in the *nos*Z gene abundance in the urine + DCD treatment as compared with the urine-only treatment.

7.4.4 Changes in denitrification of applied N in the incubated soils

There was a significant increase in N_2O-NA & N_2O-A after the application of urine to the soils. An increase in N_2O and denitrification within 24 hours of urine application has also been reported by de Klein & Van Logtestijn (1994a). The immediate increase in N_2O or denitrification just after the addition of treatments could be due to both increase in water content and addition of urine-derived C and N substrates (Uchida *et al.*, 2011), resulting in enhanced microbial activities (Monaghan & Barraclough, 1993). This increase is more pronounced with the addition of urine; however, there is not much effect of the DCD addition on N_2O production (from nitrification and denitrification) and DR in the incubated soils. Rapid increase in *nir* and *nos*Z gene copy numbers in the urine-only treatment in the MWEI soil and very high MBC content than other two soils resulted in very high N_2O-NA and N_2O-A during the incubation. In the TM and OH soil these changes were gradual.

The three soils used in this study produced variable N_2O-NA and N_2O-A with the application of three treatments at variable incubation times. MWEI and TM soils with comparatively higher DEA, Olsen P, MBC, denitrifier gene richness, and denitrifier gene abundance than the OH soil had higher denitrification during incubation. The immediate high N_2O-A in the MWEI soil could be due both to very high *nos*Z gene copy numbers in this soil and to inherently higher DEA in this soil than in the other two soils. Immediate high denitrification was also observed in the MWEI soils during the earlier experiments reported in the previous chapters of this thesis. In the TM soil, due to gradual changes in the NO_3-N and NH_4-N contents, the increase in N_2O production was also gradual and remained higher in the U than in the control, even at the end of the incubation.

The percentage of added urine N present in the soils as mineral N at the end of the incubation on Day 28 was the least in the OH soil. Despite higher N transformation of added N in this soil, N_2O production during the incubation was lower than in the MWEI and TM soils. This suggested that in the OH soil lower denitrification, due to the least DEA of the three incubated soils and N transformations other than denitrification, such as nitrification and ammonia volatilization, might be contributing to a loss of urine N more than in the MWEI and TM soils.

The reduction of 23 to 67 % of total N_2O-NA with the addition of DCD to urine is within the range of the values reported earlier. In selected studies (lab incubations or lysimeter studies) in New Zealand soils reduction in N_2O emission with DCD addition to urine application has been observed to vary between 19 and 80 % (Luo *et al.*, 2010; Singh *et al.*, 2008; Zaman & Nguyen, 2012). In the OH soil there was little difference between any of the treatments in N_2O-NA production, therefore lack of DCD effect could be due to relatively less N_2O emitted from this soil. Another reason for a lower effect of DCD in restricting denitrification in the OH soil could be due to its allophanic origin. High organic carbon and CEC of allophanic soils result in high adsorption of DCD and lower availability for restricting N_2O production (Singh *et al.*, 2008; Watkins *et al.*, 2013).

The proportion of added urine-N produced as N_2O-NA or N_2O-A from both the U and the UI treatments in the current study are comparable to values reported in earlier studies (Luo *et al.*, 2008a; Zaman *et al.*, 2009). The percentage of added N recovered as N_2O produced during denitrification varies according to soil type, incubation duration (Clough *et al.*, 1998; Uchida *et al.*, 2011), and the amount of urine N added to soils. Various studies measuring denitrification after urine application to soil have found DR_N ranging from 0.2 to 22 % in various soils under variable experimental conditions (Clough *et al.*, 1998; Colbourn, 1992; de Klein & Van Logtestijn, 1994a; Whitehead & Bristow, 1990; Zaman *et al.*, 2007). Although the DR_N reported in the current study for the three soil types falls within this range, three soils incubated in similar conditions presented contrasting results, which very well reflects the variability in their inherent biochemical and molecular characteristics.

7.4.5 Inter-relationships between soil characteristics and N_2O production

Changes in denitrification in the current study with the application of treatments and incubation time are variable with soil type. Inherent soil properties, geographical location, and management practices followed on farm resulted in variable N_2O-NA and N_2O-A during incubation. Long-term effluent-receiving MWEI soil that had significantly higher MBC, Olsen P, and DEA, also had the greatest denitrifier gene richness and denitrifier gene abundance, suggesting the regular effluent irrigation of this soil favoured the formation of a large and active denitrifier community.

Soils with an inherently rich denitrifier population showed a slight response to urine addition but DCD application was ineffective on denitrifiers.

Patterns of increase or decrease in the N_2O production in the incubated soils were reflected by their changes in NH_4-N, NO_3^--N, soluble C, and MBC contents. In the MWEI and TM soils there were significantly lower N_2O productions in the urine treatments with the addition of DCD than in the urine-only treatments. Application of urine gradually increased the denitrifier gene abundances in the soils; however, this increase was greater in the MWEI soil than in the other two soils. None of the treatments affected denitrifier gene richness. There was also no significant effect of DCD application with urine on denitrifier gene abundances in the incubated soils.

N_2O-A with urine application were higher in soils with higher DEA (MWEI and TM) than in the soil with the least DEA (OH). Although OH soil has higher TC, TN, and soluble C content than the other two soils, due to overall least DEA and Olsen P content, it showed lower cumulative N_2O-A production, suggesting overall lower microbial activity in this soil than in the MWEI and TM soils.

The results of this study indicated the biochemical (pH, mineral N, MBC, soluble C, Olsen P, TC, TN, and DEA) and molecular measurements (denitrifier gene richness and denitrifier gene abundance) complemented each other, making it possible to describe the differences in N_2O productions in three soils incubated under common conditions. Differences in gaseous emissions in soils at common incubation condition could be due to differences in their inherent soil properties and original microbial populations in three soils. Although DEA has been criticised in the literature (Qin *et al.*, 2014) for underestimating N_2O reductase (N_2OR) activity, in this study the differences in denitrification among three soils were clearly differentiated by the variations in their inherent DEA. This assay of quantifying N_2OR activity provided a fair estimate of the denitrification potentials of the three soils, and the results from the molecular analysis of the soils substantiated that finding. While information on denitrifier gene richness and denitrifier gene abundance indicated the existence of denitrifier populations in soils these measurements do not exactly point to the real-time activities of denitrifiers present in soils. The measurements of gene expressions of denitrifiers might provide a more direct estimate of production and reduction of N_2O in particular soil conditions. Therefore, more direct measurements of denitrifier gene expressions are recommended for future work.

7.5 SUMMARY AND CONCLUSIONS

This study showed changes in chemical condition, denitrifier population, and denitrification in soils with the addition of urine with and without DCD. The effect of DCD in soils was short-lived and comparable to urine application. Results showed that the effects of urine addition on overall changes in soils were variable with soil type. These differences in effectiveness of urine and DCD application arise from inherent differences in soil properties and their geographical location.

Application of urine resulted in higher C and N substrate availability for microorganisms, leading to higher microbial growth and activity. Although application of DCD with urine did not influence overall denitrifier abundance, it may have affected denitrifier activity indirectly by influencing higher NH_4-N, and lower NO_3^--N, soluble C, and MBC contents in DCD-treated urine soils compared with urine-only soils. The magnitude of increases in denitrification during the incubations and the reduction of denitrification by DCD application were variable among the three soils and reflected differences in their inherent soil properties such as DEA, MBC, Olsen P, TC, TN, and denitrifier gene abundance. The decline in N_2O production in the urine-applied treatments with DCD applications was higher in the MWEI soil than in the TM and OH soils, in which it was similar. However, the addition of DCD to urine application resulted in a similar reduction in N_2O+N_2 productions in the MWEI and TM soils, but was higher in these two soils than in the OH soil.

The chemical, gaseous, and molecular measurements of denitrification used in this experiment complemented each other and helped integrate the experimental results to improve understanding of the denitrification process after urination events in three soils of contrasting DEA. Soils with contrasting DEA, even under common incubation conditions, showed variable chemical changes, denitrifier gene abundance, and DR with increasing incubation time. Use of molecular techniques to measure richness and abundance of denitrifiers in the incubated soils made it possible to differentiate between their differential N_2O-NA and N_2O-A in similar incubation conditions. Soils with low denitrifier richness and, more importantly, with complete denitrifier abundance, had limited capacity to denitrify available N. Under urine application or additional inorganic N, soil with the most abundant denitrifier population responded to added treatment quickly and produced more N_2O-A than did the other two soils. The inherent

denitrifier population of the soils might reflect the overall trend under farm different conditions. Although denitrifier richness and denitrifier abundance may not be direct links to the activity of the denitrifying population, they might indicate possible N_2O production in soils with differential inherent complete or incomplete denitrifiers.

Chapter 8

Summary and recommendations for future work

8.1 INTRODUCTION

Denitrification is an anaerobic respiration process of the reduction of nitrate (NO_3^-) to dinitrogen (N_2). This process is carried out by a diverse group of organisms, including bacteria, fungi, archaea, and protozoa. Denitrification is mediated through sets of reductase enzymes, which facilitate the reduction of NO_3^- to N_2. Organic carbon provides energy for this reduction process. Nitrogen oxyanions NO_3^-, nitrite (NO_2^-), N oxides nitric oxide (NO), and nitrous oxide (N_2O) act as electron acceptors. Incomplete denitrification leads to the emission of nitrous oxide (N_2O), a potent greenhouse gas. Various factors such as availability of oxygen (O_2), C, N, optimum pH, the presence of active denitrifier population, etc., regulate the process and emission of N_2O and N_2 during denitrification.

Denitrification is the major source of N_2O production in temperate grasslands worldwide and also in New Zealand. There has been limited past research to evaluate various factors (soil, environmental, management) affecting N_2O productions during denitrification in New Zealand pasture soils. Moreover, little research has been conducted in New Zealand on the direct or indirect effects of key factors regulating denitrification on the denitrifier community composition and abundance in various soils that might lead to incomplete denitrification or N_2O production. The wide and varied information about factors influencing denitrification would be helpful to devise accurate and cost-effective mitigation techniques.

The research conducted and reported in the previous chapters (Chapters 3–7) of this thesis is summarised here and challenges of this research are discussed. Future research needs to develop further understanding to reduce N_2O production are briefly outlined.

8.2 DEVELOPMENT OF PROTOCOL FOR GAS SAMPLING AND MOLECULAR ANALYSIS

Various techniques to measure denitrification in the environmental systems are discussed and described in the chapter 2 of this thesis. The most convenient and economic technique commonly used for measuring denitrification is the acetylene (C_2H_2) inhibition technique (AIT), a technique that has been modified over the years. Thus preliminary experiments were conducted to standardize AIT and develop the protocols for denitrification enzyme activity (DEA) and denitrification rate (DR) measurements (Chapter 3).

Various molecular techniques currently are used to identify and quantify denitrifier bacterial communities in soils. These techniques mainly target the two key processes by using the *nir*S and *nir*K genes encoding conversion of nitrite (NO_2^-) to nitric oxide (NO) and *nos*Z gene encoding the reduction of N_2O to dinitrogen (N_2) as the molecular markers. The two molecular techniques: i) terminal restriction fragment length polymorphism (T-RFLP), and ii) quantitative polymerase chain reaction (qPCR), were standardised by repeating and modifying the already published protocol to obtain the specific reaction products for assessment of bacterial denitrifier richness and denitrifier abundance in soils by using these three genes as the markers.

8.3 DENITRIFICATION IN LABORATORY INCUBATED NEW ZEALAND DAIRY-GRAZED PASTURE SOILS

To address the variability in denitrification dairy-grazed pasture soils were collected from surface (0–100 mm) and sub-surface (100–200 mm) depth to represent differences in their texture/mineralogy, climatic condition and farm management practices from 10 different geographical locations in New Zealand. The collected samples were analysed for their gravimetric soil water content, pH, total C (TC), total N (TN), NO_3^--N, NH_4^+-N, Olsen P, soluble C, microbial biomass C (MBC) contents, bacterial denitrifier gene richness, and total bacterial and bacterial denitrifier gene abundance. Gas samples were collected for measuring their DEAs and DRs.

The soils were selected to differ greatly in their measured chemical characteristics and bacterial denitrifier richness and abundance (Chapter 4). The measured DEA in soils ranged from

1 to 3738 μg N_2O-N kg^{-1} soil hr^{-1} and DRs varied from 1 to 22 μg N_2O-N kg^{-1} soil hr^{-1} in surface and subsurface samples (chapter 4). The *nir*S+*nir*K gene richness in the soils ranged from 10 to 46, and *nos*Z gene richness from 12 to 27. *Nir*S+*nir*K gene copy numbers varied from 2.6×10^8 to 7.5×10^8 g^{-1} soil and *nos*Z gene copy numbers from 9.9×10^5 to 4.8×10^8 g^{-1} soil.

To identify the key soil parameters and their relationships to denitrifiers and denitrification the 10 soils were classified into three groups using the cluster analysis. Soils were completely different in soil properties from one another. Such as the effluent-irrigated Manawatu fine sandy loam (MWEI), was exclusive from other soils and formed a separate cluster from other soils. Similarly, the two allophanic soils, Otorohanga (OH) and Horotiu (OH), were separate from the other seven soils. The emaining soils with similar characteristics formed one cluster. Different soils collected from various sites with similar biochemical characteristics, denitrifier richness, and denitrifier abundance showed similar denitrification activity under field-moist conditions. The richness and abundance of bacterial genes correlated significantly ($P < 0.05$) with soil texture, Olsen P, MBC, TC, TN, and mineral N, confirming inherent soil characteristics influencing denitrifier populations in soils. The soils richer in MBC, Olsen P, and higher pH than other soils also had more abundant denitrifier community. *Nir*S and *nir*K gene copy numbers correlated positively with N_2O emissions (from nitrification and denitrification).

8.4 DENITRIFICATION AT FIELD CAPACITY AND STAURATION SOIL WATER CONTENT (SWC)

Incubation of five selected dairy-grazed pasture soils with contrasting DEA (and representing all the three groups described in chapter 4) at field capacity (FC) and then to saturation, demonstrated higher denitrification as a function of increasing SWC (Chapter 5). There was a 1.31 to 1.97-fold increase in N_2O-A (nitrous oxide production in C_2H_2 jars) in the incubated soils from FC to saturation in this study. The increase in N_2O with increasing SWC was variable in the five soils tested in the study. Among the five soils tested, the MWEI soil showed maximum increase in the denitrification, with an increase in SWC from field condition to FC, and had the highest N_2O-A of all the five soils. After increasing the SWC from FC to saturation, there were variable increases in denitrification in five tested soils. The differences in denitrification in the

five studied soils at common SWC were due to variabilities in their NO_3-N, TC, TN, MBC, Olsen P, DEA, denitrifier gene richness, and denitrifier gene abundance.

In another incubation study three selected soils with contrasting DEA and representing groups I and II of the four groups described in chapter 4 were incubated at saturation for 28 days to examine the changes in denitrification at saturation with increasing incubation time (Chapter 6). The results of this incubation supported the results described in chapter 5, illustrating higher N_2O-NA (N_2O production rates in non-C_2H_2 jars) and DRs in soils at saturation than at field-moist condition. In the saturated samples, *nir*S+*nir*K gene T-RFs (terminal restriction fragments) richness varied from 17 to 47, and *nos*Z T-RFs richness varied from 12 to 27 during the entire incubation. The increases in denitrification rates were variable in three soils and depended on their TC, TN, Olsen P, MBC, and denitrifier community structure. The highest cumulative DR measured from Day 0 to Day 28 was 102.4 mg N_2O-N kg^{-1} soil in the MWEI soil, followed by 77.2 mg N_2O-N kg^{-1} soil in the Tokomaru silt loam, and 54.3 mg N_2O-N kg^{-1} soil in the Otorohanga silt loam. N_2O-NA and DR in the saturated soil during the 28 days incubation were positively correlated with the denitrifier gene T-RFs richness but did not correlate with their denitrifier gene copy numbers.

8.5 DENITRIFICATION IN URINE APPLIED SOILS

Urination events by grazing animals on pasture soils when accompanied with rainfall or otherwise lead to very high N_2O emissions from soil. An incubation experiment was conducted to assess the influence of urine addition with and without DCD at saturation on the changes in soil pH, NO_3-N, NH_4-N, soluble C, MBC contents, denitrifier gene richness (numbers from gene T-RFs), denitrifier gene abundance, and denitrification (Chapter 7). Results suggested that application of urine at saturation in the current study influenced higher N_2O-NA and DRs and also have higher *nir*S+*nir*K and *nos*Z gene richness and gene copy numbers compared with samples applied only with water. The denitrifier gene richness and copy numbers either remained unchanged or were lower under urine +DCD treatments than under urine-only, and related with N_2O-NA during the incubation.

There was overall increase in DR with application of urine and urine +DCD to soils compared with the control. Comparatively higher N_2O-NA productions were observed in urine-

only treatments than in urine + DCD. The cumulative N_2O-NA from Day 1 to Day 28 after application of treatments ranged from 20.9 to 33.0 mg N_2O-N kg^{-1} dry soil in urine-only treatments and from 19.6 to 21.7 mg N_2O-N kg^{-1} soil in urine + DCD treatments in three soils.

The estimated proportion of total N added as urine-N denitrified during the entire incubation ranged from 6.3 to 22.4 % in urine treatments and 3.6 to 5.6 % in urine + DCD treatments in three soils. The addition of DCD with urine was more effective in restricting cumulative denitrification (43–77 %) than cumulative N_2O-NA (23–67 %). Overall, in urine-applied samples the N_2O-NA production (positively) was significantly correlated with their NO_3^--N, MBC, denitrifier gene richness, and denitrifier gene copy numbers. Cumulative total denitrification in both urine and urine + DCD treatments was positively and significantly correlated with their DEA and Olsen P contents and negatively with TN and TC. MWEI soil with the highest DEA and Olsen P content had higher total denitrification during the incubation.

This research produced information on the physicochemical characteristics, denitrifier richness, denitrifier abundance, and their influence on denitrification among the New Zealand dairy-grazed pasture soils. The chemical, gaseous, and molecular measurements during the various experiments complemented each other and enhanced our understanding of the denitrification process under various incubation conditions in soils with contrasting characteristics. Soils with inherently abundant denitrifier population showed higher N_2O-A production rates under common incubation conditions. DEA, denitrifier gene phylotypes, and abundance collectively explained variabilities in denitrification rates in the tested soils; in the urine-applied soils particularly these parameters explained 74 % variability in the measured DR (section 7.3.6). This suggests high denitrification potential soils with more microbial communities contributing to denitrification respond to urine addition positively by converting added N more to N_2O, especially under saturated conditions.

8.6 RECOMMENDATIONS FOR FUTURE WORK

The major challenge of this study was to measure N_2 production using the acetylene (C_2H_2) inhibition technique (AIT). AIT, being an indirect measure of N_2 production, can lead to underestimation of complete denitrification in the soils and, due to possible nitrification in non-C_2H_2 jars, calculations of N_2 production may not be reliable. Because of its affordability, convenience, and the correspondence between N_2O production with and without C_2H_2, AIT was used in conjunction with molecular techniques during preliminary experiments. When available, a more direct method to quantify N_2O and N_2 productions, such as Denitrification Dynamics Gas Chromatography (DDGC) recently developed in Landcare Research (McMillan *et al.*, 2014), may be recommended.

In this current study, Dicyandiamide (DCD), one of the most commonly used nitrification inhibitor, was tested for its impact on denitrification. Future work could look into changes in denitrifier community abundance, total denitrification, and, more importantly, into N_2O and N_2 productions (employing direct gas measurement technique) in New Zealand pasture soils using other mitigation methods such as the addition of organic matter, liming, Cu addition, and biochar application.

This study focussed on the role of bacterial denitrifier population, so the contribution of a fungal community responsible for N_2O production through denitrification was not ascertained. Future experiments involving measurements of both bacterial and fungal denitrifier communities might enhance our current understanding of the process. Quantification of fungal genes hydrazine oxidase (*hzo*) genes and p450 *nor* gene and composition of fungal communities using sequencing techniques are recommended.

Quantification of the *nos*Z community in the current study involved the use of primers targeting only typical (clade I) *nos*Z communities. However, as literature describes the presence and quantification of atypical (clade II) *nos*Z denitrifiers, further measurements involving both typical and atypical *nos*Z primers are recommended to obtain complete information about the *nos*Z denitrifier community involved in reduction of N_2O to N_2 in the system.

The measurement of denitrifier gene abundance did not always correlate with denitrification during this study. This lack of correlation is attributed to the qualitative nature of the data on abundance of denitrifiers measured on extracted DNA. To determine the active contribution of denitrifier gene abundance in the denitrification mRNA transcription technique expression of denitrifier genes would be helpful to trace the real-time activity of denitrifiers in soils.

In this study biochemical and molecular changes in soil after the addition of only cattle urine were measured. While urination by grazing animals adds a very high concentration of active N to the soil, there are other means, such as animal dung, commercial fertilizers, and the fixation of N_2 by leguminous plants, by which N enters pasture soils. Changes in denitrifier population and their activities with addition of each of these N forms individually and together would be helpful to integrate the information on denitrification on a typical farm.

The incubation experiments conducted in this study provided fundamental insights about the changes occurring under soil incubations. However, on a farm the presence of plants or a variety of plant types might affect the presence and activity of denitrifiers in soil or the N transformations in soils, and thus N_2O production. Glass house experiments under controlled conditions by growing grasses in pots with the application of various amendments are recommended to investigate the effects of applied treatments on denitrifiers and denitrification.

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References

APPENDICES

Appendix i

PowerSoil® DNA Isolation Kit

Instruction Manual

Introduction

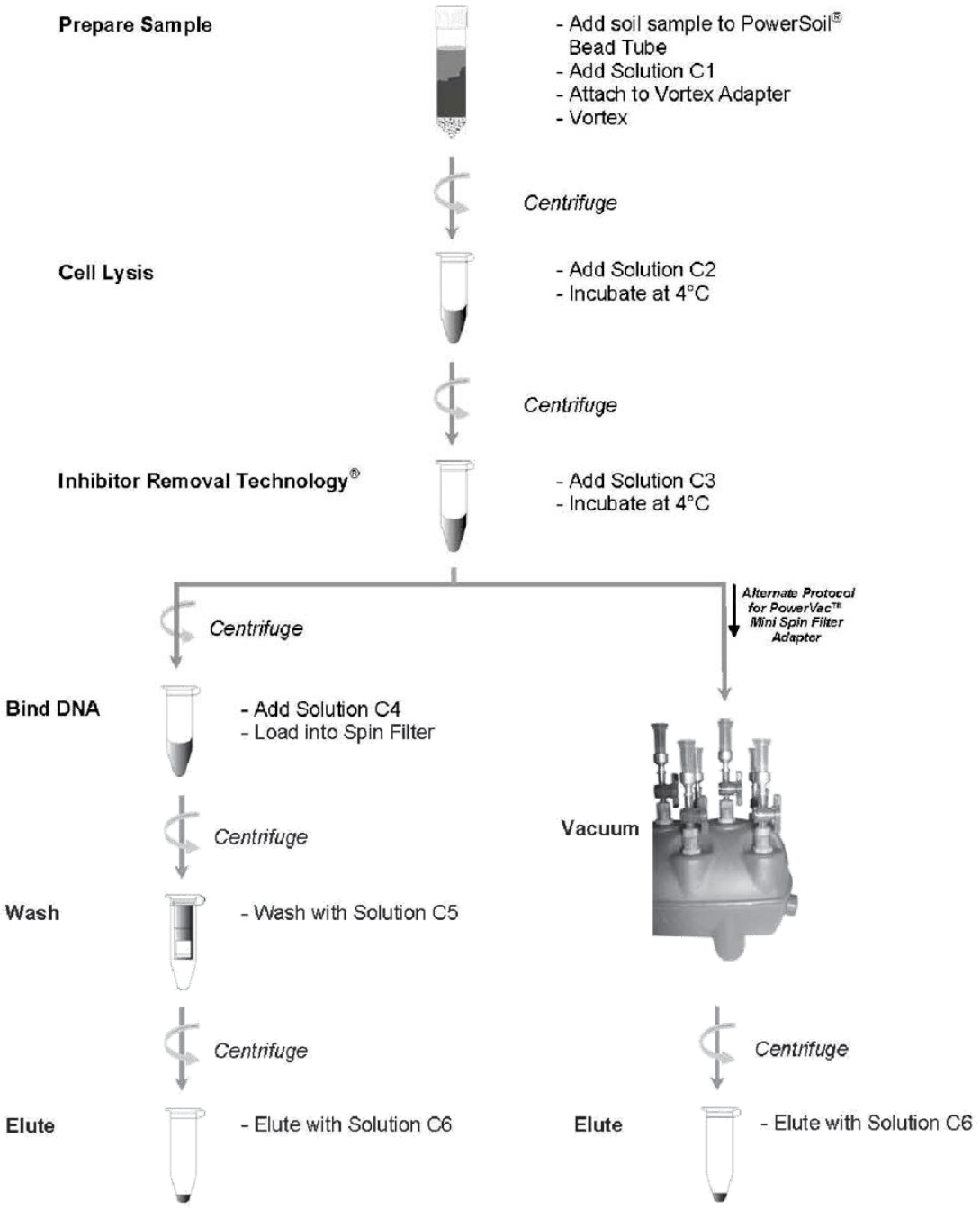
The PowerSoil® DNA Isolation Kit is comprised of a novel and proprietary method for isolating genomic DNA from environmental samples utilizing our patented Inhibitor Removal Technology® (IRT). The kit is intended for use with environmental samples containing a high humic acid content including difficult soil types such as compost, sediment, and manure. Other more common soil types have also been used successfully with this kit. The isolated DNA has a high level of purity allowing for more successful PCR amplification of organisms from the sample. PCR analysis has been performed to detect a variety of organisms including bacteria (e.g. *Bacillus subtilis, Bacillus anthracis*), fungi (e.g. yeasts, molds), algae and Actinomycetes (e.g. *Streptomyces*).

Protocol Overview

The PowerSoil® DNA Isolation Kit distinguishes itself from MO BIO's UltraClean® Soil DNA Isolation Kit with a humic substance/brown color removal procedure. This procedure is effective at removing PCR inhibitors from even the most difficult soil types. Environmental samples are added to a bead beating tube for rapid and thorough homogenization. Cell lysis occurs by mechanical and chemical methods. Total genomic DNA is captured on a silica membrane in a spin column format. DNA is then washed and eluted from the membrane. DNA is then ready for PCR analysis and other downstream applications.

MO BIO Laboratories, Inc.

PowerSoil® DNA Isolation Kit



Equipment Required
Microcentrifuge (10,000 x *g*)
Pipettors (50 µl - 500 µl)
Vortex-Genie 2 Vortex (MO BIO Catalog# 13111-V or 13111-V-220)
Vortex Adapter (MO BIO Catalog # 13000-V1)

Reagents Required but not Included
100 % ethanol (for the PowerVac™ Manifold protocol only)

Kit Contents

	Kit Catalog # 12888-50		**Kit Catalog # 12888-100**	
Component	**Catalog #**	**Amount**	**Catalog #**	**Amount**
PowerBead Tubes (contain 750 µl solution)	12888-50-PBT	50	12888-100-PBT	100
PowerSoil® Solution C1	12888-50-1	3.3 ml	12888-100-1	6.6 ml
PowerSoil® Solution C2	12888-50-2	14 ml	12888-100-2	28 ml
PowerSoil® Solution C3	12888-50-3	11 ml	12888-100-3	22 ml
PowerSoil® Solution C4	12888-50-4	72 ml	12888-100-4	144 ml
PowerSoil® Solution C5	12888-50-5	30 ml	12888-100-5	2 × 30 ml
PowerSoil® Solution C6	12888-50-6	6 ml	12888-100-6	12 ml
PowerSoil® Spin Filters (units in 2 ml tubes)	12888-50-SF	50	12888-100-SF	100
PowerSoil® 2 ml Collection Tubes	12888-50-T	200	12888-100-T	400

Kit Storage
Kit reagents and components should be stored at room temperature (15-30°C).

Experienced User Protocol
Please wear gloves at all times

- To the **PowerBead Tubes** provided, 0.25 grams of soil sample.
- Gently vortex to mix.
- **Check Solution C1.** If **Solution C1** is precipitated, heat solution to 60□C until dissolved before use.
- Add 60 □l of **Solution C1** and invert several times or vortex briefly.
- Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

  Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.

1) Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
2) Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

   Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.

8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix Solution C4 before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.
15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature.

    Note: A total of three loads for each sample processed are required.

16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.
19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
20. Add 100 µl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20 ° to -80 ° C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

Detailed Protocol (Describes what is happening at each step)

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.

   What's happening: After your sample has been loaded into the PowerBead Tube, the next step is a homogenization and lysis procedure. The PowerBead Tube contains a buffer that will (a) help disperse the soil particles, (b) begin to dissolve humic acids and (c) protect nucleic acids from degradation.

2. Gently vortex to mix.

   What's happening: Gentle vortexing mixes the components in the PowerBead Tube and begins to disperse the sample in the PowerBead Solution.

3. **Check Solution C1**. If **Solution C1** is precipitated, heat solution to 60°C until the precipitate has dissolved before use.

   What's happening: Solution C1 contains SDS and other disruption agents required for complete cell lysis. In addition to aiding in cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms. If it gets cold, it will form a white precipitate in the bottle. Heating to 60 ° C will dissolve the SDS and will not harm the SDS or the other disruption agents. Solution C1 can be used while it is still warm.

4. Add 60 ml of **Solution C1** and invert several times or vortex briefly.

5. Secure **PowerBead Tubes horizontally** using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes. **Note:** If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.

Note: *The vortexing step is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical agents from steps 1-4 and mechanical shaking introduced at this step. By randomly shaking the beads in the presence of disruption agents, collision of the beads with microbial cells will cause the cells to break open.*

What's happening: The MO BIO Vortex Adapter is designed to be a simple platform to facilitate keeping the tubes tightly attached to the vortex. It should be noted that although you can attach tubes with tape, often the tape becomes loose and not all tubes will shake evenly or efficiently. This may lead to inconsistent results or lower yields. Therefore, the use of the MO BIO Vortex Adapter is a highly recommended and cost effective way to obtain maximum DNA yields.

6. Make sure the **PowerBead Tubes** rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.

7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

Note: *Expect between 400 to 500 µl of supernatant at this step. The exact recovered volume depends on the absorbency of your starting material and is not critical for the procedure to be effective. The supernatant may be dark in appearance and still contain some soil particles. The presence of carry over soil or a dark color in the mixture is expected in many soil types at this step. Subsequent steps in the protocol will remove both carry over soil and coloration of the mixture.*

8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4 ° C for 5 minutes.

What's happening: Solution C2 is patented Inhibitor Removal Technology® (IRT). It contains a reagent to precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

10. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).

What's happening: The pellet at this point contains non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4 ° C for 5 minutes.

What's happening: Solution C3 is patented Inhibitor Removal Technology® (IRT) and is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

13. Transfer up to 750 µl of supernatant to a clean **2 ml Collection Tube** (provided).

What's happening: The pellet at this point contains additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

14. Shake to mix Solution C4 before use. Add 1.2 ml of **Solution C4** to the supernatant (be careful solution doesn't exceed rim of tube) and vortex for 5 seconds.

What's happening: Solution C4 is a high concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this will adjust the DNA solution salt concentrations to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low levels, to the Spin Filters.

15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature.

Note: A total of three loads for each sample processed are required.

What's happening: DNA is selectively bound to the silica membrane in the Spin Filter device in the high salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.

16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.

What's happening: Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.

17. Discard the flow through from the **2 ml Collection Tube**.

What's happening: This flow through fraction is just non-DNA organic and inorganic waste removed from the silica Spin Filter membrane by the ethanol wash solution.

18. Centrifuge at room temperature for 1 minute at 10,000 x *g*.

What's happening: This second spin removes residual Solution C5 (ethanol wash solution). It is critical to remove all traces of wash solution because the ethanol in Solution C5 can interfere with many downstream DNA applications such as PCR, restriction digests, and gel electrophoresis.

19. Carefully place Spin Filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.

Note: *It is important to avoid any traces of the ethanol based wash solution.*

20. Add 100 µl of **Solution C6** to the center of the white filter membrane.

Note: *Placing the Solution C6 (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in a more efficient and complete release of the DNA from the silica Spin Filter membrane. As Solution C6 (elution buffer) passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6 (10 mM Tris) which lacks salt.*

Alternatively, sterile DNA-Free PCR Grade Water may be used for DNA elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10). Solution C6 contains no EDTA. If DNA degradation is a concern, Sterile TE may also be used instead of Solution C6 for elution of DNA from the Spin Filter.

21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.

22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20 ^{o} to -80 ^{o} C). **Solution C6** does not contain any EDTA. To concentrate DNA see the Hints & Troubleshooting Guide.

AxyPrep PCR Clean-up Kit

For the purification of amplicons from PCRs

Kit contents, storage and stability

AxyPrep PCR Clean-up Kit

Cat. No.	AP-PCR-4	AP-PCR-50	AP-PCR-250
Kit size	4 preps	50 preps	250 preps
PCR column	4	50	250
1.5 ml Microfuge tube	4	50	250
2 ml Microfuge tube	4	50	250
Buffer PCR-A	2 ml	20 ml	100 ml
Buffer W2 concentrate	2.4 ml	24 ml	2×72 ml
Eluent	1 ml	5 ml	25 ml
Protocol manual	1	1	1

All buffers are stable for at least 12 months from the date of receipt when stored under ambient conditions. Please avoid exposure to direct sunlight and extremes in temperature.

Buffer PCR-A: DNA binding buffer. Store at room temperature.

Buffer W2 concentrate: Desalting buffer. Before the use of the kit, add the volume of ethanol specified on the bottle label and mix well. Either 100 % or 95 % denatured ethanol can be used. Store at room temperature.

Eluent: 2.5 mM Tris-HCl, pH 8.5. Store at room temperature.

Introduction

The AxyPrep PCR Cleanup kits employ a special Binding Solution in combination with an Axyprep PCR column and 96-well plate having an optimized chromatographic membrane to achieve high selectivity and recovery of linear DNA fragments. This product is designed to purify DNA fragments longer than 75 bp from PCRs and other enzymatic reactions, with an expected recovery of 70-90 %. Each column/well has a binding capacity of up to 8 μg. It is not necessary to remove mineral oil overlays from the PCRs before purification. This protocol will remove unincorporated primers (<50 nt), enzymes and unlabeled mononucleotides. The purified DNA fragments are suitable for a variety of applications, such as sequencing, ligation, restriction analysis, *in vitro* transcription, microinjection and microarrays.

Caution

Buffer PCR-A contains a chemical irritant. When working with the buffer, always wear suitable protective clothing such as safety glasses, laboratory coat and gloves. Be careful to avoid contact with eyes and skin. In the case of such contact, wash immediately with water. If necessary, seek medical assistance.

Equipment and supplies required but not provided

- Microcentrifuge capable of 12,000×g
- Table top centrifuge with swinging bucket rotor and plate carriers (for 96 preparation only)
- Vacuum manifold with luer-type fittings (#AP-VM)
- Vacuum source and regulator (-25-30 inches Hg required)
- Heated water bath
- 95-100 % ethanol

Preparation before experiment

3) Before using the kit, add the specified volume of ethanol to the Buffer W2 concentrate. Either 100 % or 95 % (denatured) ethanol may be used.
4) Check Buffer PCR-A for precipitation before each use. If precipitation occurs, warm at 65°C to dissolve the precipitate.
5) Pre-warming Eluent to 65°C will generally improve elution efficiency.

Protocols

I. AxyPrep PCR column

PCR Clean-up Spin Protocol

1. Add a 3× reaction volume of Buffer PCR-A to the sample. If the required volume of Buffer PCR-A is less than 100 µl, add 100 µl of Buffer PCR-A. Vortex briefly to mix the contents.
   Note: It is not necessary to remove mineral oil from the PCRs. Do not include the mineral oil volume in calculating the required volume of Buffer PCR-A.
   Examples: For a 20 µl PCR (with or without oil overlay) add 100 µl of Buffer PCR-A. For a 40 µl PCR, add 120 µl of Buffer PCR-A.

17. Place a PCR column into a 2 ml Microfuge tube (provided). Pipette the reaction from Step1 into the PCR column. Centrifuge at 12,000×g for 1 minute.

18. Discard the filtrate from the 2 ml Microfuge tube. Return the PCR column to the 2 ml Microfuge tube. Pipette 700 µl of Buffer W2 into the column and centrifuge at 12,000×g for 1 minute.
    Note: Make sure that the volume of ethanol specified on the bottle label has been added to the Buffer W2 concentrate.

4. Discard the filtrate. Return the PCR column to the 2 ml Microfuge tube. Pipette 400 µl of Buffer W2 into the column and centrifuge at 12,000×g for 1 minute.
   Note: Two washes with Buffer W2 are used to ensure the complete removal of salt, eliminating potential problems in subsequent enzymatic reactions.

5. Transfer the PCR column into a clean 1.5 ml Microfuge tube (provided). To elute the DNA, add 25-30 µl of Eluent (pre-warmed at 65°C) to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000×g for 1 minute.
   Note: Deionized water can also be used for elution.

Overviews

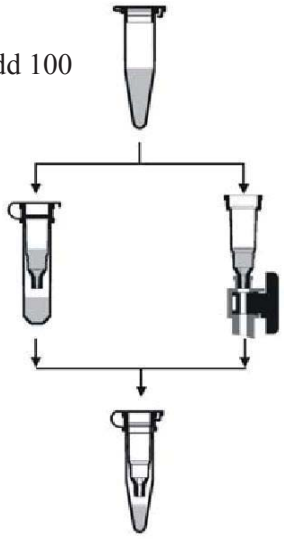
AxyPrep PCR column

Add 3× sample volume of Buffer PCR-A.
If the required volume is less than 100 µl, add 100 µl of Buffer PCR-A

Binding

Add 700 µl of Buffer W2
Repeat wash with Buffer W2

Washing



Add 25–30 µl of Eluent or deionized water

Elution

Appendix iii

Table 1: Eigenvalues and percentage of explained variability in PCA of soil characteristics (0–100 mm depth)

PC	Eigenvalue	% Total	Cumulative %
1	1.95	37	37
2	1.47	21	58
3	1.18	14	72
4	0.95	9	81
5	0.86	7	88
6	0.68	5	93
7	0.57	3	96
8	0.51	2	98
9	0.42	2	100

Table 2 Principal Component Scores for soil characteristics (0–100 mm depth)

Variables	**Component 1**	**Component 2**	**Component 3**
Soil moisture	-0.06	0.47	-0.36
pH	-0.28	0.07	0.10
NO_3-N	-0.52	0.07	0.13
NH_4-N	0.43	0.12	-0.07
TN	0.013	0.68	0.52
TC	0.048	0.51	-0.14
Olsen P	-0.38	0.12	-0.54
DEA	-0.43	-0.11	0.43
MBC	-0.34	0.02	-0.25