NITRIFICATION ACTIVITY IN NEW ZEALAND SOILS AND THE VARIABLE EFFECTIVENESS OF DICYANDIAMIDE

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

Doctor of Philosophy (PhD)

in

Soil Science

at Massey University, Palmerston North, New Zealand

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2011

Abstract

A perfusion technique was developed by which the rate of nitrification could be monitored as it changed over time following one or more additions of a nitrification inhibitor called dicyandiamide (DCD) in two contrasting soils - namely Manawatu Silt Loam (MSL) and Manawatu Fine Sandy Loam (MFSL). The modes of action of DCD in both soils were similar but the effectiveness of DCD varied between the two soils, with greater inhibition of nitrification in the MFSL than in the MSL when expressed as a percentage of the control soil. However when expressed in actual nitrification rates (absolute terms), greater inhibition of nitrification was obtained in the MSL as compared to MFSL. The actual reductions in nitrification rates between the two soils were almost similar, but the effect of DCD on the NO_3^--N reduction in the MSL was slightly higher than in the MFSL. The nitrification rates in both soils gradually recover following the addition of DCD, but it didn't return to the initial levels in either soil. This ongoing inhibition effect was more obvious in the MFSL. The effect of DCD on the ammonia oxidising bacteria (AOB) populations in both soils followed a similar pattern to the nitrification activities, with an inhibition of nitrifier population in the presence of DCD and a recovery of the temporarily suppressed nitrifier populations when the DCD solution was removed from the system and was replaced with a fresh nitrogen source. Again, there was a residual effect of DCD on AOB numbers and this appeared to be greater in the MFSL than in the MSL.

In a separate experiment the effectiveness of DCD in the two soils was similar to that obtained in Chapter 3, in which it differed when expressed as percentage and absolute terms. DCD was more effective, with higher inhibition was obtained, in the MFSL than in the MSL when expressed as a percentage of the control. This was probably due to the differences in the rate of DCD degradation in both soils, in which DCD degraded two times slower in the MFSL than in the MSL. The effectiveness of DCD was also different between the two soils, when the same amount of DCD remained in both soils, with higher inhibition was obtained in the MSL than in the MFSL. Thus, in absolute terms DCD was more effective in the MSL.

In a further experiment it was demonstrated that soils collected from steep slopes (SS) in a hill country paddock had low nitrification rates compared to soils collected from adjacent camp sites (CS). These low nitrification rates were associated with similarly low populations of AOB in the SS soils. Of interest was the observation that the numbers of AOB and the nitrification rate in absolute terms in the SS did not increase greatly over the time, even with a plentiful supply of NH_4^+ substrate from added urea and the associated higher pH. It was not clear whether the low initial population of AOB in SS resulted from low inputs of NH_4^+ substrate over many years, or whether in addition there was an inhibitory effect that may have prevented a build-up of the nitrifiers. A subsequent investigation suggested that the low nitrifying SS soil may exert a small inhibiting effect when mixed with high nitrifying CS soils.

In conclusion DCD was found to vary in its effectiveness in soil types. The effectiveness of DCD in reducing NO_3-N production in grazed pasture systems is a

function of both its half life in the soil and also the extent of inhibition of nitrification at a given concentration of DCD in the soil.

Acknowledgements

This thesis would not have been possible without the guidance and the support of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study.

I would like to thank The Agriculture and Marketing Research and Development Trust (AGMARDT) for providing me with a doctoral scholarship for the duration of my study in Massey University. I am grateful to the Department of Soil Science Massey University which made this study possible by providing me with the support and research facilities.

I am heartily thankful to my chief supervisor, Professor Emeritus Russ Tillman, for many insightful conversations during the development of the ideas in this thesis, and for helpful comments on the text. Your supervision, support and encouragement from the preliminary to the concluding level enabled me to develop an understanding of the subject. Beyond that, your cool mentoring and teaching brings cheer to each conversation. To my co-supervisor, Professor Peter Kemp, I appreciate your time and contribution during the development of the work carried out in this thesis and final review of this thesis. To Mr. Andrew Carran and Dr. Saman Bowatte thank you for your time, ideas and contributions during the initial discussions of this research.

I am grateful to Professors Mike Hedley and Nanthi Bolan for your guidance and support to pursue my study in Soil Science. To all the technical team of Soil Science, thank you so much for your technical assistance throughout this study. Thanks also to Michelle Tamehana and Warwick Johnson, technician of Food Science and Technology Massey University for allowing me to use the laboratory and assisting me with the HPLC for the DCD analysis. I thank Moira, Liza, Denise Brunskill and Denise Stewart for being accommodating and their continuous moral support. To Andy Wood I appreciate your time for helping me with the formatting of this thesis.

To all the past and present fellow post graduate students, Bambang, Peter, Jeya, Noveline, Uttara, Indika, Saman, Saleem, Sam, Erwin, Amandeep and Neha, my sincere thanks for your continuous moral support throughout this study and thank you for sharing your thoughts.

Special thanks also to my parents and family for all the constant moral support and encouragement when I couldn't see the light at the end of the tunnel.

Last but not least, to my husband, Peter, the love of my life. Thanks for believing in me and inspiring me to greater things. Your love, friendship and your kind gentle spirit make living with you each day a gift. I am blessed and thanks for finding me so many jokes!

Most of all, I am exceedingly grateful to God for all His abundance blessings in each day of my life and for giving me the strength in completing this study, thank you so much Dear Lord.

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Chapter 1

General introduction

1.1 The issue

In New Zealand the major forms of pastoral farming are sheep, beef and dairy cattle (PCE 2004). There are number of ways that nutrients are added to New Zealand farming systems. In the case of nitrogen (N) these include synthetic fertilisers, symbiotic N fixation and the importation of supplementary feeds from outside the system. Once within the system, N is then recycled back into the soil by return of animal excreta (dung and urine) (Haynes and Williams 1993) and spraying of dairy shed effluent onto pasture (Longhurst *et al.* 1999, Longhurst *et al.* 2000, Monaghan *et al.* 2007).

New Zealand agricultural soils annually receive about 3 million tonnes of N with approximately 0.9-1.1 million tonnes of this coming from symbiotic N fixation (Saggar 2004). As farming in New Zealand (and particularly dairy farming) has become more intensive in recent decades the use of synthetic N fertiliser has increased significantly (Association NZFMR 2011). For example, approximately 340,000 tonnes of fertiliser N was applied in New Zealand in the year ending June 2010, which was more than ten times that used thirty years earlier in the year ending June 1980 (Association NZFMR 2011). Of the N fertiliser applied in 2002, 54% was used in dairy farming, 31% in sheep and beef-cattle farming, 4% in vegetable growing and 2% in deer farming (PCE 2004).

In intensively grazed pasture systems, animals graze on N-rich grass-clover pastures, but only a small amount of the N ingested is actually converted to milk, meat or wool and removed from the farm. The remainder is excreted in dung and urine, and returned to the soil (Jarvis *et al.* 1995; Haynes and Williams 1993). With the intensification in dairy farming, the increases in stocking rates have led to greater returns of animal excreta, placing greater pressure on the environment (MAF 2007). The amount of N recycled or returned in this way is estimated to be around five times (1.58 million tonnes) the amount of N added to the land as N fertilisers (0.33 million tonnes) (Saggar 2004). Thus, the largest input of N to soils under grazed pastures in New Zealand is from animal excreta, particularly urine. Within the urine patch this can add rates of N equivalent to 500 kg N ha^{-1} (sheep) and 700-1200 kg N ha^{-1} (cattle), which greatly exceeds the ability of the pasture plants to absorb N (Haynes and William 1993, Jarvis *et al.* 1995, Di and Cameron 2000b).

In recent years, effluent has been increasingly sprayed onto pasture in New Zealand as a way to add nutrients for pasture growth and dispose of the waste. For example, in 2002, effluent was sprayed to almost 170, 000 hectares of pasture in New Zealand (Statistics New Zealand 2003) and almost 80% of dairy farms in the Waikato apply effluent to the land (Environment Waikato 2003).

These large and increasing N inputs to agricultural land are resulting in environmental degradation from N losses. For example, nitrous oxide (N_2O) emissions contribute not only to global warming but also to the destruction of the ozone layer (Bouwman 1990; Crutzen 1981). The largest source of N_2O emissions in New Zealand is from animal excreta deposited during grazing (80% of agricultural N_2O emissions), while N fertiliser use contributes to 14% of agricultural emissions (de Klein and Ledgard 2005). In

addition, nitrate (NO_3^-) can leach through soil into groundwater, eventually ending up in lakes, rivers and coastal waters. This can lead to deterioration of groundwater and drinking water supplies, with risks to human health, and eutrophication of fresh and coastal waters (Addiscott and Benjamin 2004; Eriksen *et al.* 1999; Gupta *et al.* 2000; Kowalchuck and Stephen 2001). More details on the effect of reactive N on human health and ecosystems are given in Chapter 2. Because of this there has been an increasing interest in understanding the N cycle in agricultural systems. A key process in the N cycle is nitrification.

Nitrification is the oxidation of reduced forms of N, such as ammonium (NH_4^+) ultimately to NO_3^- with N_2O being a by-product. It is carried out by autotrophic nitrifying bacteria (Prosser 1989). While NH_4^+ in soils is quite stable and binds tightly to soil particles, its conversion to NO_3^- leads to significant losses of soil N, thereby increasing environmental pressure due to NO_3^- leaching and gaseous losses of N_2O (Bolan *et al*. 2004). N_2O is produced during the microbial processes of nitrification and denitrification. Denitrification is the stepwise biological reduction of NO_3^- to gaseous nitrogen (N_2), with N_2O being an obligatory intermediate (Figure 2.1). Denitrification is generally accepted as the main source of N_2O from grazed pastoral soils (Stevens and Laughlin 1998). Although N_2O production from nitrification is less significant, nitrification provides the NO_3^- substrate and is often a critical prerequisite for denitrification. In the late 1970's, a wide range of nitrification activities in New Zealand soils was observed (Sarathchandra 1978a, 1978b). More recently, Bowatte (2003) observed that nitrification rates in soils collected from flat campsite areas in grazed hill country were very much greater than in soils collected from adjacent steep slopes. The

reasons for the variation in nitrification rates observed by both Sarathchandra (1978a, 1978b) and Bowatte (2003) are not completely understood.

With the increased interest in nitrification mainly because of the environmental impacts of N losses via gaseous emissions and leaching from grazed pastures, together with New Zealand's commitment to the Kyoto Protocol, there has been an increasing interest in the use of nitrification inhibitors such as dicyandiamide (DCD) to slow down the nitrification process and mitigate leaching and gaseous losses of N. DCD helps to delay the oxidation of NH_4^+ to NO_3^- by depressing the activities of nitrifying bacteria in soil (Rajendra Prasad and Powell, 1995). In doing this, it reduces N_2O emissions directly by reducing nitrification or indirectly by reducing the amounts of NO_3^- which could subsequently be leached or denitrified. At the same time DCD can increase the N use efficiency (NUE) by increasing plant growth and N uptake. The majority of research to date on DCD in New Zealand is confined to quantifying the effects of DCD on N losses from urea fertiliser (Zaman *et al.* 2007), urine deposition in legume-based pasture (Di and Cameron 2003, 2004c; Zaman and Blennerhassett 2009) and dairy shed effluent (Williamson and Jarvis 1997). These studies have shown that DCD has great potential in reducing NO_3^- leaching and N_2O emissions, however, the effect varies between soils and the reasons for these variations are not well understood.

1.2 The aim and thesis structure

The research presented in this thesis aims to increase our understanding of nitrification in New Zealand soils with a major focus on understanding the reasons for both the natural variation in nitrification rates within the landscape and also the variable effectiveness of DCD.

Chapter 2 is a review of the literature which highlights the importance of nitrification and describes the nitrifying bacteria that carry out the process. The environmental impact of nitrification and the factors that control the nitrification activity are also discussed. The review also describes the various methods of estimating the populations of nitrifying bacteria and discusses the advantages and limitations of these different methods. Finally, research on the use of DCD to reduce the losses of N via nitrification and denitrification processes in agricultural soils is reviewed.

The work described in Chapter 3 aimed to develop a technique by which the rate of nitrification could be monitored as it changed over time following one or more additions of DCD. A "perfusion" technique was developed in which a dilute solution of ammonium sulphate $(NH_4)_2SO_4$ was continuously percolated or "perfused" through a column of soil. The nitrifying organisms in the soil converted the ammonium to nitrate over time, and by regularly analysing small samples of the circulating solution the rate of nitrification could be determined. The populations of autotrophic AOB, and the effect of DCD on these populations, were also quantified.

In Chapter 3, it was observed that the effectiveness of DCD in reducing nitrification rates varied, not only between soils, but also over time within a soil. The work in Chapter 4 was designed to further explore the link between the presence of DCD and the nitrification rate. In particular, we wanted to explore whether the variable effectiveness of DCD between soils and over time was due to the disappearance of DCD from the soil through microbial breakdown, or whether there were other factors affecting the effectiveness of DCD. The objectives of this study were therefore to: i) quantify the rate of DCD degradation in 2 different soils, namely Manwatau silt loam

(MSL) and Manawatu fine sandy loam (MFSL) and (ii) to determine the effect of DCD addition on the rate of nitrification in both soils.

As noted earlier in this Chapter, nitrification rates vary markedly between soils in the field. In particular, soils from steep slopes in hill country pastures have very low rates of nitrification, and these rates do not increase, even when the soils are incubated in ideal conditions in the laboratory, with non-limiting amounts of ammonium available. The question arises as to whether these soils contain some naturally-occurring inhibiting factor. The experiments in Chapter 5 explored this possibility a little further. The first step was to check whether the low nitrification rates previously reported in the literature could be reproduced in this study. This was done by incubating soils in the laboratory, with and without added urea, and measuring the rate of nitrification. The soils used were collected from steep slopes (SS) and campsites (CS) at Ballantrae and from a lowland Manawatu dairy farm (MSL). The populations of AOB in these soils were also measured.

The final part of this study followed on from the measurement of nitrification activity in the hill country soils carried out in Chapter 5. It aimed to provide additional evidence as to whether there is a possible inhibition or "poisoning effect" on nitrifiers in the soil collected from steep sites in hill country. The results obtained in Chapter 5 had confirmed that soil collected from steep slopes had low nitrification rates and a low population of nitrifiers. It was not however clear whether the low population of nitrifiers in soils from steep slopes resulted from low inputs of ammonium substrate over many years or whether, in addition, there was an inhibitory effect that may have prevented a build-up of the nitrifiers. The experiment described in Chapter 6 investigated the effect

of mixing soils with initially low nitrification activity (collected from steep slopes) with soils with initially high nitrification activity (collected from camp sites) on the nitrification activity in the resulting soil mixtures. The nitrification activity was assessed with and without urea amendment under laboratory conditions over a 15-day period. Measurements were made after 0, 5, 10 and 15 days of incubation. In this study, it was hypothesized that if the soil collected from the steep slope had some characteristic that inhibits nitrification activity, then mixing that soil with another soil that initially had high nitrification activity would result in a suppression of nitrification activity in the soil mixture.

The findings of all these experiments are then summarised in Chapter 7 along with the main conclusions drawn from this PhD research and the direction of future research is discussed.

Chapter 2

Review of literature

2.1 Introduction

Soil microorganisms are important for the functioning and stability of ecosystems. A number of microbial communities including nitrifiers, denitrifiers and dinitrogen (N_2) fixers determine nitrogen (N) availability in soils and control the fluxes of biogenic trace gases such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and nitric oxide (NO) (Patra *et al.* 2005).

In grazed pastures, as much as 50 to 70% of the cycling N can be lost by ammonium (NH_4^+) volatilisation, leaching and denitrification (Ball and Ryden 1984; Field *et al.* 1985; MfE 2010) thereby causing a significant decrease in the nitrogen use efficiency (NUE) and adversely affecting the environment. The environmental impacts include surface and groundwater nitrate (NO_3^-) pollution, N_2O emissions contributing significantly to global warming and ozone layer destruction in the stratosphere, and appreciable N losses through ammonia (NH_3) emissions which act as a secondary source of N_2O emissions and contribute to acid rain (Di and Cameron 2002b; Jarvis *et al.* 1995; Whalen 2000).

In many countries around the globe including New Zealand, increasing inputs of N fertilisers, increasing application of waste effluents to soils and continued return of animal urine and dung to agricultural land has increased the interest in nitrification.

Nitrification involves two steps of biological oxidation. The first step is the conversion of NH_4^+ to nitrite (NO_2^-). The second step is oxidation of NO_2^- to NO_3^-. Both steps are carried out by aerobic nitrifying bacteria (Prosser 1989). These bacteria belong to genera with the prefix *Nitroso-* (converts NH_4^+ to NO_2^-) and *Nitro-* (converts NO_2^- to NO_3^-) (Spieck and Bock 2005) (Figure 2.1). Ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) are the two enzymes that accelerate the ammonia (NH_3) oxidation in the nitrification process (Figure 2.1) (Kowalchuck and Stephen 2001). The AMO enzyme catalyses the oxidation of NH_3 or NH_4^+ to hydroxylamine (NH_2OH), while HAO catalyses the oxidation of NH_2OH to NO_2^-.

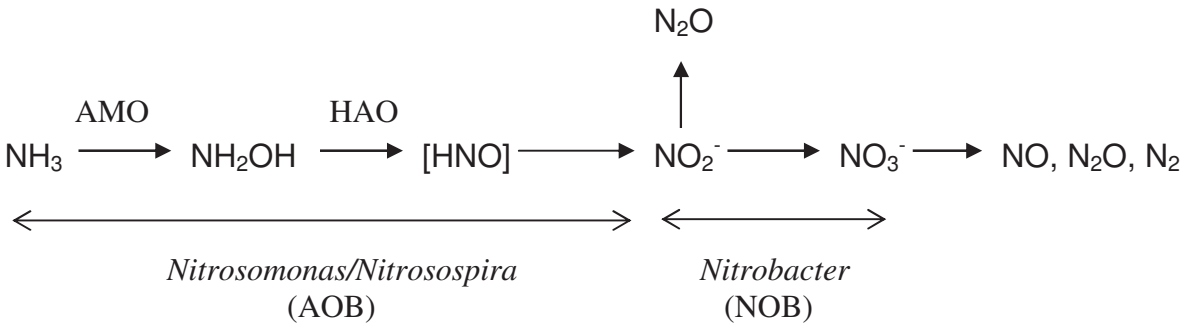


Figure 2.1 Bacteria and enzymes involved in the nitrification process. Abbreviations: AOB, ammonia oxidising bacteria; NOB, nitrite oxidising bacteria; AMO, ammonia monooxygenase and HAO, hydroxylamine oxidoreductase.

Nitrification can occur in a wide range of environments, including agricultural soils (Bruns *et al.* 1999; Mendum and Hirsch 2002; Mendum *et al.* 1999), grasslands (Kowalchuck *et al.* 2000a, 2000b; Webster *et al.* 2002), forest soils (Backman and Klemedtsson 2003; Carnol *et al.* 2002; Laverman *et al.* 2000; Mintie *et al.* 2003; Nugroho *et al.* 2005) , and treated sewage and waste-water (Ibekwe *et al.* 2003; Mota *et al.* 2005; Painter 1986). Nitrification also occurs widely in aquatic environments which include freshwater (Hall 1986a; Hovanec and DeLong 1996; Speksnijder *et al.* 1998),

marine (Hall 1986b; Stephen *et al.* 1996), rooted aquatic plants, algae, on rock surfaces and in sediments (Matulewich and Finstein 1978).

In the late 1970's, some studies were carried out on the activities and populations of nitrifying bacteria in New Zealand soils (Sarathchandra 1978a, 1978b). In these studies, a wide range of nitrification activities in New Zealand soils was observed. More recently, Bowatte (2003) observed that nitrification rates in soils collected from flat campsite areas in grazed hill country were very much greater than in soils collected from adjacent steep slopes. The reasons for the variation in nitrification rates observed by both Sarathchandra (1978a, 1978b) and Bowatte (2003) are not completely understood.

The large quantities of NO_3^- accumulating in ecosystems due to nitrification processes, can, if not taken up by a crop, be lost as NO_3^- through leaching or surface runoff, which could contribute to eutrophication (Pierzynski 1994) and is a source of public health concern; under some circumstances NO_3^--N concentrations are close or exceed the current acceptable guidelines for drinking water (11.3 mg L^{-1} of NO_3^--N) (MfE 2007). In addition, excess of NO_3^- in the soil often leads to losses of N via N_2O emission via the denitrification process. Because of these environmental concerns there has been an increasing interest in New Zealand in the use of nitrification inhibitors (NIs) as a way to mitigate N losses. Nitrification inhibitors are compounds that suppress the activity of AOB and inhibit the oxidation of NH_4^+ to NO_2^-, resulting in a slowing down of the conversion rate of NH_4^+ to NO_3^-. This inhibition of nitrification helps to reduce N losses through leaching and denitrification by reducing the amounts of NO_3^- produced.

As already mentioned in the Chapter 1, the majority of research on NIs in New Zealand has been confined to quantifying the effects of dicyandiamide (DCD) on N losses from urea fertiliser and urine deposition in legume-based pasture (Di and Cameron 2002c, 2003, 2005; Zaman *et al.* 2007). Most of the studies have concluded that the application of DCD was able to reduce NO_3^- leaching and N_2O emissions. However, the effect varies between soils and the reasons for these variations are not well understood.

This review discusses the importance of nitrification in the N cycle, the occurrence of nitrification in soils and which nitrifiers are responsible for the oxidation of NH_4^+ in soil. Because nitrification is sensitive to environmental conditions, the factors controlling nitrification are also briefly discussed. The role of NIs, including DCD, as a possible means of controlling nitrification in agricultural systems is also discussed in this chapter. Finally, the methods used to quantify the numbers of AOB in the soil environment are described and discussed.

2.2 Nitrification in soil

2.2.1 Autotrophic nitrifiers

Autotrophic nitrification is a two-step oxidation process that involves the conversion of NH_4^+ to NO_2^- by AOB from a number of genera followed by the conversion of NO_2^- to NO_3^- by nitrite oxidising bacteria (NOB) of the genus *Nitrobacter* (Figure 2.1). The autotrophic AOB are comprised of two phylogenetically distinct sub-groups (gamma (γ) and beta (β)) of the *Proteobacteria* (Prosser 2007).

The γ-*Proteobacteria* are represented by only two species of the genus *Nitrosococcus,* and have been isolated from marine environments (all strains of *N. oceani*) and from

salt lakes (*N. halophilus*) (Koops *et al*. 2006). Both species have a similar morphology but are different in salt requirement, salt tolerance and NH_3 tolerance (Koops *et al*. 2006). On the other hand, β-*Proteobacteria* include members of the genera *Nitrosomonas*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* (Head *et al*. 1993; Teske *et al*., 1994; Utåker *et al*. 1995). It has been suggested (Head *et al*. 1993) that the latter three genera should be reclassified in the single genus of *Nitrosospira*. The *Nitrosomonas* group consist of six distinct lineages of *N. oligotropha, N. marina, N. europaea/Nitrosococcus mibilis*, *N. communis*, *N. cryotolerans* and *N.* sp. Nm143 (Koops *et al*. 2006). These lineages can also be called as clusters, whereby the *Nitrosomonas* lineages are grouped into clusters 5 to 9 (Koops *et al.* 2006; Kowalchuck and Stephen 2001; Stephen *et al.* 1996), and the *Nitrosospira* lineages are grouped into clusters 0 to 4 (Koops *et al.* 2006; Kowalchuck and Stephen 2001; Purkhold *et al.* 2003; Stephen *et al.* 1996). According to Avrahmi and Conrad (2003) however, based on the *amoA* gene (encoding the active subunit of ammonia monooxygegenase, AMO) sequences, the *Nitrosospira* lineages can be grouped into clusters 1, 2, 3a, 3b, 4, 9, 10, 11 and 12. Clusters 2, 3 and 4 can be related to corresponding 16S rDNA gene clusters as defined earlier (Stephen *et al.* 1996). Table 2.1 shows the diverse lineages associated to *Nitrosospira* and *Nitrosomonas* groups.

Table 2.1 The diverse lineages associated to γ- and β-subgroups of the *Proteobacteria* group described by Purkhold *et al.* (2003) on the basis of 16S rDNA and *amoA* gene sequences. Information for *Nitrosospira* clusters 9-12 was obtained from Avrahmi and Conrad (2003), based on *amoA* gene sequences. Other information provided in this table was obtained from Koops and Pommerening-Röser (2001) and Prosser (2007).

Sub-division of AOB	**Lineage**	**Cluster**	**Salt requirement**	**Preffered habitat**	**Isolation/enrichment obtained from**	**Sequences derived from**
γ- *Proteobacteria*	*Nitrosococcus oceani*	-	Obligately halophilic	Seawater	Seawater	Seawater
	Nitrosococcus halophilus	-	Obligately halophilic	Salt lakes	Sediment	-
β- *Proteobacteria*	*Nitrosospira*	0	No salt requirement	Soil	Soil	Soil, sand dune, freshwater
		1	Obligately halophilic	Seawater	Marine sample	Sand dune, seawater
		2	No salt requirement	Acidic soil	Soil, sewage treatment	Sand dune, soil
		3	No salt requirement	Fertilised neutral pH soil	Soil, peatbog	Soil, freshwater, sand dune
		4	No salt requirement	Soil without fertiliser use	Soil	Soil, sand dune, freshwater
		9, 10, 11 and 12	No salt requirement	Soil	-	Soil

Table 2.1 (Cont.)

Sub-division of AOB	***Lineage***	***Cluster***	***Salt requirement***	***Preffered habitat***	***Isolation/enrichment obtained from***	***Sequences derived from***
β- *Proteobacteria*	*Nitrosomonas*	5	Obligately halophilic	Seawater	Seawater	Seawater, freshwater, sand dune
	Nitrosomonas oligotropha	6a	No salt requirement	Freshwater, occasionally found in moderately acidic soils	Estuary, soil, activated sludge	Freshwater, soil, waster water
	Nitrosomonas marina	6b	Obligately halophilic	Seawater	Seawater	Seawater, freshwater, sand dune
	Nitrosomonas europaea/ Nitrosococcus mobilis	7	Halotolerant or moderately halophilic	Sewage disposal plants, eutrophic freshwater and brackish water, soil	Activated sludge, biofilm, seawater, brackish water, concrete wall (animal house)	Wastewater, seawater, freshwater, soil
	Nitrosomonas communis		No salt requirement	Soils with moderately eutrophic and neutral pH	Soil, sewage, estuary	-
	Nitrosomonas sp. Nm 143	9	Obligately halophilic	Seawater	Seawater	Marine estuary
	Nitrosomonas cryotolerans		Obligately halophilic	Seawater	Seawater	-

The AOB and NOB are autotrophs, which are able to use energy released during the oxidation of NH_4^+ and NO_2^-, and use inorganic carbon dioxide as their sole source of carbon for growth (Havlin *et al*. 2005). These organisms are aerobes although some species may be highly tolerant of low oxygen or anoxic environments (Bodellier *et al.* 1996). The autotrophic nitrifiers are slow growing bacteria, with generation times that vary from 8 h for *Nitrosomonas* and 10 h for *Nitrobacter* to 60 h for *Nitrosospira* (Bock *et al.* 1986; Koops *et al.* 1991).

Ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) are the two enzymes that accelerate the ammonia (NH_3) oxidation in the nitrification process (Figure 2.1) (Kowalchuck and Stephen 2001). The AMO enzyme catalyses the oxidation of NH_3 or NH_4^+ to hydroxylamine (NH_2OH), while HAO catalyses the oxidation of NH_2OH to NO_2^-.

2.2.1.1 Distribution of AOB in different environments

Members of β-*Proteobacteria* are widely distributed in natural environments, while species of γ-*Proteobacteria* are mostly found in marine environments. Several species or groups of species have been observed predominantly in specialised sites, such as oceans (Ward and Carlucci 1985; Ward and O'Mullan 2002), salt lakes (Koops *et al*. 1990, Sorokin *et al*. 2001), freshwater lakes (Koops and Pommerening-Röser 2001; Speksnijder *et al*. 1998; Whitby *et al*. 1999), brackish waters (Koops et al. 1976), sewage water treatment plants (Okabe *et al.* 1999; Schramm *et al*. 1996; Urakawa *et al*. 2006) and soils with acidic (Nugroho *et al.* 2005; Stephen *et al.* 1996, 1998), neutral and alkaline conditions (Bruns *et al.* 1999; Mendum *et al.* 1999; Kowalchuck *et al.* 2000a, 2000b). Overall, there is evidence (discussed in the following sections) that

some AOB species have an obligate salt requirement, some prefer eutrophic or oligotrophic environments and others tolerate either low or high temperatures (Koops *et al.* 2006; Freitag *et al*. 2006). Different NH_3 concentrations in activated sludge, fresh water lakes and sediments have caused adaptive evolution of different subgroups of ammonia oxidisers (Okabe *et al*. 1999; Kowalchuck and Stephen 2001; Koops *et al.* 2006).

2.2.1.1.1 Marine environments

Nitrosocystis oceanus was the first marine AOB strain isolated from seawater (Watson 1965). This species is now known as *Nitrosococcus oceani*, and along with *Nitrosococcus halophilus*, belongs to the γ-subdivision of the *Proteobacteria*. All other AOB species are in the β-subdivision, and while some β-*Proteobacteria* AOB has been isolated from and detected in both marine environments and freshwater, γ-*Proteobacteria* have only been found in marine environments. *N. oceani* has been detected in many marine environments by immunofluorescence at concentrations in the order of 10^3 to 10^4 cells ml^{-1} (Ward *et al.* 1982; Ward and Carlucci 1985; Zaccone *et al*. 1996) and this has been confirmed by molecular analysis (Ward and O'Mullan 2002). *Nitrosomonas marina* (a member of the β-*Proteobacteria*) has also been detected by immunofluorescence at similar concentrations (Ward and Carlucci 1985). In Chesapeake Bay however, both *N. oceani* and *Nitrosomonas marina* have been detected at abundances several orders of magnitude greater than those reported above (Ward *et al.* 1982).

Molecular analysis has also revealed wide distribution of *Nitrosospira* and *Nitrosomonas* AOB in marine environments. Stephen *et al*. (1996) provided the first

evidence of the presence of *Nitrosospira* cluster 1 in marine sediments. From then on, there have been many findings of *Nitrosospira* (clusters 1-4) and *Nitrosomonas* (clusters 5-7) in Dutch coastal sand dunes (Kowalchuk *et al*. 1997). In addition, Phillips *et al*. (1999) demonstrated that *Nitrosospira* cluster 1 were dominant in planktonic samples whereas members of *Nitrosomonas* were mostly detected in particle-associated samples, showing that there was separation by genus in distinct niches of marine environments. This may result from differences in physiological properties and environmental conditions associated with these niches. A shift of *Nitrosomonas* cluster was observed within estuaries according to the level of salinity. For example, de Bie *et al*. (2001) have reported a transition from *Nitrosomonas* cluster 6a in the freshwater part of the Schelde estuary to the marine-associated *Nitrosomonas* cluster 5 in more seaward regions, coincident with observed gradients in salinity, oxygen and NH_3 in the estuary.

2.2.1.1.2 Salt lakes

In salt lakes, the pH as well as the salt concentration varies significantly (Ward *et al*. 2000), thus creating different niches for AOB. *Nitrosococcus halophilus* of the γ-*Proteobacteria* subgroup has been isolated from sediment samples from a lake in Saudi Arabia (Koops *et al*. 1990) and *Nitrosomonas halophilus* and *Nitrosomonas europaea* (both cluster 7 lineages and members of *β-Proteobacteria*) have been observed in the Mongolian soda lakes (Sorokin *et al*. 2001) and in the hypersaline Mono Lake in California (Ward *et al*. 2000).

2.1.1.1.3 Freshwater environments

In natural oligotrophic freshwater environments (rivers and lakes), members of the *Nitrosomonas oligotropha* and *Nitrosomonas ureae* (Cluster 6a) are generally the

dominant AOB present (Koops and Pommerening-Röser 2001; Koops *et al*. 2006; Prosser 2007; Speksnijder *et al.* 1998). On the other hand, strains of *Nitrosomonas europaea*, *Nitrosomonas eutropha* (cluster 7) and *Nitrosomonas nitrosa* (cluster 8) have been isolated from eutrophic rivers and lakes (Koops and Pommerening-Röser 2001). In other studies Whitby *et al*. (1999) found *Nitrosospira* in sediment and lakewater samples throughout the seasonal cycle, but could only detect the presence of *Nitrosomonas* cluster 7 during the summer months when the NH_4^+ concentrations were increased.

2.1.1.1.4 Waste water treatment systems (WWTS)

Nitrosomonas europaea strains are most frequently recovered in pure culture when using high N culturing conditions (Prosser 1989), and it has therefore been assumed that *Nitrosomonas* are the key AOB in high N waste treatment systems (Kowalchuck and Stephen 2001).

A number of studies have confirmed the dominance of the *N. europaea* lineage in most WWTS (Mobarry *et al*. 1996; Okabe *et al.* 1999; Schramm *et al*. 1996; Urakawa *et al*. 2006). However, other studies have reported that *Nitrosococcus mobilis* and *Nitrosospira* are the dominant AOB species found in the activated sludge and fluidized bed reactors (Juretschko *et al*. 1998; Schramm *et al.* 1998). Multiple AOB populations either with one genus or across genera have been detected in some WWTS, and shifts in the relative proportions of AOB populations have been observed in response to the system conditions (Juretschko *et al*. 1998; Okabe *et al*. 1999; Sakano and Kenkhof 1998).

2.1.1.1.5 Distribution of autotrophic AOB in different soils

The community structures of AOB have been studied in various soils that differ in environmental conditions such as pH, NH_4^+ concentration, type of fertiliser application, water content and soil temperature. This work is summarised in (Table 2.2).

Table 2.2 The presence of autotrophic AOB in soils in relation to environmental factors and soil management.

Factors contributing to diversity of AOB in the soil	**Clusters by 16S rDNA/ *amo A* and the type of AOB present**	**Reference**
1. Agricultural and forest soils, acidic pH	16 S rDNA *Nitrosospira* cluster 2	Stephen *et al.* 1996, 1998; Nugroho *et al*. 2005
2. Limed acid forest soil,	16S rDNA *Nitrosomonas* (dominant), *Nitrosospira*-like (minority)	Carnol *et al*. 2002
3. High NH_4^+ concentration		
a) Early succesional grassland soil with neutral pH	a) 16S rDNA *Nitrosospira* cluster 3	Kowalchuck *et al*. 2000a, 2000b
b) Incubated agricultural soil, over a period of 16 weeks	b) *amoA* *Nitrosospira* cluster 3	Avrahmi *et al*. 2003
4. Low NH_4^+ concentration		
a) Older succesional grassland soil with acidic pH	a) 16S rDNA *Nitrosospira* clusters 2 and 4	Kowalchuck *et al*. 2000a, 2000b
b) Incubated agricultural soil, over a period of 16 weeks	b) *amoA* *Nitrosospira* cluster 1 (disappeared after >16 weeks of incubation and *Nitrosospira* cluster 9	Avrahmi *et al*. 2003
5. Moisture limitation, forest soil (pH 4.1-5.2)	16S rDNA *Nitrosomonas*, exhibit rapid recovery after rewetting dried soil	Hastings *et al*. 2000
6. Temperature effect (incubated agricultural soil)		
a) 4-10 ^oC	*amoA* *Nitrosospira* cluster 1	Avrahmi *et al*. 2003
b) 25-30 ^oC	*amoA* *Nitrosospira* clusters 3 and 9	Avrahmi *et al*. 2003
7. Irrigated soils		Avrahmi *et al*. 2003
a) with liquid fertiliser (NPK)	*amoA* *Nitrosospira*-like	Oved *et al*. 2001
b) with effluent	*amoA* *Nitrosomonas*-like	Oved *et al.* 2001
8. Long term (16 years) application of mineral fertiliser (NPK) and organic manure	*amoA* *Nitrosospira* cluster 3	Chu *et al*. 2007
9. Agricultural soils amended with pig slurry	*amoA* *Nitrosomonas europaea* 16S rDNA *Nitrosospira*	Hastings *et al.* 1997

Most of the molecular studies have suggested a predominance of *Nitrosospira* species (particularly clusters 2 and 4) in acidic soil environments, while other members of both *Nitrosospira* (particularly cluster 3) and *Nitrosomonas* have been detected in soils with a more neutral pH. For example, molecular surveys of AOB in a forest soil with low pH identified *Nitrosospira* cluster 2 (Nugroho *et al*. 2005) and Stephen *et al.* (1996, 1998) found *Nitrosospira* cluster 2 was dominant in an acidic agricultural soil. In another study, there was a high representation of *Nitrosospira* clusters 2 and 4 in older grassland soils which were acidic and had low concentrations of NH_4^+ (Kowalchuck *et al.* 2000a, 2000b).

In contrast to the studies described above, one study has found that *Nitrosomonas europaea* (cluster 7) species dominated in an acidic forest soil with and without liming (Carnol *et al.* 2002) and in oligotrophic moderately acidic soils, a strain of *Nitrosomonas oligotropha* lineage has also been isolated (Koops and Pommerening-Röser 2001).

Kowalchuck *et al.* (2000a, 2000b) reported that *Nitrosospira* cluster 3 was dominant in recently established (within the previous 5 years) grassland fields with neutral pH and with relatively high NH_4^+ concentrations. This was supported by enrichment cultures using different NH_4^+ concentrations, which revealed biases towards *Nitrosospira c*luster 3 in high and *Nitrosospira* cluster 4 in low NH_4^+ concentrations (Kowalchuck *et al.* 2000a). Apart from this, many other researchers have reported that members of *Nitrosospira* cluster 3 were the dominant AOB in arable fields which had been fertilised and had neutral pH (Bruns *et al.* 1999; Mendum *et al.* 1999; Phillips *et al.* 2000; Stephen *et al.* 1996, 1998). In a more recent work, Di *et al.* (2009), demonstrated that

all the AOB identified from all the six pasture soils sampled across New Zealand were *Nitrosospira a*nd no *Nitrosomonas* species were observed. The identified *Nitrosospira* species were aligned in clusters 3a and 3b with some in clusters 4.

In a study under laboratory incubation conditions the community structure of AOB in soil didn't change with different NH_4^+ concentrations in low (LF) and high (HF) fertiliser treatments in a short (4 weeks) period (Avrahami *et al.* 2002). However, obvious community shifts occurred over a longer period (>16 weeks) and fertilisation (LF, HF and slurry treatments) and temperature affected the pattern of community shift (Avrahami *et al.* 2003). In a further study, Avrahmi *et al*. (2003) reported that in soil incubation studies *Nitrosospira* cluster 1 was initially present in the LF treatment, but disappeared completely after a long period of incubation (> 16 weeks). The disappearance of *Nitrosospira* cluster 1 was probably due to the inability of this nitrifier to grow below a certain threshold of NH_4^+ concentration. In the HF treatment, *Nitrosospira* cluster 1 became more dominant with time. On the other hand, *Nitrosospira* cluster 3 was detected in the LF, HF and slurry treatments indicating that this nitrifier is not necessarily present at only high NH_4^+ concentrations, as suggested in the previous field studies (Bruns *et al.* 1999; Kowalchuck *et al.* 2000a, 2000b). Avrahami *et al.* (2003) also showed the presence of *Nitrosospira* cluster 9 could only be detected in the soil incubated with low NH_4^+ concentrations.

As mentioned above, *Nitrosomonas* species have also been found in both fertilised and unfertilised soils with neutral pH. For example, *Nitrosomonas* cluster 7 was reported in both improved (with addition of N fertiliser) and unimproved (no additions of N fertiliser) soils in grassland pastures in Scotland (Webster *et al.* 2002). In Italian

agricultural soils that had been fertilised with swine manure, members of the genus of *Nitrosospira* were found in both unamended and amended soils, regardless of the quantity of pig slurry applied. In contrast, *Nitrosomonas europaea* (cluster 7) were detected only in the soil plots that had received high loadings of slurry and could not be detected in soil from the untreated plot (Hastings *et al.* 1997). But Ceccherini *et al.* (1998) detected both *Nitrosospira* and *Nitrosomonas* species in treated (with pig slurry) and untreated plots, although the genus *Nitrosospira* was more abundant than *Nitrosomonas*. Members of *Nitrosomonas communis* (Cluster 8) have also been isolated from agricultural soils with a neutral pH (Koops and Pommerening-Röser 2001).

One study has investigated the effect of water limitations on AOB numbers and their diversity in an acid forest soil (Hastings *et al.* 2000). The effect of water limitation (drought) and rewetting of three different organic layers of forest soil (litter (top layer), fermentation layer (middle layer) and humus layer (bottom layer)) was investigated. In this study a simulated drought regime in the field was imposed for 10 weeks by placing a transparent polyvinylchloride (PVC) roof below the forest canopy to exclude rainfall but allowing sunlight penetration to the underlying soil. Samples of litter, fermentation and humus layers to a depth of 10 cm were collected manually from the experimental site before the PVC roof was placed, to act as controls. After the 10 weeks of drought simulation, the PVC roof was removed, exposing the underlying soil to the rain. Rainfall occurred a few days after exposure and rehydrated soils were sampled 18 days later. These are referred to here as the rewetted samples. A decrease in AOB numbers was observed in the litter, fermentation and humus layers during the drought period, but the numbers of AOB increased after rewetting the layers. Species of *Nitrosospira* were present in nearly all the soil layers in each of the control, drought and rewetted

treatments, suggesting that they predominated over *Nitrosomonas* species in that acid forest soil - a finding common to other studies of *Nitrosospira* in acid soils (Nugroho *et al.* 2005; Stephen *et al.* 1996, 1998). In contrast, *Nitrosomonas europaea-eutropha lineages* (Cluster 7) were only detected in the control fermentation layer and in all three soil layers after rewetting. Their results suggested that the litter and humus layers of soil did contain populations of *Nitrosomonas europaea-eutropha lineages* that were not detectable during the drought, but became active after stimulation by the re-wetting.

Avrahami *et al.* (2003) reported that temperature was another factor that affects the community structure of AOB. In their LF, HF and slurry treatments, *Nitrosospira* clusters 3 and 9 were dominant at 25-30^{o}C and, *Nitrosospira* cluster 1 was dominant at low temperatures (4-10^{o}C) in the HF treatment and slurry but had completely disappeared at high temperature (30^{o}C). Overall, they concluded that shifts in AOB communities occurred only after a long period of incubation (> 16 weeks). These community shifts were affected by different fertiliser treatments and incubation temperatures. The observed community shifts were mostly within the different phylogenetic clusters of *Nitrosospira*.

2.2.1.2 Ammonia oxidising bacteria as an indicator of environmental quality

Ammonia oxidising bacteria (AOB) have been extensively studied and have been suggested as model organisms in microbial ecology (Kowalchuck and Stephen 2001). It has also been demonstrated that the activity of AOB communities can be studied as part of an integrated approach to assess soil health and they are often used as an indicator in

studies of soil perturbations and soil toxicity (Chang *et al.* 2001; Nielsen *et al.* 2004; Nyberg *et al.* 2006; Remde and Hund 1994).

As already detailed in the previous sections, communities of AOB have been isolated and studied in a wide variety of environments such as agricultural soils, forest soils, grasslands, marine and freshwater environments and waste water treatment systems. The advantage of using AOB as an indicator is that they are known as a monophyletic group of bacteria that are the major performers within their functional guild. Thus, if a pollutant has negative effects on AOB it is likely to be detected as a reduced NH_3 oxidation rate (Nyberg *et al.* 2006). Although AOB represent only a small fraction of the microbial biomass in soil, their sensitivity to different pollutants and their importance in N cycling has resulted in AOB having frequently been used as indicator organisms for disturbances caused by herbicides and metals, or effects due to agronomic practice (Hastings *et al.* 1997; Oved *et al.* 2001; Phillips *et al.* 2000; Stephen *et al.* 1999).

2.2.2 Heterotrophic nitrifiers

2.2.2.1 Nitrifiers involved in heterotrophic nitrification

The assumption that nitrification in soils was carried out by autotrophic AOB was challenged when it was first demonstrated that some heterotrophs could also carry out the oxidation of NH_3 to NO_2^- and NO_3^- (Eylar and Schmidt 1959). At that time, the main factor favouring heterotrophic nitrification was thought to be an acidic soil environment.

Heterotrophic nitrification is defined as the oxidation of NH_3 or NH_4^+ or of organically bound N of the oxidation state -3 to hydroxylamine, NO_2^- and NO_3^- by heterotrophs under aerobic conditions (Papen and Berg 1998). More details of these processes are given in Section 2.2.2.2. In contrast with autotrophic nitrification, the process of heterotrophic nitrification is not coupled to energy generation. The growth of heterotrophic nitrifying organisms is completely dependent on the oxidation of an organic substrate for both carbon and energy sources (Focht and Verstraete 1977).

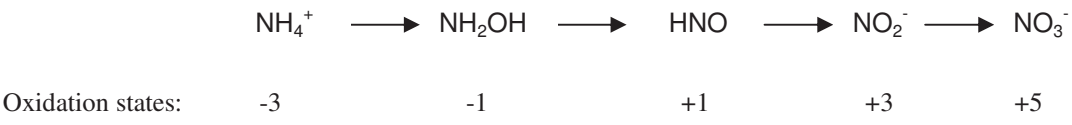
Heterotrophic nitrifiers include various species of prokaryotes and eukaryotes including bacteria, fungi, algae and animal cells (Sprent 1990). From pure laboratory culture studies of all the heterotrophic nitrifiers in soil, the fungi are considered the most dominant and most efficient (Eylar and Schmidt 1959; Odu and Adeoye 1970; Schimel *et al.* 1984) - although bacteria (Nelson 1929) and actinomycetes (Remacle 1977) have also been identified as potential nitrifiers in the soil. Earlier work carried out by Eylar and Schmidt (1959) on screening 751 soil fungi for their ability to nitrifiy identified three species. *A. flavus* was the most active nitrifier in culture, while *Penicillium* spp. and a *Cephalosporium* sp. produced low concentrations of NO_3^-.

Since then, many researchers have isolated fungi such as *Motrierella pulchella* Linnem. and *Mucor hiemalis* (Johnsrud 1978), *Veticillium lecanii* (Lang and Jagnow 1986), *Penicillium* spp (Stams *et al.* 1990) and bacteria identified as *Micromonospora* sp (Johnsrud 1978), mainly from acid forest soils, that were responsible for heterotrophic nitrification. Tate (1977) isolated from an organic soil bacteria such as *Arthrobacter* sp. that were capable of producing NO_2^- and NO_3^-. One study (Stroo *et al.* 1986) has reported the presence in an acid forest soil of an acid-tolerant fungus called *Absidia*

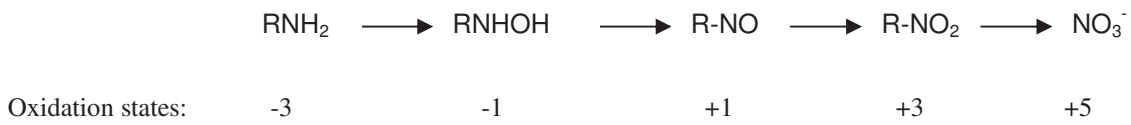
cyclidrospora, from which the isolates were able to nitrify rapidly at pHs between 4 and 5.

2.2.2.2 The biochemistry of heterotrophic nitrification

There is evidence of two pathways of heterotrophic nitrification ammonia oxidation (Paul and Clark 2007). The first pathway is similar to that of autotrophic oxidation, in which the heterotrophs have similar ammonia- and hydroxylamine- oxidising enzymes. The heterotrophs are capable of producing NO_3^- from inorganic N sources with the following intermediates and associated oxidation states:



There is also some evidence of an organic pathway for heterotrophic nitrification that appears limited to fungi (Paul and Clark 2007) and that involves oxidation of amine or amides to a substituted hydroxylamine, followed by oxidation to nitroso- and to nitro-compounds and finally produces NO_3^-:



In contrast with autotrophic nitrification, these two pathways are not coupled to ATP, and therefore do not produce energy. To date there are few clues as to why heterotrophs carry out this non-ATP coupled nitrification. It may be that nitrification confers some competitive advantage on the heterotrophs. Verstraete (1975) suggested that certain heterotrophic nitrification by-products are known to be toxic and mutagenic which might inhibit many forms of competition to the nitrifier in the soil environment. He

also suggested that a key role of heterotrophic nitrification may lie in the production of hydroxamic acid intermediates which have been identified as microbial growth factors as well as important agents in microbial uptake of iron (Waid 1975). Acetylene does not inhibit heterotrophic nitrification, and therefore provides a means for differentiating autotrophic from heterotrophic nitrification in soil (Hynes and Knowles 1982; Pedersen *et al.* 1999).

As mentioned earlier, heterotrophs can use inorganic or organic energy sources to perform nitrification. In other studies, Honda *et al*. (1998) and de Boer and Kowalchuck (2001) found that heterotrophs can use both organic and inorganic N compounds for nitrification, but these findings were not in agreement with the recent work carried out by Islam *et al*. (2007). In their (Islam *et al.* 2007) incubation experiment using ^{15}N-labeling techniques to measure heterotrophic and autotrophic nitrification rates in two acid pasture soils with pH ranges from 4.8-5.3, heterotrophic nitrifiers used organic N compounds as a substrate for nitrification and did not use NH_4^+-N. Their results were in accordance with the observations made by Schimel *et al*. (1984) that NH_4^+ was not an important substrate for heterotrophic nitrification and that unidentified organic-N compounds were the main N source.

2.2.3 Is nitrification in soils caused by autotrophic or heterotrophic nitrifiers?

In soil, nitrification is most rapid in neutral or slightly alkaline conditions and at those pHs was assumed to be mostly carried out by autotrophic AOB (Belser 1979; Belser and Schmidt 1978). This was supported by studies in pure culture that found the pH for optimum growth of AOB nitrifiers to be in the range of 6.5 to 8.5 and typically growth

did not occur below a pH of 6.5 (Allison and Prosser, 1993). Even more recent work by Burton and Prosser (2001) found that growth of *Nitrosospira* was possible at pH 7 but not at pH 6.2 or lower.

It was therefore suggested that perhaps active nitrification under acid conditions was the result of heterotrophic nitrification (Lang and Jagnow 1986; Papen and Berg 1998; Stroo *et al.* 1986). These researchers suggested that the NO_3^- production in acid forest soils was unlikely to be catalysed by autotrophic AOB as no AOB could be isolated from this soil, while fungi and heterotrophic bacteria were isolated and were able to nitrify in such soil, although their specific nitrifying activity was low.

Despite this however, many studies have reported that nitrification by autotrophic AOB can in fact occur in a wide range of acid soils with pH values as low as 3.0: including tea plantations (Walker and Wickramasinghe 1979), heathlands (de Boer *et al.* 1988), grasslands (Bramley and White 1990; Islam *et al.* 2007) and forest soils (Bottomley *et al.* 2004; Nugroho *et al.* 2005). Bhuiya and Walker (1977) detected autotrophic AOB in several moderately acid (pH range from 4.0 to 6.2) soils from Bangladesh and Sri Lankan tea estates. Their pure culture isolates revealed the presence of members of β-*Proteobacteria* identified as *Nitrosolobus*, *Nitrosomonas* and *Nitrosospira*. Walker and Wickramasinghe (1979) then isolated AOB from several soils from the same countries, which were more acidic (pH range from 4.0-4.5). The pure culture isolates demonstrated the presence of *Nitrosospira* in the Bangladesh soils, while *Nitrosolobus*, *Nitrosospira* and *Nitrosovibrio* were present in the Sri Lankan soils. Further studies by de Boer *et al.* (1989a) showed that additions of nitrapyrin (NP) to the suspensions of fertilised acid heath soils supplied with urea completely inhibited the nitrification,

indicating that autotrophic nitrifiers were the main contributors to nitrification in the low pH soil. A similar result was obtained by de Boer *et al.* (1991). In this study they demonstrated that NO_3^- production in the enrichment cultures was likely to be by autotrophic AOB as it was completely inhibited by acetylene at a concentration as low as 1 µmol/litre, and was strongly retarded under conditions of CO_2 limitation (in this experiment the presence of AOB was tested via NO_3^- production in the subcultures for its sensitivity to limitation of CO_2. The depletion of CO_2 was carried out via absorption by NaOH. The limited availability of CO_2 had a pronounced delaying effect on NO_3^- production, showing a direct dependency of the AOB on the availability of CO_2). de Boer *et al*. (1992) have further shown that AOB were adapted to the low pH of acid heathland soils (pH 4.0) and were responsible for active nitrification. In another study, Pennington and Ellis (1993) observed that nitrification occurred at pH as low as 3.4 and concluded that acidophilic autotrophic AOB rather than heterotrophs were responsible for the nitrification in both acidic forest and grassland soils.

Stams *et al*. (1990) studied the role of autotrophic and heterotrophic nitrifiers in the oxidation of atmospheric NH_4^+ in two acid and one calcareous soil of a Dutch forest. They showed that both autotrophic NH_4^+- and NO_2^- oxidisers were present in high numbers and that these nitrifiers were able to nitrify at pH values below 4. Heterotrophic nitrifiers were also present in the two acid soils. To assess the contribution of the heterotrophic nitrifiers in these two acids soils, about 200 strains of heterotrophic bacteria were isolated from the acid soils and tested for their ability to form NO_2^- and NO_3^- in the test media with either with NH_4^+ or yeast extract-peptone as the N source. None of the strains appeared to produce significant amounts of NO_2^- and NO_3^- after the two weeks of incubation. Based on phenotypic appearance, 23 different

fungal strains were also isolated from the acid soils and were further tested for their ability to nitrify. Only one fungus (*Penicillium*) isolated from one of the acid soils was able to form NO_3^- in a glucose-NH_4^+ medium with initial pH values of as low as 3.3. In the same study, Stams *et al*. (1990) carried out soil slurry incubations in the presence and absence of acetylene to elucidate the contribution of autotrophic and heterotrophic nitrifiers to the forest soils' nitrification activity. They showed that the addition of acetylene completely inhibited the NO_3^- formation. Overall, they concluded that heterotrophs were of minor importance in the nitrification activity in the acid forest soil and autotrophic AOB were the main nitrifying organisms involved in the nitrification.

Analysis of autotrophic AOB populations by sequencing their 16S rDNA and *amoA* genes has similarly supported the importance of autotrophic AOB in nitrification in acid soils. For example *Nitrosospira* clusters 1, 2 and 4 are more prevalent in soils with low pH, such as forest and agricultural soils (Nugroho *et al.* 2005; Stephen *et al.* 1996, 1998), soils with low pH that have been retired from agriculture use (Kowalchuck *et al.* 2000a, 2000b) and soils that have never been exposed to tillage and/or application of N fertiliser (Bruns *et al.* 1999; Webster *et al.* 2002). The effect of pH on nitrification and the mechanisms that allow autotrophic nitrification in acid soil will be discussed in more detail in Section 2.4.2.1.1.

Very recently, the assumption of AOB being the main contributor to nitrification activity in soils has been questioned. Recently, a role for ammonia oxidising archaea (AOA) in ammonia oxidation was identified (Leininger *et al.* 2006). Several studies have shown that AOA are present abundantly in natural environments such as the water column and ocean sediments (Beman and Francis 2006; Francis *et al.* 2005), activated

sludge bioreactors (Park *et al.* 2006) and soils (Adair and Schwartz 2008; Herrman *et al.* 2008; Ji-zheng *et al.* 2007; Leininger *et al.* 2006). It has also been reported that AOA exist in three New Zealand soils tested by Bowatte *et al*. (2009). More recently Di *et al*. (2010) investigated the presence of AOB and AOA and their nitrification activities in two different layers (topsoils and subsoils) of three grassland New Zealand soils treated with animal urine applied with and without DCD. These authors suggested that AOA may favour low NH_3 substrate conditions (low fertility soils), while AOB are abundant in high NH_3 substrate conditions (high fertility soils). The AOB population increased substantially when supplied with high doses of urine in all three topsoils, while AOA only grew in the controls without the urine-N substrate. The addition of DCD significantly reduced the numbers of AOB and AOA. Their results also showed that nitrification rates were higher in the topsoils than in the subsoils and were significantly related to AOB abundance, but not to AOA abundance.

In another study on agricultural soils in China, it was reported that AOA contribute to soil nitrification, but that AOB were still the dominant microorganisms catalysing the soil nitrification activity (Jia and Conrad 2009; Jia 2010). How widespread AOA are in agricultural, pastoral and forest soils and how much they contribute to the overall soil nitrification activity still remain unknown. What soil conditions favour these nitrifiers and whether AOA are sensitive to nitrification inhibitors such as DCD still need to be further investigated.

2.3 The potential effects of nitrification

The nitrifiers that are responsible for nitrification can adversely effect the environment and indirectly pose a risk to plant, human and animal health. Nitrification of NH_4^+ is

known to cause soil acidification and soil acidity is enhanced with the application of NH_4^+-based N fertilisers such as ammonium nitrate, ammonium sulphate and urea (Biederbeck *et al.* 1996; Black 1992; Bouman *et al.* 1995; Bolan *et al.* 1991; He *et al.* 1999; Neilsen *et al.* 1994; Parchomchuck *et al.* 1993; Tachibana *et al.* 1995).

The impact of the nitrification process on soil chemical properties consists of two components. These include the acidifying effect from the internal ion hydrogen (H^+) release through nitrification of NH_4^+ and the acceleration of cation leaching associated with the mobile NO_3^- (Bouman *et al.* 1995; Neilsen *et al.* 1994).

The extent to which percolating solutions are acidified by this nitrification process is depending on the rate of such H^+ release relative to the rate of H^+ neutralisation, which frequently involves the cation exchange reactions (McFee *et al.* 1977; Wiklander 1980). As the H^+ move through the soil profile, they can gradually displace nutrient bases from the cation exchange complex, thus resulting a drop in soil pH and base saturation. The cations displaced from the exchange complex can be either be taken up by plants, by micoroorganims or be carried further down as counter-ions to mobile NO_3^- (Kinjo and Pratt 1971) which can be easily leached below the plant root zone when not immobilised biologically (Vitousek and Melillo 1979). Thus the nitrification process has the potential to acidify the soil and/soil solution while removing from the system some of its exchangeable bases. This in turn can increase the risk of metals such as aluminium and manganese being released which can damage the plant root system and shoot growth (Desmond 1993; Delhaize and Ryan 1995). At the same time the NO_3^- can increases the NO_3^- concentrations in the groundwater and potentially in freshwater (Addiscott and Benjamin 2004; Eriksen *et al.* 1999) which may stimulate the growth of

aquatic algae and lead to eutrophication (Pierzynski 1994). This can create anoxic conditions in the water when the dead plant material decomposes, causing other organisms to die and therefore leading to a decrease in biodiversity (Kowalchuck and Stephen 2001).

High concentrations of NO_3^- in drinking water (ranging from 40-100 mg NO_3^--N L^{-1}) can be toxic and are suspected of causing "blue baby syndrome" (methaemoglobinaemia; MHb) (Addiscott and Benjamin 2004; Gupta *et al.* 2000). Infants and people aged above 45 years are most susceptible to NO_3^- toxicity. Concerns about human cancer due to NO_3^- toxicity have also been reported (Gupta *et al.* 2000; Umar and Iqbal 2006).

The production and emission of N_2O through nitrification and denitrification processes (Williams *et al.* 1992; Wrage *et al.* 2001) has contributed significantly to global warming and destruction of the ozone layer in the stratosphere (Skiba *et al.* 1997). High rates of nitrification may intensify problems resulting from acid rain. It has been reported that the production of nitric acid as a result of the microbial transformation of nitrogeneous compounds has caused deterioration in natural stone used as a building material and in historical monuments (Meincke *et al.* 1989; Spieck *et al.* 1992). Thus, a high nitrification rate can have several environmental drawbacks, especially in agricultural environments with large inputs of N fertilisers.

2.4 Factors that control nitrification in soils

The obvious prerequisite for nitrification to take place in soil is the presence of a nitrifying population together with the availability of NH_4^+ substrate (Sahrawat 2008;

Subbarao *et al.* 2006). However, because the nitrifiers involved in nitrification are quite specialised, the amounts of NO_3^- formed and the nitrification rates in soils are usually variable, and are influenced by a combination of many environmental and chemical factors (Kowalchuck and Stephen 2001; Sahrawat 2008). These factors are discussed below.

2.4.1 Environmental factors

2.4.1.1 Soil moisture and aeration

Nitrifiers are sensitive to soil moisture and aeration (Havlin *et al.* 2005; Killham 1990; Rajendra Prasad and Power 1997). Both soil water content and rate of oxygen (O_2) consumption determine the O_2 availability (Tiedge 1988) and therefore the interactions between moisture and O_2 supply in the soil matrix have a major influence on nitrification. Both too much and too little soil moisture may limit the activity of nitrifiers and influence the rate of nitrification. High levels of soil moisture create anaerobic conditions, thereby inhibiting the aerobic processes of N mineralisation and nitrification (Ohte *et al.* 1997).

Oxygen affects nitrification activity through its roles as a substrate for the AMO enzymes and as the terminal electron from cytochrome *c* oxidases (Arp *et. al.* 2002). The availability of O_2 is controlled by the interaction of O_2 consumption and diffusion from the surface through the air-filled pores. Sufficient O_2 diffuses into most soils that are at field capacity to maintain nitrification, although microsites lacking O_2 may frequently occur inside the soil aggregates (Sexstone *et al.* 1985). In soils that remain wetter than field capacity for several days, nitrification rates generally decline. In three arable soils with increasing clay contents at 108, 224 and 337 g clay kg^{-1} soil, maximum

net nitrification was observed at -14, -17 and -43 kPa water potential respectively (Schjønning *et al*., 2003), with rates decreasing at higher water potentials presumably due to O_2 depletion. In earlier work by Saby (1969) on the influence of moisture tension on NO_3^- accumulation in silt loam soils, a maximum rate for net nitrification occurred at approximately -10 kPa. Nitrification rates are generally highest at field capacity (Havlin *et al.* 2005; Myers *et al.* 1982).

In soils at field capacity and drier, water availability impacts nitrification rates through its direct effect on cell physiology and metabolic activity and through indirect effects on substrate availability (Stark and Firestone 1995). They suggested that restricted diffusion of NO_3^- substrate and adverse physiological effects associated with cell dehydration can explain the decline in the activity of nitrifiers at low moisture content. In soil from a California oak woodland-annual grassland ecosystem, the relative importance of these two factors shifted over the range of water potentials; substrate diffusional limitation was the dominating factor at water potential wetter than -0.6 MPa, while effects due to dehydration and its physiological impacts were more pronounced in soils drier than -0.6 MPa (Stark and Firestone 1995). As discussed in Section 2.1.1.1.5, moisture limitation decreased the numbers of AOB and their diversity in a forest soil. Singh and Kashyap (2006) found the highest nitrification rates and the greatest number of nitrifiers were obtained in soils during the rainy season and were lowest during the drier summer season. The significant increase in the size of the nitrifier population during the wet season may also be related in part to nutrient (NH_4^+) release as a result of a concurrent increase in N-mineralisation with the onset of rain.

2.4.1.2 Soil temperature

Temperature is one of the most important factors that influence nitrifier populations (Belser 1979). The optimum temperatures for nitrifiers in pure cultures (Focht and Verstraete 1977) and soils (Mahendrappa *et al.* 1966; Myers 1975) range from 25 to 35 ^{o}C, but nitrification activity has also been observed at temperatures as low as 0^{o}C (Focht and Verstraete 1977).

In Section 2.1.1.1.5 it was reported that work carried out by Avrahmi *et al.* (2003) showed that temperature also affects the community structure of AOB. The effect of temperature on the community structure of AOB was further investigated in three different meadow soils (Avrahmi and Conrad 2003). Two of the soils (OMS and GMS) were obtained from Germany with pH 5 to 5.8, while the KMS soil was obtained from Israel with slightly alkaline pH (7.9). The effect of temperature (4 to 37^{o}C) was investigated by incubation of the soils for up to 20 weeks in a buffered slurry containing urea and in moist soil treated with fertiliser. Two patterns of community change due to temperature were observed in this study.

The first pattern was observed in the KMS soil and showed a community shift predominantly within a single *Nitrosospira* cluster with temperature (4 to 37^{o}C). The soil slurries showed similar trends but exhibited a lower diversity compared to the incubation experiments with moist soils. Although the sequences of the individual DGGE bands exhibited different trends with temperature, they were all grouped within *Nitrosospira* cluster 3a. *Nitrospsira* sp. strain B6 was detected at low and intermediate temperatures in moist soil samples but not in the slurry. The second pattern of community change due to temperature was observed in moist and slurry incubations of

OMS and GMS soils, and the change was between different *Nitrosospira* clusters. *Nitrosospira* cluster 1 was mainly detected below 30°C, while *Nitrosospira* cluster 4 was predominant at 25°C. *Nitrosospira* cluster 3a, 3b and 9 dominated at 30°C.

2.4.2 Chemical factors

Nitrification in soil is influenced by the soil pH and the availability of NH_4^+ ions to the population of nitrifying organisms (Sahrawat 2008; Subbarao *et al.* 2006).

2.4.2.1 Soil pH

As already mentioned in Section 2.2.3, nitrification in soils is most rapid in neutral or slightly alkaline conditions. Liming and liming plus N amendment have been shown to increase nitrification in soils that were known to have low nitrification rates (Nugroho *et al.* 2007).

2.4.2.1.1 Mechanisms of autotrophic nitrification at low pH

In Section 2.2.3, it was pointed out that growth of pure cultures of autotrophic AOB in liquid culture were optimal within the pH range 7.0-8.5 and typically did not occur below pH < 6.5 (Allison and Prosser, 1993; Burton and Prosser 2001). However, many studies have confirmed the contribution of autotrophic AOB to nitrification in acidic soil with pH values as low as 3.0 (Bhuiya and Walker, 1977; de Boer *et al.* 1991; de Boer et al 1992; Kowalchuck *et al.* 2000a, 2000b; Nugroho *et al.* 2005; Pennington and Ellis, 1993; Stephen *et al.* 1996, 1998; Walker and Wickramasinghe, 1979).

Given this apparent inconsistency, one of the prevailing mechanisms explaining autotrophic nitrification in acid soils is the ability of many AOB to hydrolyse urea

(Allison and Prosser 1991; Burton and Prosser 2001; de Boer *et al.* 1989a). Urea can be taken up by cells at low pH through diffusion and then hydrolysed intracellularly to NH_3 (Burton and Prosser 2001). Autotrophic AOB are incapable of growing on organic substrates. However, many species of AOB, but not all, are able to hydrolyse urea to NH_3 and use the NH_3 as their sole energy source. This group of AOB are described as urease positive, while the group that is incapable of hydrolysing urea are described as urease negative AOB (Koops and Pommerening-Röser 2001). Urease positive and negative strains exist in both *Nitrosospira* (Koops and Pommerening-Röser 2001) and *Nitrosomonas* clusters (Koops *et al.* 2006).

Burton and Prosser (2001) demonstrated the inability of pure cultures of AOB (*Nitrosospira* strains) to grow in a medium containing NH_4^+ at pH < 6.2, but growth of AOB did occur at pH 7. However, growth of AOB on a medium containing urea occurred at pH values in the range of 4 to 7.5, but ceased when urea hydrolysis was complete, even though NH_3 released during the hydrolysis remained in the medium. There were two reasons given by the author for these observations.

Firstly, nitrification in pure cultures of AOB rarely occurs at pH less than 7 because of ionisation of NH_3 to NH_4^+ and the consequent energy requirement for active transport of NH_4^+ by the AOB, rather than diffusion of NH_3. Secondly, growth of AOB on a urea medium occurred at pH values as low as 4 due to the mechanism of urea-linked ammonia oxidation, whereby urea enters the cells by diffusion and is then hydrolysed intracellularly to NH_3. The AOB then use this NH_3 a source of energy and nitrification occurs independently of the external pH in the range of 4 to 7.5. They therefore concluded that ureolysis provides a mechanism for nitrification in acid soils. Similar

findings were reported by de Boer *et al.* (1989a) and Allison and Prosser (1991) in which ammonium oxidisers were able to hydrolyse urea at low pH and therefore allow autotrophic nitrification in acid soils.

A further explanation for autotrophic nitrification at low pH might be the existence of acid-sensitive (de Boer *et al.* 1989b) or acid-tolerant AOB (de Boer *et al.* 1989b; de Boer *et al.* 1991; de Boer and Kowalchuck 2001; Hayatsu 1993). These two types of nitrifiers and their different nitrifying capacities were demonstrated in the study carried out by de Boer *et al*. (1989b) in the organic horizons of two heathland soils of the Netherlands. In this study they observed that there were two types of autotrophic nitrification in the acid heathland humus.

The first type involved acid sensitive nitrifiers that appeared to be more dominant in slow-NO_3^--producing humus, and the NO_3^- production at low pH could be stimulated by urea. It has also been proposed that autotrophic nitrification by acid-sensitive nitrifiers in acid soil might be restricted to microsites of alkaline pH (Hankinson and Schmidt 1984; Overrein 1967; Prosser 1989). The occurrence of microsites enabling acid-sensitive nitrification in the upper organic layers of acid soils has been attributed to the relatively high pH of water falling through the tree canopy and the presence of hot-spots of ammonification (Overrein 1967; Stams *et al.* 1990). de Boer *et al.* (1988) proposed that nitrification occurring in acid heath soils by AOB was due to the coupling of autotrophic nitrification and net N mineralisation - implying ammonifying microorganisms and AOB nitrifiers are closely associated. This association was thought to be achieved by liberation of NH_3 which induces pH favourable for nitrification close to the ammonifying organisms.

The second type of AOB nitrifiers in acid heathland humus were acid-tolerant nitrifiers, which were present in rapid-NO_3^--producing humus and the nitrification activity was not stimulated by urea (de Boer *et al.* 1989b). It is not clear however, whether acid-tolerant AOB comprise different species or whether they are acid-sensitive strains that in some way have become adapted to low pH conditions. de Boer *et al.* (1995) showed that an acid-sensitive *Nitrosospira* strain isolated from acid heathland soil could adapt to grow and nitrify at pH 4.

Another experiment was carried out by de Boer *et al*. (1990) on the distribution of acid-sensitive and acid-tolerant nitrifiers in 41 humus samples from Dutch heathland with pHs ranging from 3.5 to 4.6. The sensitivity of the nitrifiers towards acidity was determined by comparing the production of NO_3^- in 5% suspensions that were maintained at pH 4 and pH 6. From this experiment, four patterns of results were observed.

Firstly, there was no NO_3^- production at either pH 4 or pH 6 in some soils. Secondly, three soil samples showed NO_3^- production at pH 6 but not at pH 4, suggesting the presence of acid-sensitive AOB. Thirdly, in some humus samples NO_3^- production occurred at both pH 6 and pH 4, but the production of NO_3^- at pH 6 was at least 1.5 times faster than at pH 4. The difference in response to a pH-rise may be due to the presence of predominantly acid-tolerant AOB which were pH-dependent. The final category of soils produced NO_3^- at the same rate at both pH 6 and pH 4. This suggested the presence of acid-tolerant AOB which were pH-independent. They concluded that acid-tolerant AOB are widespread in the Dutch heathland soils. Although there was also

nitrification contributed from acid-sensitive AOB, it was limited to only small numbers of soil samples.

As already pointed out above, the occurrence of autotrophic nitrification in acid soils by acid-tolerant autotrophic nitrifiers can perhaps be partly explained by the adaptation of the indigenous nitrifier population to the prevailing soil pH and thereby enabling nitrification at low pH without the need for special NH_3-generating mechanisms. Hayatsu (1993) concluded that the nitrification activity in acid soils from a tea field in Japan was due to the presence of acid-tolerant nitrifiers that had become adapted to the acidic conditions. This conclusion was made from a study carried out to evaluate the effect of acidity on nitrification with the addition of various amounts of $CaCO_3$ to two types of strongly acid tea soils, namely a "Red and yellow soil" and an Andosol soil. Their results showed that both soils had very high nitrification activity despite the low pHs of 3.6 and 3.5 respectively. However, when the pHs of both soils were increased with the addition of $CaCO_3$, to moderately less acidic pHs of 5.3 and 5.4 and to near neutral pHs of pH 6.6 and 7.7, the nitrification activity did not increase significantly.

2.4.2.2 Organic matter and C:N ratio

In many ecosystems, the availability of NH_4^+-N controls the activity of nitrifying bacteria (Martikainen 1985; Mendum *et al.* 1999). The availability of substrate can be limited by protection of N from microbial attack through sorption or fixation of organic N and NH_4^+ by soil clay minerals and immobilisation of NH_4^+. Where decomposition and thus N mineralisation is low, or where NH_4^+ uptake and thus N-immobilisation by heterotrophs or plants is high, nitrification rates will be low (Robertson and Groffman 2007). A high C:N ratio usually leads to immobilisation of NH_4^+-N, thus suppressing

nitrification activity (Focht and Verstraete 1977). Most arable soils have a C:N ratio of about 10, at which immobilisation of NH_4^+-N is limited and nitrification proceeds at a normal rate (Vitousek *et al.* 2002). In contrast, lower nitrification rates have been obtained in forest soils with low atmospheric N deposition, high C:N ratios and with initially low NO_3^--N concentrations (Nugroho *et al.* 2005).

2.4.3 Allelopathy

Several researchers have suggested that allelopathy, or the release from plant roots of inhibitory compounds such as tannins, phenolic acids and phenolic glycosides, can suppress nitrifier activity in mature grassland ecosystems (Lata *et al.* 1999, 2004; Munro 1966; Rice and Pancholy 1973).

Natural grasslands dominated by *Brachiaria humidicola* have low NO_3^--N concentrations and the nitrifier populations have been reported to be generally lower than in soils under grasslands dominated by other *Brachiaria* species such as *B. brizantha* and *B. desumbens* or *Andropogon gayanus* (Slyvester-Bradley *et al.* 1988). Legume pastures in the same study had high nitrification rates and the nitrifier populations were several-fold greater than in the pastures containing *B. humidicola.* The addition of fertilisers to *B. humidicola* pasture did not result in any NO_3^--N accumulation and as no immobilisation of NH_4^+-N was observed this suggested that *B. humidicola* may have the ability to suppress nitrification in soils by releasing inhibitory compounds from the root. Further work on isolating the inhibitory compounds from the root tissue of *B. humidicola* confirmed that two methylated phenolic acids (methyl-p-coumarate and methyl ferulate) were produced from the *B. humidicola* root and these had the characteristic of inhibiting nitrification (Gopalakrishnan and Subbarao 2007).

Another grass that exhibits an inhibitory effect on soil nitrification is *Hyparrhenia diplandra* (Lata *et al.* 2004). In their study the transplantation of individual *H. diplandra* originating from high (HN) and low (LN) nitrifying sites demonstrated a clear plant effect on nitrification (Lata *et al.* 2004). The LN plants decreased nitrifying enzyme activity (NEA) in the HN site to values found in the LN site, whereas HN plants restored the NEA in the LN site to values found in the HN site.

2.4.4 Population of nitrifying organisms

As already mentioned in Section 2.1.1.1.5, land use can influence the diversity of AOB via changes in NH_4^+-N availability from adding N fertiliser (Chu *et al.* 2007; Oved *et al.* 2001) or through changes in soil characteristics that influence rates of N cycling. These include soil pH (Kowalchuck *et al.* 2000a, 2000b), soil moisture (Hastings *et al.* 2000) and temperature (Avrahami *et al.* 2003; Avrahmi and Conrad, 2003).

In addition, N fertilisation, soil liming and soil water content have been demonstrated to increase the size of the nitrifier population as indicated by cell counts (most probable number (MPN), competitive polymerase chain reaction (cPCR) and real time PCR) and nitrification potential measurements (Chu *et al.* 2007; Chu *et al.* 2008; Hastings *et al.* 2000; Hermansson and Lindgren 2001; Jha *et al.* 1996a, 1996b; Martikainen 1985; Mendum *et al.* 1999; Singh and Kashyap 2006). In a laboratory experiment, the population of AOB was larger and the rate of NO_3^--N production was higher at a pH of 7.5 compared to when the pH was lowered to 5.5 (Sarathchandra 1978a). Krave *et al*. (2002) in their study on potential nitrification and the factors controlling nitrifying activity in tropical pine forest and agricultural soils in Central Java, Indonesia reported that fertilised coffee plantation soils had higher potential nitrification rates than the pine

forest soils and these results correlated with higher MPN counts of AOB obtained in the coffee plantation soils. They also showed that soil moisture content, pH and NH_4^+-N availability were the factors best correlated with nitrification potential and the numbers of AOB.

2.5 Methods used to estimate the numbers of nitrifiers

The most probable number (MPN) technique has commonly been used as a means to provide an indirect statistical estimate of the number of cells involved in particular oxidative steps (Deni and Penninckx 1999; Ghosh and Dhyani 2005; Johnson *et al.* 2005; Krave *et al.* 2002; Martikainen 1985; Meiklejohn 1968; Sarathchandra 1979; Singh and Kashyap 2007; Yuan *et al.* 2005).

In the MPN technique, one can only enumerate culturable AOB. Generally, the nitrifying bacteria that are active in soils are firstly extracted with phosphate buffer and then serially diluted. These dilutions are then used to inoculate 3, 4, 5 or 10 culture tubes of sterilised growth medium containing ammonium as the sole energy source. After prolonged incubation at 25-30^{o}C, the dilutions are screened for ammonium oxidation (Schmidt and Belser 1994). A positive result shows at least one organism capable of growth was present in that sample. The range of probable AOB numbers can be calculated based upon assumptions that the cells are randomly distributed in the initial and subsequent dilutions and that the single nitrifying cell is capable of growing, multiplying and nitrifying and thereby producing a positive result (Cochran, 1950).

There are two requirements to achieve high counting efficiency in the MPN technique (Belser 1979; Tate 2000). Firstly, the living cells extracted from the soil must be

sufficiently separated from any particulate matter, that each cell will be individually and independently dispersed in the phosphate buffer or diluent. Secondly, the growth media and growth conditions used must able to allow the extracted active living cells present in the soil sample to grow and multiply to a population density that can be detected. However, it has been reported that no MPN technique meets either of these requirement and therefore populations are often underestimated (Tate 2000).

All media are selective and one parameter that could cause this selectivity is the pH. Belser (1977) reported that media used at pH 7.1 were more effective in counting NO_2^--oxidisers than media at pH 8.3. Further work reported by Belser and Schmidt (1978) confirmed that maximal counts of NH_4^+-oxidisers depended on the media used in the one soil type tested. This soil had been treated with ammonium nitrate and sewage. From this study they reported that the genera *Nitrosomonas* and *Nitrosospira* occur more commonly than the genus *Nitrosolobus*. The three different MPN media gave approximately the same overall counts of NH_4^+-oxidisers within statistical error after 6 weeks of incubation but differed markedly in the ratios of *Nitrosomonas* and *Nitrosospira*. A further study on the effect of the three media on the MPN enumeration of the three AOB genera, and the time required to reach the maximal counts, showed that the growth of *Nitrosomonas*, *Nitrosospira* and *Nitrosobolus* strains differed considerably with the media used and the incubation time. The times to reach the maximal counts varied between 2 and 9 weeks depending on the culture and medium used.

Various lengths of incubation period have been proposed for full development of nitrifier counts obtained from waters, rooted aquatic plants, sediments and slimes

(Matulewich *et al.* 1975). These authors reported that maximum MPN counts for NH_4^+-oxidisers took between 20 and 55 days (median, 25 days) to develop, whereas the incubation time required for maximum NO_2^-- oxidisers numbers was at least 100 days. However, other researchers have used MPN methods with shorter incubation times to estimate both NH_4^+- and NO_2^- oxidisers. For example, 4-week incubations have been used to estimates the numbers of both NH_4^+ and NO_2^- oxidisers in forest soils (Singh and Kashyap 2006), while 3-week incubations were used to determine the numbers of *Nitrosomonas* and *Nitrobacter* from plant roots (Molina and Rovira 1964).

Another study conducted by Sarathchandra (1979) estimating AOB numbers based on the MPN method, showed that an incubation period of 8 weeks at 25 ^oC was sufficient to obtain maximum estimates of AOB in pasture soils, although the incubation time could be shortened to 1 week by incubating at a higher temperature such as 28^oC (Sarathchandra 1978a). From the above review, it appears that an incubation time that is too short and/or the use of inappropriate types of media may lead to only a proportion of the nitrifiers being detected – leading to an underestimation of the AOB population.

The MPN technique has a low order of precision when few replicates are employed. However, the precision of the MPN estimates can be improved by increasing the numbers of replicates (five or more) at each dilution and using a tenfold dilution ratio especially when the microbial population is not known. Increases in replicate number result in narrower confidence intervals than less replicated MPN procedures (Cochran 1950; Colwell 1979; Wellington *et al.* 1997).

Despite the potential problems with the MPN technique cited above, the method does offer a relatively simple measurement with no specialised apparatus required and it is cheaper than more recent procedures using molecular techniques. The MPN method may not be the best method in all situations, but it is certainly a useful means of estimating the nitrifier populations (Wellington *et al.* 1997).

The advances in molecular techniques which do not rely on laboratory culture, have been applied to the study of soil microbial communities (Bruns *et al.* 1999; Head *et al.* 1993; Kurola *et al.* 2005; Mendum and Hirsch 2002; Satoh *et al.* 2007). However, the suitability of molecular procedures for determining the composition of the soil nitrifier community is still questionable (Kowalchuck and Stephen 2001).

Briefly, the molecular technique involves DNA extraction from soil followed by the use of a PCR to amplify individual nucleic acid sequences to a million copies of the DNA strand for easier analysis (Liesack *et al.* 1997; Tate 2000). This is followed by denaturing gradient gel electrophoresis (DGGE), that separates the PCR-generated DNA products (Fisher and Lerman 1979; Heuer and Smalla 1997). Differing sequences of DNA from different bacteria will denature at different denaturant concentrations, resulting in patterns of bands. Each band represents a different bacteria population present in the community. Once these bands are generated, the patterns or fingerprints can be uploaded into databases in which fingerprint similarity can be assessed to determine the differences in microbial structural between environments or among treatments (Heuer and Smalla 1997; Nakatsu 2005). As is the case with the MPN method, each of the steps in the molecular technique is subject to bias and may affect the downstream analysis but this will not be discussed further in this review.

2.6 History and role of nitrification inhibitors

The use of nitrification inhibitors (NIs) in agriculture as a means of increasing N use efficiency (NUE) and decreasing N losses through nitrification and denitrification is not new. The production of synthetic NIs gained importance during the 1960's in the United States, Japan and West Germany (Amberger 1989; Rajendra Prasad 1995). Research on the use of NIs to increase NUE was first carried out in laboratory studies during the 1960s in the USA. The research then progressed to field experimentation in the late 1960s and 1970s and has continued to the present day (Amberger 1989; Di and Cameron 2004c; Irigoyen *et al.* 2003; Sharma and Kumar 1998; Singh *et al.* 2008; Vogeler *et al.* 2007).

Generally, NIs are compounds that delay the oxidation of NH_4^+ to NO_3^- by depressing the activities and populations of NH_4^+-oxidising bacteria in soil (Edmeades 2004; Rajendra Prasad 1995). As far as it is known, all NIs act on the AMO of AOB. Nitrification inhibitors have beneficial effects in decreasing N losses via NO_3^- leaching and N_2O emissions by reducing the fraction of NH_4^+ oxidised to NO_3^-. The role of NIs in controlling N transformations and N losses is shown in Figure 2.2.

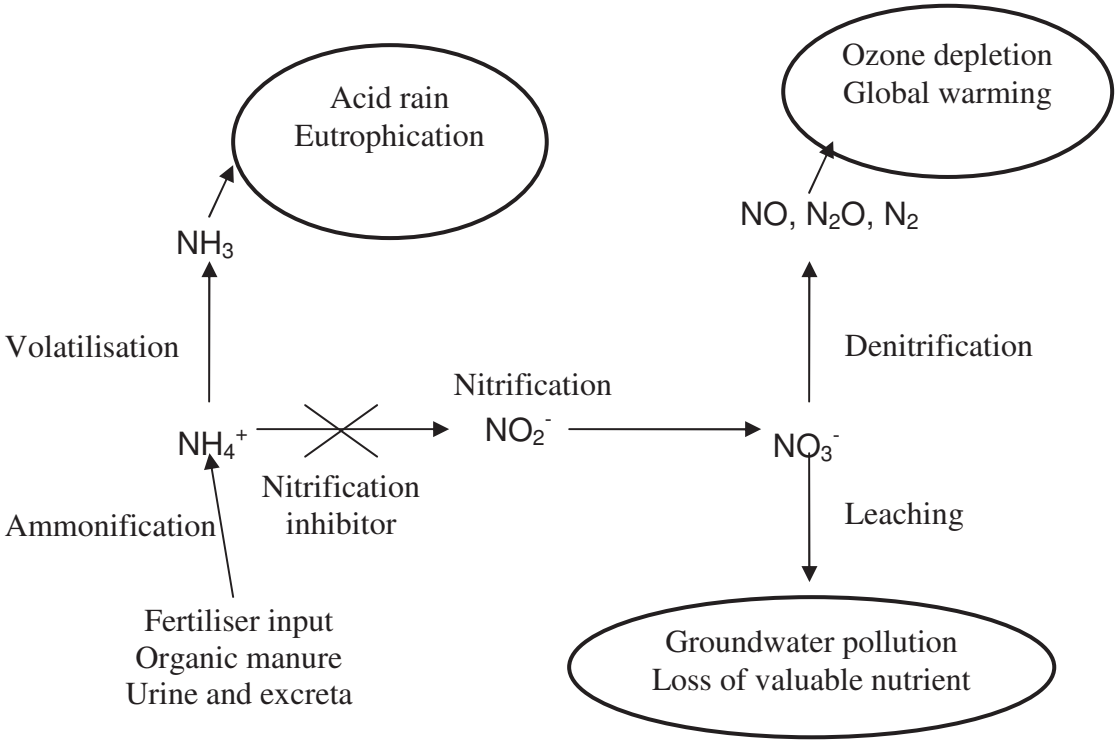


Figure 2.2 The role of nitrification inhibitors in soil.

A large number of compounds, including some pesticides and chelating agents, have been identified as specific inhibitors of the biological oxidation of NH_4^+ and NO_3^- in soils. Although most of these compounds show nitrification-inhibiting capabilities (Rajendra Prasad 1995), only NP (chemical name, 2-chloro-6-(trichloromethyl)-pyridine; trade name N Serve), etridiazole and dicyandiamide (DCD) have received significant attention due to their high inhibition characteristics. Among these three compounds, NP and etridiazole volatilise much faster than DCD due to their high vapour pressures and are therefore less suitable for use with solid fertilisers. It has also been reported that NP is seldom as effective as DCD because of sorption on soil colloids and hydrolysis to 6-chloropicolinic acid which is toxic to plants. In contrast, DCD is more favoured as an inhibitor because it has fewer limitations to its use than NP

and the cost of production of DCD is also lower than NP and etridiazole (Bronson *et al.* 1989; McCarty and Bremner 1989).

Recently a new NI called DMPP or ENTEC (3,4-dimethylpyrazol phosphate) has been produced by BASF in Germany and has been widely used in Europe for agricultural and horticulture purposes (Zerulla *et al*. 2001). It appears to be an effective NI at low concentrations of 0.5 to 1.0 kg ha^{-1}. In addition to the development of synthetic NIs, a number of natural products have been reported to show nitrification inhibiting properties and have been widely evaluated in South Asian countries. These products include neem (Azadirachta indica) and karanjin (a furanoflavonoid), obtained from "karanja" (*Pongamia glabra Vent*.) seeds (Rajendra Prasad 1995; Deepanjan 2002). Deepanjan (2002) demonstrated that karanjin was a more effective NI than DCD, resulting in reduction of total N_2O-N emission by 92-96%, compared with 60-71% obtained from DCD. In contrast, neem has been shown to reduce the total N_2O-N emissions by only by 9% in wheat (Deepanjan 2002). Of all the inhibitors reviewed, DCD and DMPP appear most suitable for pastoral systems (Helen *et al.* 2006).

In New Zealand, the application of NIs has been relatively recent. The majority of the research has been confined to quantifying the effect of DCD on reducing NO_3^- leaching and N_2O emissions from urea based fertiliser and urine patches (Di and Cameron 2003, 2004a; Singh *et al.* 2003; Vogeler *et al.* 2007; Zaman *et al.* 2007). Currently there are two types of DCD formulations that are available in the New Zealand market. These include N-care, a granulated urea fertiliser containing DCD, designed for use during autumn and winter, when the risk of NO_3^- leaching is highest. Another product is Eco-N (a DCD suspension), which reduces NO_3^- leaching and emissions of N_2O from cattle

urine. It is designed to be sprayed onto pasture twice a year to slow down the nitrification activity (McKervey *et al.* 2005; PCE 2004). SustainN, which contains a urease inhibitor called Agrotain®, is a product that slows down the formation of NH_4^+ ions due to inhibition of urea hydrolysis. This then reduces NH_3 volatilisation to the atmosphere and slows down the conversion of NH_4^+ into easily leached NO_3^-.

2.7 Characteristics and performance of DCD

DCD is a dimeric form of cyanamide. It has a dual function. It acts as an inhibitor and also as a slow release N fertiliser due to its high N content (65-67% N). DCD has high water solubility (23 g/L at 13 ^oC), is non-volatile and is chemically and physically stable. It can therefore be mixed with fertilisers and can be dissolved in liquid manures (Amberger 1989). DCD is degraded in soil to guanylurea and then guanidine and urea, which then completely decompose to non-toxic products such as to CO_2, NH_3 and H_2O.

DCD specifically affects and mainly inhibits the activity of NH_4^+-oxidising bacteria (Figure 2.3). This effect is due to the C≡N group of DCD inhibiting the active copper-site of the AMO enzyme, thus affecting the cytochrome oxidase in the respiratory electron transport system of the AOB (Amberger 1989; Subbarao *et al.* 2006). Thus, DCD inhibits the oxidation of NH_4^+ to NO_2^-, resulting in a slowing down of the conversion rate of NH_4^+ to NO_3^- (Amberger 1989; Merino *et al.* 2001; Merino *et al.* 2002). This helps to reduce N losses through leaching or denitrification by reducing the amounts of NO_3^- substrate, and hence improves the N fertiliser efficiency.

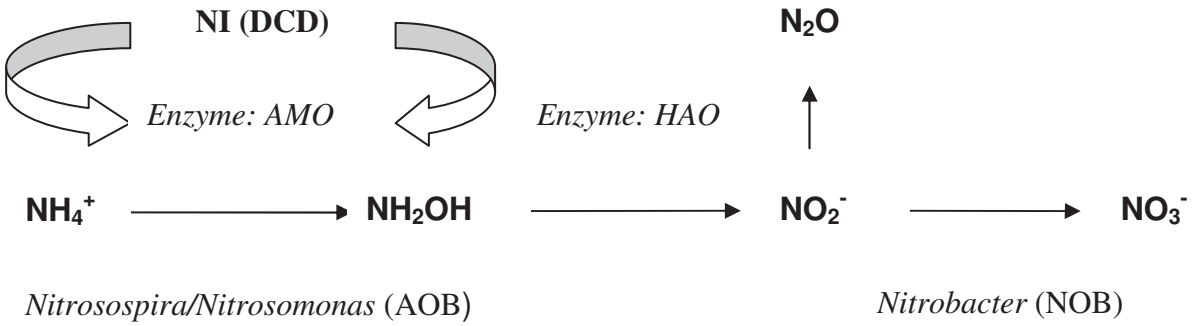


Figure 2.3 Specific inhibition of DCD on nitrification. Modified from (Amberger 1989).

2.7.1 Bacteriostatic or bacteriocidal effect of DCD?

Zacherl and Amberger (1990) and Rodgers and Ashworth (1982) distinguished between bacteriostatic and bactericidal actions of several NIs. Zacherl and Amberger (1990) showed that DCD has a bacteriostatic action that temporary suppresses the activity of nitrifiers but does not kill them. In addition, Di and Cameron (2004a) showed that DCD does not effect the activity and biomass of other soil microorganisms. On the other hand, other NIs such as NP and thiourea (TU) have been demonstrated to be bactericidal, meaning that they significantly reduce the numbers of AOB, or eliminate them completely (Rodgers and Ashworth 1982; Zacherl and Amberger 1990).

Zacherl and Amberger (1990), in their first trial, investigated the effects of NP, TU and DCD on the growth of AOB (*Nitrosomonas europea)* as measured by the production of NO_2^--N in a nutrient solution. This study was carried out for up to 12 days in pure culture. They reported that the addition of two concentrations of each inhibitor (1 and 10 ppm of NP, 0.5 and 1 ppm TU and 200 and 300 ppm of DCD) significantly reduced the production of NO_2^--N compared to the control in all the treatments.

In the same study (Zacherl and Amberger 1990), a second trial was carried out examining the bacteriostatic effect of DCD on the growth of *Nitrosomonas* as measured by the NO_2^--N produced. In this study the concentration of DCD used was 100 ppm. Two treatments were applied to the inoculated pure cultures - without (control) and with DCD. The production of NO_2^--N was monitored in the two treatments for 6 days. When the NO_2^--N production in the untreated samples had reached a stationary phase, the untreated and DCD-treated bacteria were transferred to separate fresh DCD-free media containing nutrient solution. The NO_2^--N production in these second incubations was measured after 5 days. In the first incubations the addition of 100 ppm DCD reduced NO_2^--N production, to 61% of that in the control. However, in the second incubations using DCD-free media, production of NO_2^--N by the bacteria from the original DCD treatment recovered to be 89% of the control. They concluded that the addition of 100 ppm of DCD had a bacteriostatic effect on the growth of *Nitrosomonas*. However, it should be noted that the effect of DCD on the actual population of AOB wasn't assessed in this study.

In another study, Rodgers and Ashworth (1982) examined the short term effects of five types of inhibitors on AOB to assess whether these inhibitors have bacteriostatic or bactericidal effects on the nitrifiers. Their first experiment was a laboratory experiment with nitrifying cultures. Exposure for 48 hours to 2,6-dimethylbezoquinone, DCD or potassium ethyl xanthate at a concentration of 100 mg inhibitor/L of culture medium and NP at the lower concentrations of 1 and 10 mg/L completely inhibited nitrification activity, but the numbers of AOB were not significantly affected. On the other hand, when etridiazole was applied at two different rates (1 and 10 mg/L) or NP was applied at the higher concentration of 100mg/L, these inhibitors were apparently fatal to AOB.

Their second experiment was a laboratory incubation, in which they investigated the inhibition effect of etridiazole and NP added to a sandy soil at a lower concentration of 0.5 mg inhibitor kg^{-1} soil and incubated at 20^{o}C. Both inhibitors applied at this low concentration were apparently fatal to AOB, and were more bactericidal than appeared to be the case in pure cultures.

Their third experiment was a field experiment on the effects of etridiazole and NP on soil nitrification activity. These inhibitors were applied at the rate of 1.5 kg ha^{-1} of aqueous etridiazole or NP with 375 kg N ha^{-1} (as urea) to the Rothamsted silty clay loam during autumn or spring. The results suggested that the numbers of AOB were not affected by the addition of these two inhibitors, although both inhibitors effectively inhibited the nitrification activity. They suggested that perhaps the results obtained in the field trials differed from the amended soil in the laboratory incubations because of the different soil types and temperatures that were involved.

2.8 Factors that influence the degradation and the effectiveness of DCD

The inhibitory effect of most inhibitors, including DCD, persists for an average of one to three months depending on soil factors and application rates (Rajbanshi *et al.* 1992). The soil factors include the soil type, temperature, pH and moisture. Among these parameters, soil type and temperature are the most critical and are discussed in the next section. The effect of these parameters in controlling the degradation of DCD in soil has been studied widely and is well documented (Bronson *et al.* 1989; Di and Cameron 2002a; Irigoyen *et al.* 2003). It has also been reported that increasing the soil pH from

5.8 to 8.3 with lime reduced the efficacy of DCD due to the increase in microbial activity (Slangen and Kerkhoff 1984).

Generally, the effectiveness of most NIs increases when soil moisture contents are lower than the soil field capacity. In another study, Belser and Schmidt (1981) showed that different strains of *Nitrosomonas* AOB showed remarkable differences in sensitivity to NP inhibitor, with some strains about five times more resistant than others. They concluded that strain sensitivity may be one of the factors that contributes to the effectiveness of NIs in the soil. It seems likely that soils which show good response at low level of NP applications have very sensitive strains present. Continued application of NP however might select for less sensitive strains, leading to a need for progressively higher application rates (Belser and Schmidt 1981).

2.8.1 Soil types

Both laboratory and field experiments have shown differences in the effectiveness of NIs on different types of soil (Malzer *et al.* 1989). Most of the research under laboratory incubation conditions shows that the performance of DCD and other NIs is strongly correlated with soil texture and soil organic matter (OM) content. Generally, soil OM reduces the effectiveness of most NIs, either by stimulating microbial activity that results in rapid degradation of the inhibitors or by reducing the bioactivity of inhibitors through absorption on the soil colloids (Slangen and Kerkhoff 1984). Bioactivity is defined here as the relative inhibition of nitrification (Keeney 1980). For example, a compound may persist for a long time but have little bioactivity towards nitrifiers due to sorption on soil colloids, while a compound that rapidly disappears from a soil may

have high bioactivity while it is present. The inhibitory effects of DCD increase with lower amounts of OM and with higher sand content and vice versa.

A study conducted by McCarty and Bremner (1989) evaluating the effectiveness of DCD in three different types of soils (as detailed in Table 2.3) showed that the lowest inhibition effect (8%) was obtained in the Harps soil followed by the Webster and Storden soils. The high nitrification inhibition obtained in the Storden soil was attributed primarily to the courser texture of this soil. Another factor that may have also influenced the effectiveness of DCD was the amount of OM in the soil, as the Harps soil which had the highest OM content had the lowest inhibition of nitrification. Similar results were obtained for the other NIs tested in their study, such as 2-ethynylpyridine, etridiazole and NP.

Table 2.3 The influence of soil texture on the effectiveness of DCD in nitrification[a]. Adapted from McCarthy and Bremner (1989).

Soil	Sand	Organic C %	% inhibition of nitrification
Harps	10	6.6	8
Webster	31	3.3	20
Storden	59	0.5	41

[a]20 g of soil samples were incubated at 25 ^{o}C for 25 days with 5 µg g^{-1} soil of DCD

2.8.2 Soil temperature

The breakdown or decomposition of DCD in the soil is accelerated with increasing temperature, thus reducing the effectiveness of DCD. Most NIs including DCD are very

effective at temperatures $\leq 5^{o}C$. Under low temperatures, the inhibitory effect can last up to six months, and therefore inhibitors are suitable for application in autumn and winter (Slangen and Kerkhoff 1984; Zerulla *et al.* 2001).

Bronson *et al*. (1989) investigated the decomposition rates of two inhibitors (DCD and NP) in two conrasting soils; namely Decatur (silt loam) and Norfolk (loamy sand). The DCD was applied at 20 mg DCD-N kg^{-1} of soil with 80 mg kg^{-1} of $(NH_4)_2SO_4$-N and the NP was applied at 5 mg NP kg^{-1} of soil with 100 mg kg^{-1} of $(NH_4)_2SO_4$-N. Their results showed that the half-lives of DCD in the Decatur and Norfolk soils were 7.4 and 14.7 days respectively at a soil temperature of $22^{o}C$ but that at a soil temperature of $8^{o}C$ the half-lives of DCD extended to 25.8 and 52.2 days in the two soils respectively. They concluded that DCD was not effective in inhibiting nitrification in Decatur silt loam at the higher temperature of $22^{o}C$. Similar results were obtained when Harps, Webster and Storden soils with different textures and OM contents were incubated at four different temperatures of 15, 20, 25 and $30^{o}C$. The inhibition of nitrification decreased from 72 to 19%, 78 to 25% and 90 to 23% in the three soils respectively as the temperature increased from 15 to $30^{o}C$ (McCarty and Bremner 1989).

Further evidence that soil temperatures markedly affect the effectiveness of DCD as a soil nitrification inhibitor has been provided by several authors (Di and Cameron 2004a; Irigoyen *et al.* 2003; Rajbanshi *et al.* 1992). For example, Irigoyen *et al.* (2003) concluded that the inhibitory effect of DCD would last for one month and more than three months when the soil was incubated at temperatures of $20^{o}C$ and $10^{o}C$ respectively. Di and Cameron (2004a) showed that when DCD was applied to the Lismore silt loam at two rates (7.5 kg and 15 kg DCD/ha) with additions of urea at 25

kg N ha^{-1} and urine at 1000 kg N ha^{-1}, the half life was 111-116 days when the soil was incubated at 8^{o} C but only 18-25 days when incubated at 20^{o}C. They also concluded that both application rates of DCD were effective in reducing the nitrification rate, particularly in the cooler conditions of 8^{o}C.

2.9 Research on DCD in New Zealand and elsewhere

Most of the early research work carried out on DCD throughout New Zealand has involved laboratory studies and small-plot field trials, treated with high rates of either urine, fertiliser N (urea) or dairy shed effluent. These include small and large lysimeter studies, static or manual gas sampling chambers, field trials using tile drains and laboratory incubation experiments (Edmeades 2004; Helen *et al.* 2006).

The majority of these studies have shown that DCD has great potential in reducing NO_3^- leaching and N_2O emissions, however the effects vary between soils and the soil temperature appears to be one of the main determinants of the effectiveness of DCD. In other studies, the efficacy of DCD been somewhat inconsistent and there have been some questions concerning its performance in reducing NO_3^- leaching and N_2O emissions and increasing crop yields (Davies and Williams 1995; Fox and Bandel 1989; Gioacchini *et al.* 2002). In the following section the effects of DCD in reducing NO_3^- leaching and N_2O emission are discussed.

2.9.1 Effectiveness of DCD in reducing NO_3^- leaching

The effects of DCD on NO_3^- leaching losses in New Zealand were first reported by Francis *et al.* (1995). This work investigated the effects of DCD on NO_3^- leaching losses over the winters of 1991 and 1992 following the ploughing-in of temporary

grass/clover pastures in either early (March) or late autumn (May) and left fallow over winter, and the subsequent effects of these treatments on the yield and N uptake of a spring wheat crop sown in October, 1992. They reported that the application of DCD did not affect the extent of net N mineralisation but it did inhibit the nitrification activity when applied to pasture before ploughing. Nitrification inhibition in spring was greater when DCD was applied in May rather than in March due to its reduced degradation over the winter. These two-year studies showed that the cumulative NO_3^- leaching losses in each year were about 100 kg N ha^{-1} and shortening the fallow period reduced the losses by an average of 70 kg N ha^{-1}. They concluded that the most reliable way to reduce NO_3^- losses was to delay ploughing, but in cases where early cultivation was required, the application of 20 kg DCD ha^{-1} was effective in reducing NO_3^- losses by 30-45%. Their results also showed that the harvest grain yields, gain in N concentration and total N of the wheat crop, were not significantly different between the March and May fallow treatments.

In another lysimeter study, Williamson *et al.* (1998) investigated the effectiveness of DCD in reducing NO_3^- leaching in pasture that had been irrigated with a high N loading of dairy shed effluent (1100 kg N ha^{-1}) to a freely draining soil (Horotiu loamy silt) during the winter season. Three treatments were applied in this study. Three lysimeters received only dairy farm effluent, a further three received effluent amended on one occasion with DCD at 12 kg ha^{-1}, and another three lysimeters received an equivalent volume of water. Irrigation of the effluent dramatically increased the amount of N leached from the lysimeters, in comparison with the water treatment. Of the total effluent-N applied, 620 kg N ha^{-1} (56%) was leached over a period of 19 weeks. DCD reduced the cumulative amount of total N leached from effluent-irrigated lysimeters by

18%. The authors concluded that the application of DCD didn't totally inhibit the nitrification activity and the repeated high effluent N-loadings particularly during the winter compromised groundwater quality even when DCD was applied.

In other field trials examining the effects of two different rates of DCD at 10 kg and 25 kg ha^{-1}, applied with urine at 750 hg N ha^{-1} on free draining soil (Wakanui silt loam), DCD significantly reduced the peak NO_3^--N concentrations from 140 to 60 and 35 µg N g^{-1} of soil respectively (Cookson and Cornforth 2002). From this, they inferred that NO_3^- leaching could be reduced by 57-75%. The authors also observed that neither DCD rate affected the pasture yield, but they did significantly increase the soil pH.

Most of the soil lysimeter studies reported by Di and Cameron (2002c, 2003, 2004c, 2005) show that NO_3^- leaching losses were significantly reduced when DCD was applied in solution and fine particle suspension form in combination with high urine (1000 kg N ha^{-1}) and high urea inputs (200 kg N ha^{-1}). Their initial work (Di and Cameron 2002c) demonstrated that the addition of DCD reduced NO_3^- leaching losses by 76% and 42% when urine was applied in autumn and spring respectively. Their results also showed that DCD significantly increased the herbage yields by more than 30% in all treatments compared with the non-DCD treatments. In another study, they found that DCD decreased by 50-65% the leaching losses of cation nutrients such as Ca^{2+}, K^+ and Mg^{2+} (Di and Cameron 2004b).

A further lysimeter study (Di and Cameron 2004c), using Templeton fine sandy loam treated with 1 or 2 applications of eco-n™ (active ingredient-DCD) at 15 kg ha^{-1} with 1000 kg N ha^{-1} yr^{-1} as urine and 200 kg N ha^{-1} yr^{-1} as urea, showed that the NO_3^-

leaching was decreased from 85 to 20-22 kg N ha^{-1} yr^{-1}. This was equivalent to a 74-76% reduction in NO_3^- leaching. There was no significant difference in the total amount of NO_3^--N leached between the single and double eco-n treatments. In addition, the eco-n application reduced the Ca^{2+} and Mg^{2+} leaching by 38-56% and 21-42% respectively, with no significant difference observed between the single and double eco-n applications. It was also reported that the single eco-n treatment increased the pasture production from 15.9 to 18.2 t ha^{-1} yr^{-1} and pasture production further increased to 21.1 t ha^{-1} yr^{-1} when eco-n was applied twice. Their results showed that one application of eco-n immediately after urine application may be sufficiently to reduce the NO_3^- leaching from the urine patches without a need for repeated applications. However, they further suggested that a minimum of two eco-n applications, one in the autumn (April/May) and one in the spring (August/September), may be necessary to reduce NO_3^- losses from urine deposited over the whole winter season. This was due to urine deposited at different times of the year following each grazing rotation in grazed pasture with the highest potential of NO_3^- losses for urine deposited in the autumn and in early spring (Di and Cameron 2004c). Di and Cameron (2005) showed that DCD applied as a fine particle suspension (FPS) at 10 kg ha^{-1} was as effective in reducing NO_3^- leaching in grazed pasture soils as when it was applied at 15 kg ha^{-1} as a solution. However, they found that FPS DCD at the rate of 5 kg ha^{-1} was not effective in providing environmental and agronomic benefits under the conditions of the experiment.

Elsewhere, Davies and Williams (1995), reported that DCD gave no significant reduction in the amount of NO_3^- being leached. In addition, Gioacchini *et al.* (2002) have also reported that DCD was not effective at reducing NO_3^- leaching in a lysimeter

study, in which more soil-derived N was lost through leaching in the presence of DCD relative to their control treatment.

2.9.2 Effectiveness of DCD in reducing N_2O emissions

DCD has been shown to reduce the emission of N_2O. The summary of published work on the effectiveness of DCD in reducing N_2O emissions from urine patches in New Zealand showed that the relative reduction in emissions ranged from 0 to 86% (de Klein *et al.* 2011). Although the relative reduction in N_2O emissions was generally high in most of the studies, the absolute reduction in N_2O emission ha^{-1} varied and ranged from <0.5 to ~30 kg N ha^{-1} (de Klein *et al.* 2011).

A lysimeter study with a free-draining Lismore stony silt loam soil showed that when DCD was applied with urine to the soil, the total emissions of N_2O were reduced by 82% from 46 kg N_2O-N ha^{-1} to 8.5 kg N_2O-N ha^{-1} over the course of two spring urine applications (Di and Cameron 2002c). In their more recent study Di and Cameron (2006) examined the effectiveness of DCD applied in a fine particle suspension in reducing N_2O emissions from dairy cow urine patches in two different soils. The authors found that the applications of DCD at three rates (7.5, 10 and 15 kg ha^{-1}) were effective in reducing N_2O emissions by 65-73% in the Lismore soil and 56-61% in the Templeton soil. In a further field experiment (Di *et al.* 2007) large reductions (61-73%) in N_2O emissions from urine patch areas were achieved when urine and DCD were applied between May and August in four soils under different climatic and management conditions.

McTaggart *et al.* (1997) assessed the effectiveness in reducing N_2O emissions of DCD applied to grassland with urea, ammonium sulphate (AS) and ammonium nitrate (AN) fertilisers in April and August in 1992 and 1993. DCD was applied to grassland with N fertiliser applications in. They showed that for up to two months following each application of DCD in the grassland, emissions of N_2O were reduced by 58-75% when applied with urea and 41-65% when applied with AS. Annual emissions (April to March) of N_2O were reduced by up to 58% and 56% in 1992-1993 and 1993-1994. Applying DCD to AN fertilised grasslands however, didn't reduce the emissions after the April 1993 fertilisation, but emissions following the August application were reduced. Kumar *et al*. (2000), reported smaller reductions in N_2O emissions when DCD in an irrigated rice fields fertilised with urea and AS with 11% and 26% reductions respectively.

2.10 Summary of literature review

Nitrification occurs in a wide range of environments. All known AOB strains isolated from terrestrial ecosystems fall within the *Nitrosospira*/*Nitrosomonas* sub-groups of the β-*Proteobacteria* based upon 16S rDNA and *amoA* gene sequences. These two genera can be further divided into seven to nine gene clusters, and the relative abundance of certain clusters has been correlated with specific environmental conditions, including soil pH, the concentration or availability of NH_4^+, soil water limitation and agricultural use. Wide ranges of nitrification activity have been reported in New Zealand pasture soils on both flat and hill country, but the reasons for this variation are not well understood. On the other hand, many researchers have reported that various physical, environmental and chemical factors affect nitrification in soils.

More recently, there has been an increasing interest in the usage of NIs such as DCD to mitigate N losses in New Zealand and globally. These NIs slow down nitrification by AOB, thereby reducing the amounts of NO_3^- being produced and minimising the losses of NO_3^- through leaching or denitrification. Many studies have shown that the effectiveness of NIs, including DCD, in the soil is dependent on their persistence and bioactivity. The half-lives of inhibitors may vary from a few days to several weeks, depending on the nature of the compound, application rates, soil type, pH and temperature. To date, there have been few studies on the inhibitory effect of DCD on soil nitrification rates with time and the implications of DCD on the population of AOB. Furthermore, there is still only limited information on whether the repeated addition of DCD reduces the effectiveness of the nitrification inhibition.

The aim of the subsequent experimental chapters of this thesis is to explore the reasons for both the natural variation in nitrification rates within the landscape and also the variable effectiveness of DCD. Thus in Chapter 3, a perfusion technique was developed to monitor the changes of nitrification activity over time in New Zealand pasture soils. The technique was then used to evaluate the effect of one or more additions of DCD on the nitrification rates and the AOB populations in two soils with contrasting physical and chemical properties. The quantification of AOB was carried out with the MPN technique. Despite the potential problems with the MPN technique, as detailed in the Literature Review, this method does offer a relatively simple measurement with no specialised apparatus required and it is cheaper than more recent procedures using molecular techniques. The MPN method may not be the best method in all situations, but it is certainly a useful means of estimating the nitrifier populations.

In Chapter 4, soil incubation studies were carried out to further evaluate the link between the presence of DCD in the two contrasting soils and the nitrification rates, and to investigate whether two different rates of DCD applied had an effect on the soil nitrification rates.

As noted earlier in the background of this study, the nitrification rates in the steep slopes of the hill country soil appear to be considerably lower than the nitrification rates obtained in flat campsites. In addition, some studies have indicated that lower nitrification rates observed in some soils were positively correlated with the population of AOB present in that soil. Thus in Chapter 5, an experiment was designed to provide further information on why the rates of nitrification in the steep slopes were very low and whether this further correlates with low numbers of AOB.

Finally, Chapter 6 investigates whether there is a possibility of a natural poisoning effect or inhibition in soils from steep slopes that may prevent the build-up of nitrifiers, and thus influence the nitrification rate. This work was carried out through soil incubation studies by mixing soils from the lower nitrifying steep sites with soils from the high nitrifying flat campsites.

Chapter 3

Measuring soil nitrification activity over time with a perfusion technique

3.1 Introduction

In general, a perfusion system involves a continuous recirculating flow of a dilute solution that is applied at the top of a soil column, percolates down through the soil to a reservoir at the bottom and is then recycled back to the top (Figure 3.1).

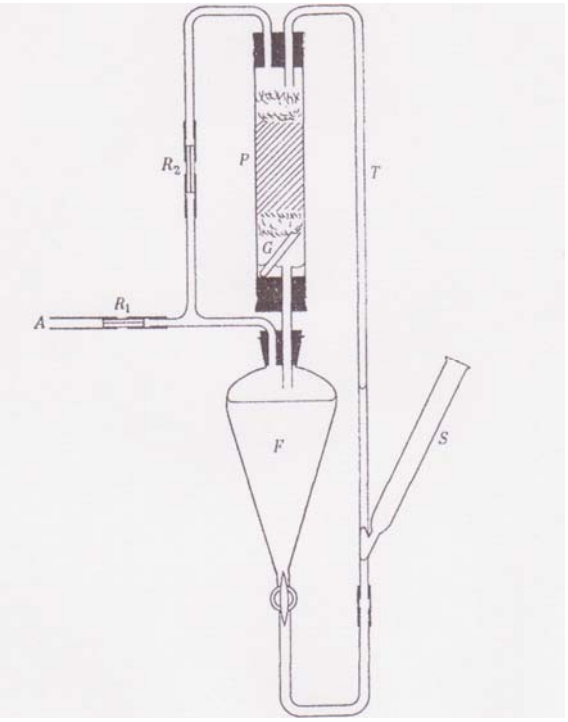


Figure 3.1 Soil perfusion apparatus (Audus 1946). The components are as follows : P, a glass tube containing soil crumbs between pads of glass wool (G); F, the solution supplied to the soil; A, a constant suction applied by a suction pump connected by flexible tubing (R_1 and R_2) to the column; T, tubing carrying the solution and S, the sample taken out for analysis.

The perfusion of soil columns with various solutions as a means of studying biological transformations by soil bacteria is a well established technique and several designs of suitable apparatus have been described (Audus 1946; Collins and Sims 1956). These devices have been proved to be very valuable for studying such microbial processes as arsenite oxidation in soil (Quastel *et al.* 1952), sulphur metabolism in soil (Gleen and Quastel 1953) and lignin removal from waste water (Joshi and Dutta 1987).

In soil nitrification studies, the perfusion technique has been widely used to examine the effects of herbicides and other agricultural chemicals on the nitrification process (Debona 1970; Lees and Quastel 1946). Lees and Quastel (1946) also concluded that this technique is of great value in studying the effect of inhibitors on soil nitrification. This technique has been adapted and used to monitor nitrification activity in New Zealand pasture soils (Robinson 1963; Sarathchandra 1978a; 1978b; 1979). In nitrification studies, a dilute ammonium sulphate ($(NH_4)_2SO_4$) solution is perfused with continuous aeration through the soil column and this process may be maintained for an indefinite period. The soil is left intact throughout the experiment and analysis of the amount of nitrate-N (NO_3^--N) formed and the amount of ammonium-N (NH_4^+-N) left in the perfusate is performed at intervals.

The study of metabolic processes occurring in soils with an open-system perfusion has many advantages (Quastel 1955). These include:

a) being able to provide a solution at a constant rate;

b) maintaining homogeneous distribution of the solution throughout the experiment;

c) maintaining maximal aeration of the soil;

d) the soil in the perfusion column is undisturbed and analysis confined to the constituents of the perfusate, however a soil sub-sample can be collected for further analysis of adsorbed ions or bacterial activity and

e) the solution can be replaced at any time and chemicals (i.e inhibitors, herbicides) can be added to the solution at any stage of the experiment, corresponding to the known metabolic activity of the soil.

The main disadvantages or shortcomings of the perfusion technique are (Lees and Quastel 1946):

a) Removal of soil solution for analysis inevitably decreases the total volume of perfusate, and there is no corresponding decrease in the amount of soil to compensate for this. The error can be reduced to a low value by removal of the smallest quantities possible for analysis, and by having a large initial volume of perfusate.

b) The apparatus is suitable only for studies conducted at a soil-moisture content close to water-logging. Whilst many microbial activities are rapid and optimal at this water content (aeration being also optimal), the impossibility of working at any other water content is a drawback.

It also needs to be recognised that by continually recycling the perfusate there will be a build up of reaction products and metabolites that would not occur in the field where the leachate originates from rainfall or irrigation. These accumulating products and metabolites may inhibit further reaction. However, this effect will be very much less than occur in experimental designs that involve incubation. On balance, the advantages of the perfusion technique for studying metabolic processes in soil often outweigh its

disadvantages, and this technique has been proven useful in monitoring the nitrification activity in soils.

Another method that has been used to evaluate nitrogen (N) transformations in soil is aerobic incubation under controlled temperature and moisture conditions in the laboratory. This technique has been used:

a) to evaluate net soil N mineralisation (Griffin *et al.* 2008; Heumann and Bottcher 2004);

b) to evaluate net N mineralisation from organic fertilizers (Griffin *et al.* 2005);

c) to model N mineralisation dynamics (Muller *et al.* 2003) and

d) to quantify soil nitrification rates in agricultural and forest soils (Backman and Klemedtsson 2003; Malhi and McGill 1982; Subler *et al.* 1995; van Kessel *et al.* 2000; van Niekerk and Claassens 2005)

Most aerobic incubation experiments have common features. Generally, soil samples are treated with the substances under investigation, brought to a suitable water content and placed in containers, where the nutrient transformation is allowed to proceed. Samples of soil are taken at intervals, shaken with an extractant, centrifuged, filtered and finally the extracts are analysed.

Other techniques have been proposed for quantifying N mineralisation, N immobilisation and nitrification under field conditions (Rees *et al.* 1994). These include field incubations of enclosed soil cores to prevent N losses during incubation (i.e. N uptake by plant roots, N leaching and denitrification losses) including capped tubes (Raison *et al.* 1987), polyethylene bags (Eno 1960; Monaco *et al.* 2010), in situ ion

exchange membranes (IEM) to measure soil mineral N dynamics in grazed pastures (Bowatte *et al.* 2008), ion exchange resins to trap N leached (Di Stefano and Gholz 1986) and the addition of acetylene to inhibit nitrification (Hatch *et al.* 1990).

Although both laboratory and field incubation methods are suitable for studying soil processes such as nitrification, there are many possible permutations of these procedures. These include the degree of soil disturbance (intact cores versus sieved soils), incubation temperatures (*in situ* temperature fluctuations versus a constant temperature), incubation moisture (field moisture at the time of sampling versus some proportion of field capacity) and substrate enrichment (absence versus presence of added ammonium). Disturbances of incubated soil by sampling may also occur during the extraction, and these disturbances will cause changes in soil aeration and nitrification rate.

Overall, no one method provides a clear and accurate assessment of the nitrification rates in agriculture and forest soils under different soil conditions. The selection of the most appropriate method for each study needs to be based on the study objectives.

The main objective of this study was to develop a perfusion system that could be used to monitor the nitrification rates of New Zealand pasture soils over time and to use this technique to study both the immediate and the residual effects of one or more additions of the nitrification inhibitor, dicyandiamide (DCD). The effect of DCD on the population of AOB was also quantified.

3.2 Materials and Methods

3.2.1 Soil samples

Three soils were used in this study. Approximately 10 kg of field moist topsoil (0-10 cm) was collected from random spots on two soil types (Manawatu silt loam; MSL and Manawatu fine sandy loam; MFSL) within Dairy Farm No.1 and from a third soil type (Tokomaru silt loam; TSL) on Dairy Farm No. 4, Massey University, Palmerston North, New Zealand. Sub-samples of the fresh soil samples were immediately passed through a 2-mm sieve in the laboratory and homogenised by hand and these soils were stored at 4^{o}C until further analysis for quantification of AOB (Section 3.2.6). The remainder of the fresh soil samples were air-dried, ground and passed through a 2-mm sieve.

3.2.2 Soil analysis

A sub-sample of each of the dried and sieved soils was used to determine total C and N as measured by combustion in a Leco FP-2000 CNS analyser (LECO Corp., St Joseph, MI, USA). The soil pH was measured at a 1:2.5 soil:water ratio and the mixture was allowed to stand overnight. The soil pH was then measured with a combined electrode pH meter (Blakemore *et al.* 1987). Other 5g sub-samples of the air-dried soils were extracted with 2 M KCl solution by shaking for 30 min (1:5 soil:extractant ratio), then centrifuged at 9000 rpm for 15 min. The extractable NH_4^+-N and NO_3^--N in the supernatant were analysed using a Technicon auto-analyser (Blakemore *et al.* 1987). Determination of the cation exchange capacity (CEC) of the soil subsamples was carried out by a semi-micro leaching method (Blakemore *et al.* 1987). All soil analyses were carried out on three replicates of each soil. The chemical properties of the collected soil samples are shown in Table 3.1.

Table 3.1 Chemical properties of the three soils studied.

Soils	pH	NH_4^+-N ($\mu g\ g^{-1}$)	NO_3^--N ($\mu g\ g^{-1}$)	Total C (%)	Total N (%)	CEC ($cmol_c/kg$)
Manawatu silt loam	6.12	7.6	37.1	4.87	0.50	25.1
Tokomaru silt loam	5.78	4.9	22.6	4.24	0.38	20.7
Manawatu fine sandy loam	5.24	6.6	11.8	1.61	0.14	10.9

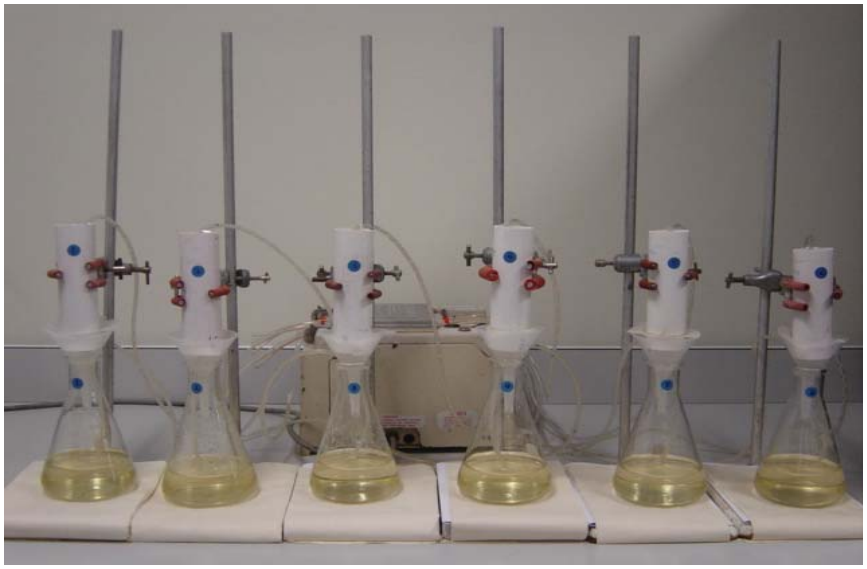
3.2.3 Perfusion apparatus

During the initial stage of developing this technique, devices similar to those described by Audus (1946) (Figure 3.1) were used, with six sets of apparatus connected to a suction pump. Soil alone (without any added sand) was used at this initial stage. However, this experimental setup suffered from the disadvantage of being relatively bulky and difficulties arose in maintaining the same constant rate of percolating fluid in all the six units simultaneously. Furthermore, this system required time to construct and used a considerable amount of glassware. The flow of the perfusate through the soil columns was variable, and at one stage the systems clogged and became anaerobic. The clogging effect was more severe with a finer textured soil such as the Manawatu silt loam (MSL) than with the free draining Manawatu fine sandy loam (MFSL).

Many attempts were made to overcome this problem. These included mixing the soil with increasing proportions of sand to assist with the percolation of the perfusate, however similar results were obtained. Therefore a simplified soil perfusion apparatus

which avoids these disadvantages was developed and was used throughout this study to examine the soil nitrification activity.

In this perfusion apparatus (Figure 3.2), mixtures of 30 g of air dried soil and 30 g of sand (0.125-0.25 mm particle size) were placed into polyvinyl chloride (PVC) pipes of 150 mm height and 42 mm internal diameter. Sand was mixed with the soil to improve the percolation of the perfusate solution. A thin layer of glass wool was placed on the top and at the bottom of the soil columns and the bottoms of the PVC pipes were tightly covered with muslin cloth. The columns were arranged vertically with plastic funnels at the bottom to facilitate the collection of the perfusate. The perfusate (initial volume 300 mL) was placed in a conical flask (F in Figure 3.2) and was then continuously pumped, using a peristaltic pump at a flow rate of 0.8 mL/min, from the flask to the top of the soil column, from where it percolated slowly through the soil back into the flask. The perfusate was a diluted 3.6mM $(NH_4)_2SO_4$ solution containing KH_2PO_4 (1.4 mM); $MgSO_4.7H_2O$ (0.8 mM) and $CaCl_2$ (0.2 mM). The apparatus was kept at room temperature (ranging from 15 to 20^oC). The flow of perfusate (0.8 mL/min) through the system was controlled by a peristaltic pump (P).



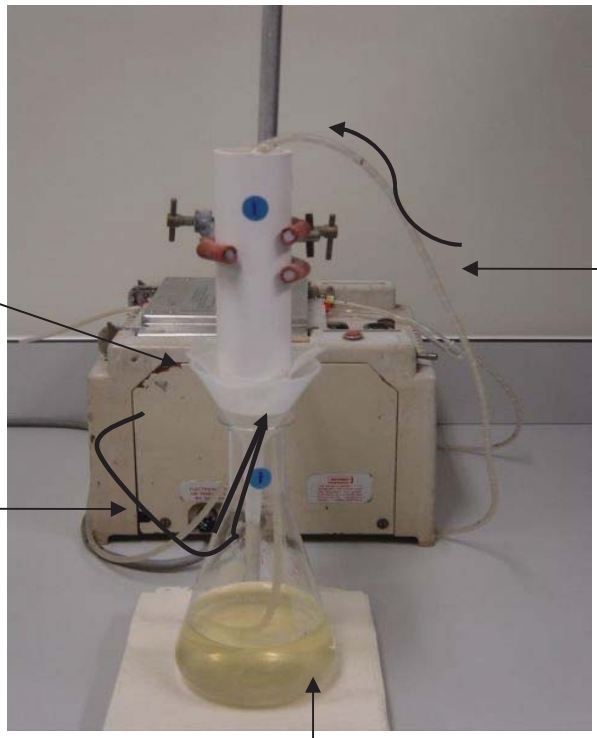


Figure 3.2 Perfusion apparatus used in this study to determine the nitrification activity in New Zealand pasture soils.

3.2.4 Rapid determination of nitrification activity over time in three pasture soils

In order to make sure the perfusion apparatus as described above was able to detect and quantify the soil nitrification activity over time, it was first tested with one soil type. Six replicates of MSL were used in this experiment. The perfusate in the conical flask was sampled on day 1 and then daily until at least half the added NH_4^+-N had been nitrified. The NH_4^+-N and NO_3^--N concentrations in the perfusate were analysed using an autoanalyser (Blakemore *et al.* 1987). Before sampling the perfusate from each of the conical flasks, the tubing carrying out the perfusate from the flask was lifted up above the solution to allow the remaining perfusate in the tubing to be pumped to the soil column, before the pump was stopped. After a delay of approximately 15 minutes to allow the perfusate to stop draining from the soil column, the solution in each of the flasks was well mixed and then sampled.

Once the perfusion apparatus developed above was shown to be suitable to demonstrate and to quantify the nitrification activity over time in the MSL, it was then used to monitor the nitrification rates in the other two soil types, namely MFSL and TSL. In this experiment, another set of perfusion apparatus was used with an additional set of six units connected to a peristaltic suction pump and a similar procedure was followed to that described above for the MSL.

In this study, and in all the experiments using the perfusion technique described in this chapter, the nitrification rate in each replicate was calculated from the increase in NO_3^--N concentration over that found on the first day. For example, suppose 30 g of soil are

perfused with an initial volume of 300 mL of $(NH_4)_2SO_4$ solution and samples (10 mL) of perfusate yield the following NO_3^--N concentrations at the indicated times:

Duration of experiment in days:	1	3	5
Concentration of NO_3^--N in perfusate (µg/mL):	5	25	75

Thus between day 1 and day 3, there was an increase in NO_3^--N concentration of 20 µg/mL in the 290 mL solution (i.e. in the original 300 mL minus the first 10 mL sample). Therefore the total NO_3^--N formed during this period is 20 x 290 = 5800 µg, and the nitrification rate (taking into account the 30 g of soil used in the experiment, and the number of days between samplings (2)) is 5800 / (2 x 30) = 96.7 µg g^{-1} soil day^{-1}. Similarly, between 3 and 5 days the total NO_3^--N formed is 50 x 280 µg = 14,000 µg and the nitrification rate is 14,000 / (2 x 30) = 233.3 µg g^{-1} soil day^{-1}. Therefore the average nitrification rate between days 1 and 5 is [(5800 + 14000 µg)/(4 days x 30 g soil)] = 165.0 µg g^{-1} soil day^{-1}. This type of calculation was used to calculate average nitrification rates over the duration of the total trial. The results in this chapter were then reported as the means of the replicates used in each treatment in the experiment.

In this study, using either the rate of disappearance of NH_4^+-N or the rate of appearance of NO_3^--N to estimate nitrification rates can lead to different conclusions, since NH_4^+-N can react with the organic matter of the soil or be fixed in clay particles, while NO_3^--N can be immobilised in microorganisms, leached or denitrified. Lees and Quastel (1946) suggested that because NO_3^--N is not adsorbed on the soil surface, a series of NO_3^--N analyses of the perfusate can yield an accurate estimate of the rate of NO_3^--N formation and this was the approach used in this study. It was that because the columns were

constantly aerated, it was unlikely that extensive denitrification would occur in the perfusion system.

3.2.5 Effects of DCD and residual effects of DCD on soil nitrification rates

Two soils (MSL and MFSL) were used in this study. The perfusion technique as described above was used for these experiments. The mixtures of 30 g of soils and 30 g of sand (0.125-0.25 mm particle size) in the columns were continuously perfused with a diluted 3.6 mM $(NH_4)_2SO_4$ solution with KH_2PO_4 (1.4 mM), $MgSO_4.7H_2O$ (0.8 mM) and $CaCl_2$ (0.2 mM) as described in the previous section. These experiments consisted of four cycles, with each cycle lasting 14 and 17 days for MSL and MFSL respectively. At the end of each cycle the remaining perfusate was discarded and a new batch of perfusate introduced.

Two treatments were applied to MSL, with three replicates in each treatment. In the first (control) treatment, the perfusate in each cycle was a dilute solution of $(NH_4)_2SO_4$ as described above. In the second treatment the perfusate in cycles 1 and 3 had DCD added to the $(NH_4)_2SO_4$ solution at a rate equivalent to 20 kg DCD ha^{-1} (amount in the perfusate was 1.2 mg DCD), while in cycles 2 and 4 the fresh perfusate contained only the $(NH_4)_2SO_4$ solution with no added DCD. The purpose of cycles 2 and 4 was to measure the recovery of nitrification activity following the removal of DCD. Since this experiment consisted of four cycles and took a longer time than the earlier experiment, at the end of each cycle all the tubing was renewed so that the flows of solution were maintained at a constant level during the experiment.

A similar procedure was followed for the MFSL, but three treatments were included with two replicates in each cycle. The first treatment was a control percolated with $(NH_4)_2SO_4$ solution in each cycle. In the second treatment, in cycles 1 and 3, the $(NH_4)_2SO_4$ solution had added DCD at the same concentration as the MSL, and in the third treatment $(NH_4)_2SO_4$ solution had added DCD at twice the concentration (equivalent to 40 kg DCD ha^{-1}) as the MSL. As for the MSL, the old perfusate from cycles 1 and 3 was replaced with fresh perfusate containing only $(NH_4)_2SO_4$ solution for cycles 2 and 4. The treatments used in both MSL and MFSL are summarised in Table 3.2. Throughout the experiment, 10-mL samples of perfusate were collected at intervals and analysed for NH_4^+-N and NO_3^--N. At the end of each cycle a small sample of soil (equivalent to 5.0 g) was taken from each column for estimation of the AOB population using the most probable number (MPN) technique.

Table 3.2 Treatments applied to the MSL and MFSL soils.

	Soil types				
	MSL		MFSL		
Cycles	Treatments		Treatments		
	1	2	1	2	3
1	Control	+DCD	Control	+DCD	++DCD
2	Control	-DCD	Control	-DCD	-DCD
3	Control	+DCD	Control	+DCD	++DCD
4	Control	-DCD	Control	-DCD	-DCD

Control and -DCD ($(NH_4)_2SO_4$ solution only); + DCD ($(NH_4)_2SO_4$ + 20 kg DCD ha^{-1}) and ++ DCD ($(NH_4)_2SO_4$ + 40 kg DCD ha^{-1}).

3.2.6 Estimation of AOB populations

The estimation of AOB numbers in the three New Zealand pasture soils and the effects and residual effects of DCD on the AOB population was carried out using the MPN technique with ammonium calcium carbonate media as described by Schmidt and Belser (1994).

3.2.6.1 MPN media

Fresh ammonium (1 mM) medium was prepared from individual stock solutions of the component reagents. The reagents used to prepare the individual stock solutions were as follows (the numbers in brackets are the weight of the chemicals in grams used to make up 100 ml of the stock solutions): $(NH_4)_2SO_4$ (5.0), $CaCl_2.2H_2O$ (1.34), $MgSO_4.7H_2O$ (4.0), bromothymol blue (0.04) and KH_2PO_4 (2.72). The chelated iron stock solution contained $FeSO_4.7H_2O$ (0.246) and EDTA disodium (0.331). The trace elements were combined in one stock solution which consisted of $Na_2MoO_4.2H_2O$ (0.01), $MnCl_2$ (0.02), $CoCl_2.6H_2O$ (0.0002), $ZnSO_4.7H_2O$ (0.01) and $CuSO_4.5H_2O$ (0.002). From each of these individual stock solutions, 1 L of media was prepared by pipetting 10 mL $(NH_4)_2SO_4$, 1.0 mL $CaCl_2.2H_2O$, 1.0 mL $MgSO_4.7H_2O$, 5.0 mL bromothymol blue, 7.5 mL KH_2PO_4 and 1.0 mL from each of the chelated iron and trace element mixtures and making the solution up to 1 L with deionised water. The pH of this media was buffered to pH 7.2 by adding 2.0% (w/v) K_2CO_3. Four-mL aliquots of this media were then added to the tubes used for the MPN assay (referred to hereafter as MPN tubes) and were sterilised for 15 min at 121^{o}C.

3.2.6.2 Dilution bottles

Fresh phosphate buffer (1 mM) was prepared by mixing 4 mL of K_2HPO_4 (from the stock solution prepared in the previous section) with 1 mL of KH_2PO_4 stock solution (prepared by dissolving 3.48 g of KH_2PO_4 in 100 mL of deionised water) and making the total volume up to 1 L. The final pH of this buffer fell in the range of 7.1-7.4. Nine-mL aliquots of this solution were then added to 25 mL glass bottles (referred to hereafter as dilution bottles) which were then sterilised for 15 min at 121^{o}C.

3.2.6.3 MPN procedure

The estimation of AOB numbers was first carried out on fresh samples of the three New Zealand soils described in Section 3.2.1. Three replicate sub-samples (2 g) of each of the 2-mm sieved soils were used. Each of the weighed soil samples was placed in 200 mL glass bottles and was homogenised well with 18 mL of 1mM sterilised phosphate buffer and then shaken for an hour in a horizontal shaker. This soil solution was referred as the 10^{-1} dilution. One-mL of the 10^{-1} dilution was then transferred into a new dilution bottle containing 9 mL of sterilised buffer by using a sterilised pipette and the solution was shaken vigorously (10^{-2} dilution). Continued dilutions were made up to 10^{-9}. To avoid contamination, starting from the highest serial dilution prepared (10^{-9}) 1-mL aliquots of each dilution were used to inoculate each of five replicate MPN tubes of sterilised growth medium containing the 1 mM NH_4^+ solution.

At the end of each cycle in the experiment described in Section 3.2.4, 2.5 g of moist soil was taken from each of the soil columns. The soil was kept in an individual plastic bottle and was stored at 4^{o}C for MPN analysis to be carried out the next day using a similar MPN procedure to that described above.

3.2.6.4 Cell counts

All the MPN tubes were incubated in the dark at 28^{o}C in an incubator. At intervals of 1 week for a maximum of 12 weeks, each series of tubes was tested for NO_2^-. Change of colour from blue green to yellow indicated a pH drop due to acid production during the nitrification process and was regarded as a positive indication of the presence of AOB. The colour change was also compared with the uninoculated or control tubes. A qualitative spot test for NO_2^- was also carried out in the MPN tubes that showed positive results by using the Griess-Ilosvay colorimetric spot test, in which colour production (pink to red) indicates that NO_2^- is present and confirmed that had tested positive for AOB (Schmidt and Belser 1994).

To the MPN tubes that didn't show any colour change from blue green to yellow (mainly in the higher dilution series), a similar procedure to that described above was carried out to test for the presence of NO_2^-. If these MPN tubes did not show a colour change from pink to red, this confirmed the negative test for AOB. A further NO_3^- test was carried with a test strip (Merckoquant 1.10020, Darmstadt, Germany) and with a diphenylamine spot test in these MPN tubes that had tested negative for NO_2^-. If a reddish colour develops on the test strip or there is development of blue/violet colour from the colourless diphenylamine spot test solution, this is taken as a positive test for AOB on the basis that the initial negative reading for NO_2^- meant only that NO_2^- formed by AOB was oxidised to NO_3^- by *Nitrobacter* (Schmidt and Belser 1994). However on all occasions in this present study, the MPN tubes that previously tested negative for NO_2^- also tested negative for the NO_3^- test and therefore these tubes were scored as negative for AOB.

The estimates of AOB population were obtained from the published MPN tables for ten-fold dilutions (initial dilution) with 5 replicate tubes per dilution. The MPN obtained was then multiplied by the actual initial dilution after taking into account the original moisture content of the 2 g subsample of soil used for the assay (Woomer 1994). The MPN values in Figures 3.7 to 3.16 are given as cells per gram soil dry weight (cells g^{-1} SDW).

3.2.7 Statistical analysis

The mean concentrations of NH_4^+-N, NO_3^--N, mean nitrification rates and AOB populations were calculated from the number of replicates in each treatment, and least significant difference (l.s.d.) values were calculated following analysis of variance using SAS v 9.3 (SAS 2011). The 5% confidence level is regarded as statistically different.

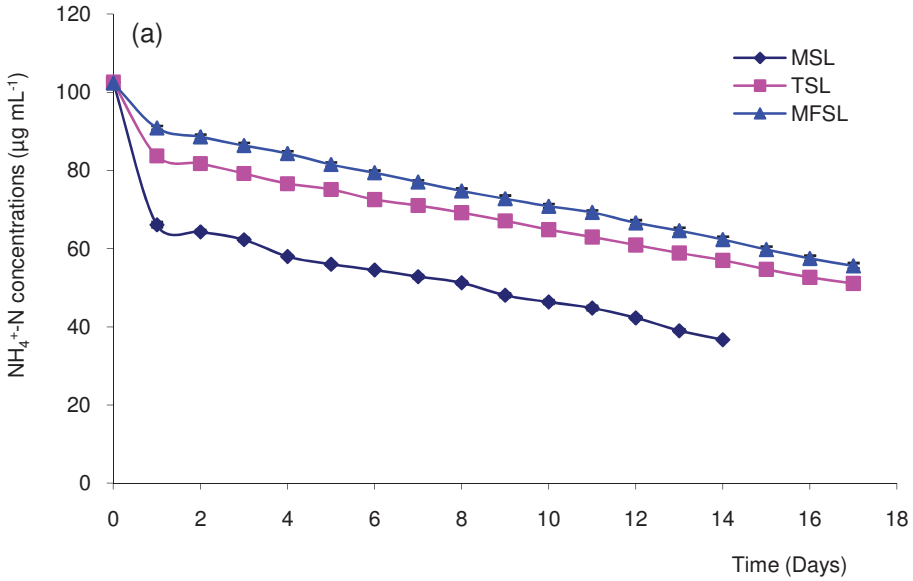
3.3 Results

3.3.1 Nitrification rates in the three pasture soils

The perfusion technique was very useful for demonstrating and measuring the changes in nitrification activity over time in the three tested soils. The rates of decrease in NH_4^+-N concentrations (Figure 3.2 (a)) and increases in NO_3^--N concentration (Figure 3.2 (b)) varied between soils. In the MSL soil, there was a more rapid decrease of NH_4^+-N and a faster build-up of NO_3^--N compared with the TSL and MFSL soils. The concentration of NH_4^+-N in the perfusate was reduced to 36% of the original concentration within 14 days of the commencement of the experiment in MSL whereas in TSL and MFSL, the concentration of NH_4^+-N had only dropped to 56 and 61% of its original level in the

same time. Even after a further 3 days, the concentrations of NH_4^+-N in the perfusate of the TSL and MFSL had only dropped to 50 and 54% of the original levels.

There was a large reduction in the NH_4^+-N concentration during day 1 of the experiment in all three soils (Figure 3.3 (a)). However, there was no correspondingly large increase in the concentration of NO_3^--N on day 1 (Figure 3.3 (b)). The decrease in the NH_4^+-N concentration during day 1 was therefore likely due to the adsorption of the NH_4^+ onto the negative charged of soil particles, and was positively associated with the soil CEC. This is also demonstrated by the decrease in the concentration of total mineral N in the perfusing solution in the three soils (Figure 3.4 (a), (b) and (c)) during the first day of the experiment. The concentrations of total mineral N thereafter in all the three soils were nearly constant although there was a slight increase in the MSL.



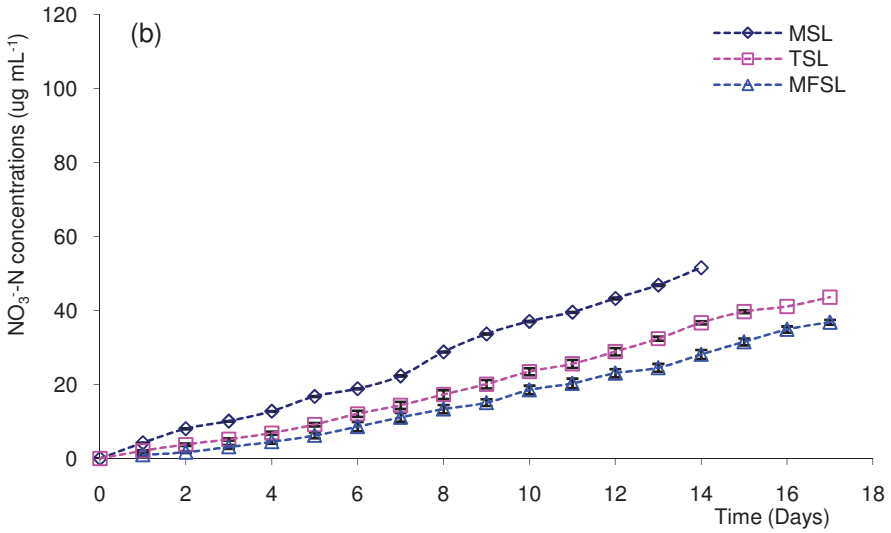
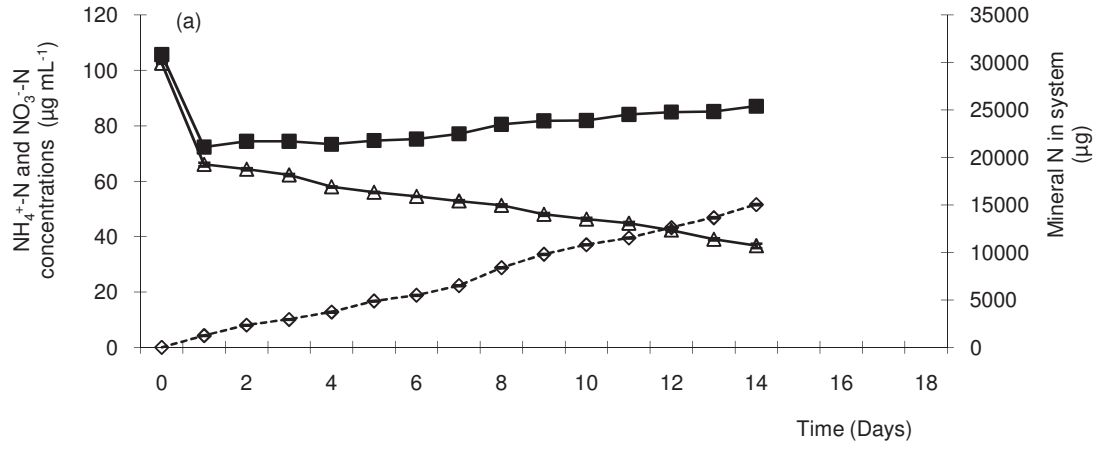


Figure 1 3.3 Decrease in NH_4^+-N concentrations in the perfusing solutions over time (a) and increase in NO_3^--N concentrations over time (b) in three New Zealand pasture soils. Vertical bars denote the standard errors of six replicates.



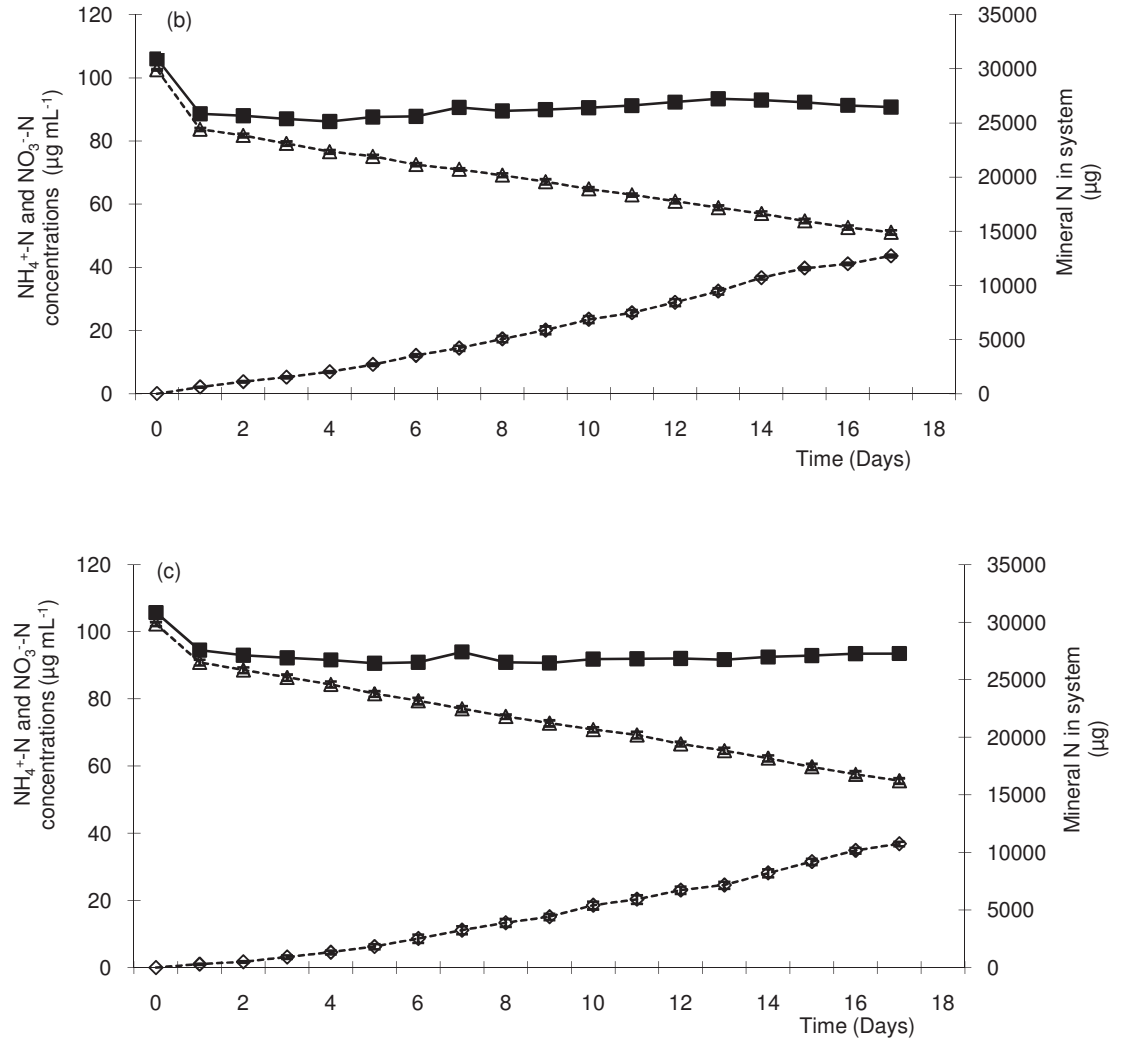


Figure 3.4 Mean changes in the concentrations of inorganic N over time in (a) MSL, (b) TSL and (c) MFSL during the perfusion experiment. Error bars represent the standard errors of the six replicates. Symbols represent total mineral N (-■-), NH_4^+-N (-△-) and NO_3^-–N (-◇-).

The mean nitrification rate in MSL (30.3 µg NO_3^--N g^{-1} soil day^{-1}) was higher (P < 0.05) and was twice that in the MFSL (14.4 µg NO_3^--N g^{-1} soil day^{-1}). The nitrification rate in the TSL (18.5 µg NO_3^--N g^{-1} soil day^{-1}) was intermediate between the other two soils (Figure 3.5). The cumulative NO_3^--N formed over the 14 day of measurement period in MSL was higher (P < 0.05) (3179 µg NO_3^--N g^{-1} soil) than in TSL (2059 µg NO_3^--N g^{-1} soil) and MFSL (1471 µg NO_3^--N g^{-1} soil). The nitrification rates in all the

three soils varied considerably from day to day (Figure 3.6 (a)) and it was difficult to see consistent differences between soils or over time. This could be due in part to the daily temperature fluctuations during the experiment, ranging from 15 to 20°C. However, when the short term daily variations (Figure 3.6 (a)) were smoothed out by re-plotting the data as a 4-day running average (Figure 3.6 (b)) (the nitrification rate for day 4 was calculated as the average of days 1-4; for day 5, the average of days 2-5; for day 6, the average of days 3-6 etc until the end of the experiment), the nitrification rates were more consistent and were highest in MSL, followed by TSL and MFSL. There was no marked change in the nitrification rates over the course of the experiment.

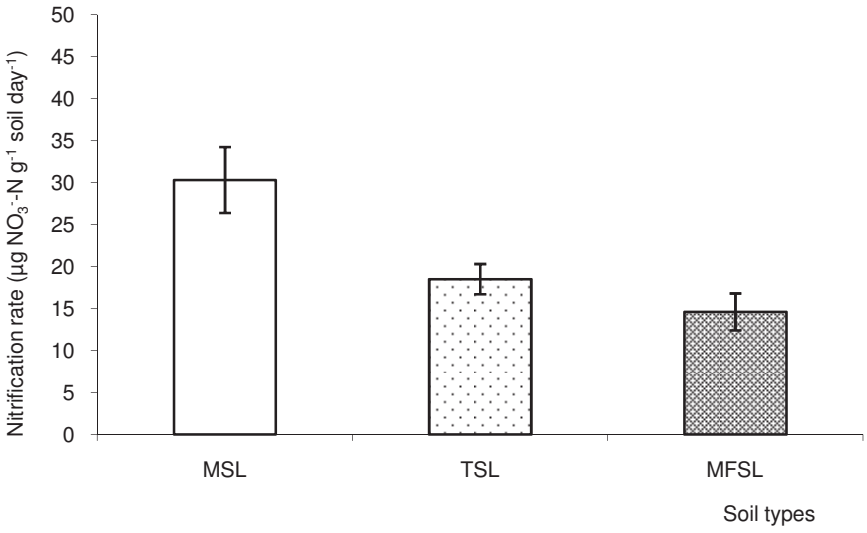


Figure 3.5 Mean nitrification rates in three New Zealand pasture soils during the experimental period. Vertical bars denote the standard errors of six replicates.

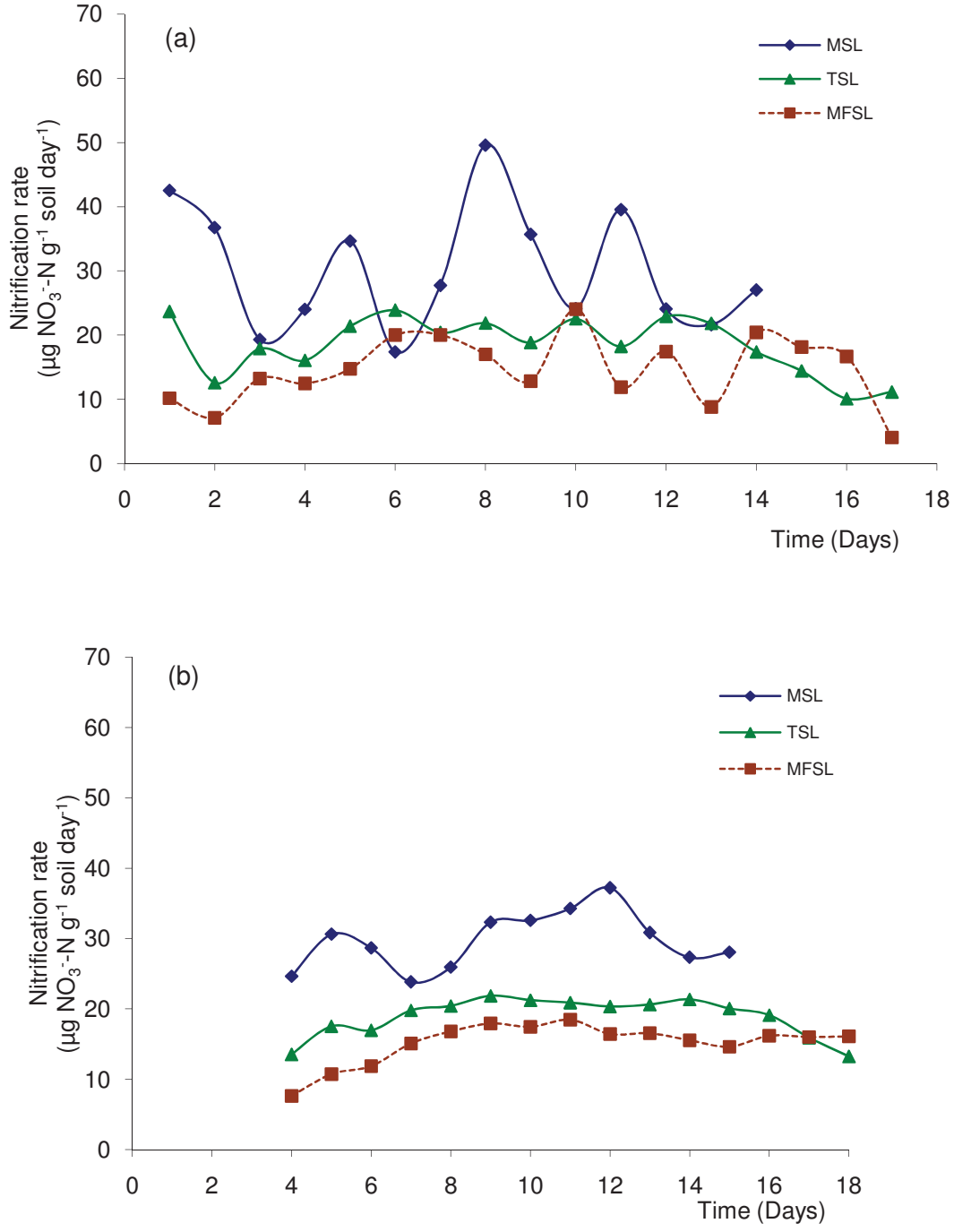


Figure 3.6 Daily (a) and 4-day moving average (b) nitrification rates in three New Zealand pasture soils during perfusion.

3.3.2 Numbers of AOB in the three original soils

3.3.2.1 Weekly AOB growth and the estimated counts in the three original soils

During the MPN incubations the estimates of AOB populations in the three original soils increased steadily from week 1 to week 8 (Figure 3.7) and then remained constant until week 12. This suggests that the 12 weeks of incubation used to estimate the populations of AOB was easily long enough to ensure that all the tubes that were inoculated with viable cells had sufficient time for the cells to multiply and produce acid from nitrification.

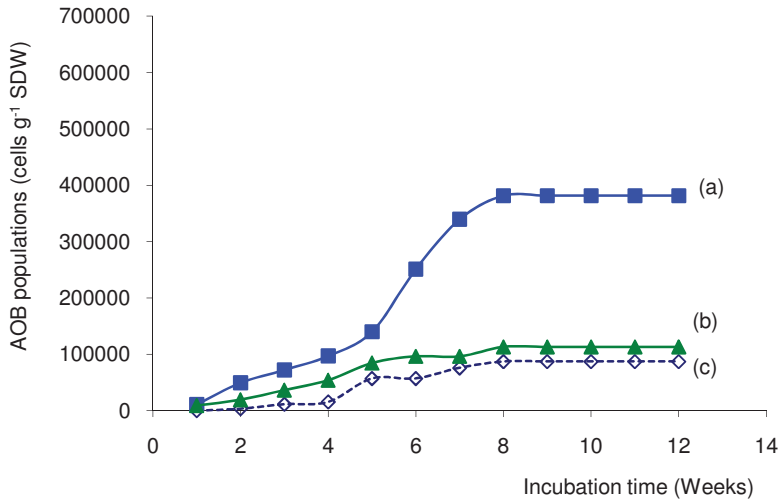


Figure 3.7 Estimate of AOB populations in (a) MSL, (b) MFSL and (c) TSL with time of incubation in growth medium after serial dilution.

The estimates of AOB population in the MSL (3.8 x 10^5 cells g^{-1} SDW), based on the maximal counts of AOB reached after 8 weeks of incubation (Figure 3.8) were 3 and 4 fold higher *($P < 0.05$)* than in the TSL (1.1 x 10^5 cells g^{-1} SDW) and the MFSL (8.7 x

10^4 cells g^{-1} SDW) respectively (Figure 3.8). The greater numbers of AOB found in the MSL corresponded to the higher nitrification rates obtained in this soil (Figure 3.9).

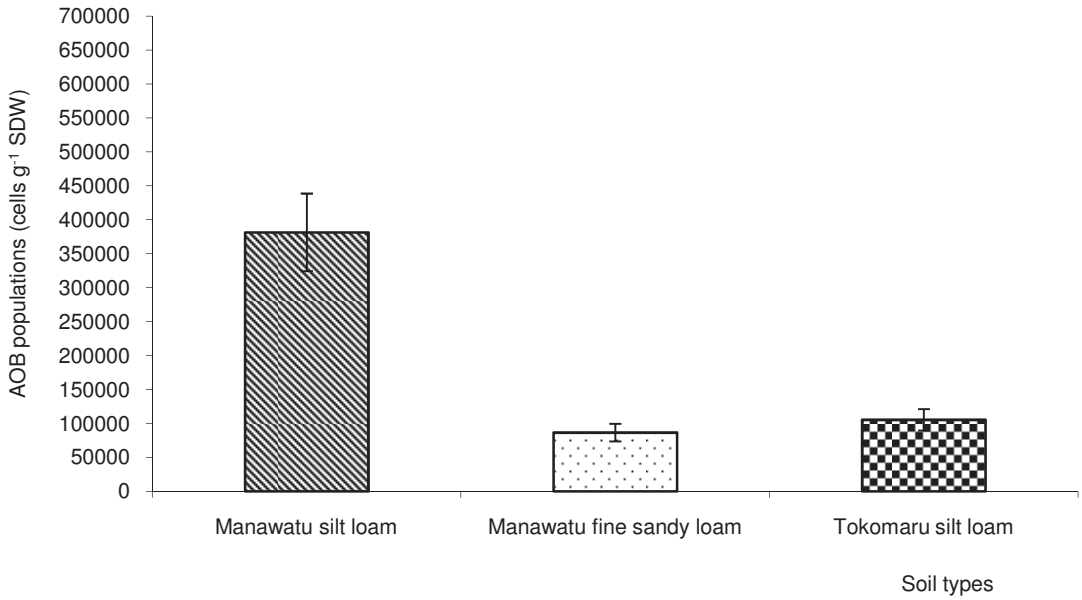


Figure 3.8 The estimated populations of AOB present in the original soil samples. Vertical bars in the columns indicate the range of AOB numbers obtained in the three replicates in each soil.

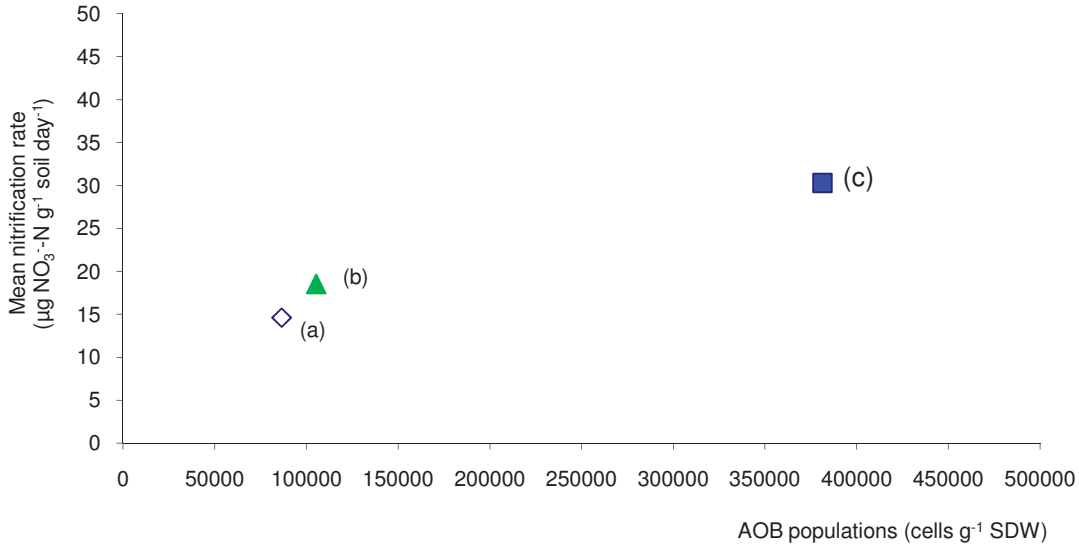


Figure 3.9 Mean nitrification rate and the estimated numbers of AOB in (a) MFSL and (b) TSL and (c) MSL.

3.3.3 Effect of DCD on soil nitrification activity in MSL

3.3.3.1 Nitrification activity

Higher concentrations of NH_4^+-N ($P < 0.05$) were obtained in the perfusate containing DCD in cycles 1 and 3 Figure 3.10 (a) and (c) compared to the without-DCD (control) treatment. In the control treatment in cycles 1 and 3, the concentrations of NH_4^+-N decreased rapidly with time and the amounts of NO_3^--N accumulated were higher in cycle 1 ($P < 0.05$) and in cycle 3 than with the addition of DCD. When the perfusates in cycles 1 and 3 were replaced with fresh $(NH_4)_2SO_4$ solution in cycles 2 and 4 (Figure 3.10 (b) and (d)), there was a recovery in the nitrification activity with an increase in the concentrations of NO_3^--N in the perfusate, but these remained lower than in the control soils.

In both treatments (with- and without-DCD) there was a large reduction in NH_4^+-N concentrations during the first day of the experiment in all the cycles, while there was no large increase in the amount of NO_3^--N during that period (Figure 3.10). As suggested in the earlier experiment, the large reduction in NH_4^+-N concentration on day 1 was likely due to the adsorption of NH_4^+ onto the MSL soil particles. This conclusion was supported by the decrease in the mass balance of total mineral N during the first day of the experiment in the perfusing solution in all the cycles (Figure 3.11 (a) – (d)).

After day 2 there were small increases in the total mineral N in cycle 1 for the control treatment, while there was a slight decline with the DCD addition. In cycle 2 slight increases in the total N mass balance for both treatments were obtained. Slight declines of total mineral-N were observed in cycles 3 and 4 for both treatments. The results suggest that although there might be slight losses of N from the system, perhaps through

volatilisation or denitrification, no major losses of N occurred. DCD added to the soil columns in cycles 1 and 3 resulted in slightly higher total mineral N (Figure 3.11 (a) and (c)) than in the control soil column.

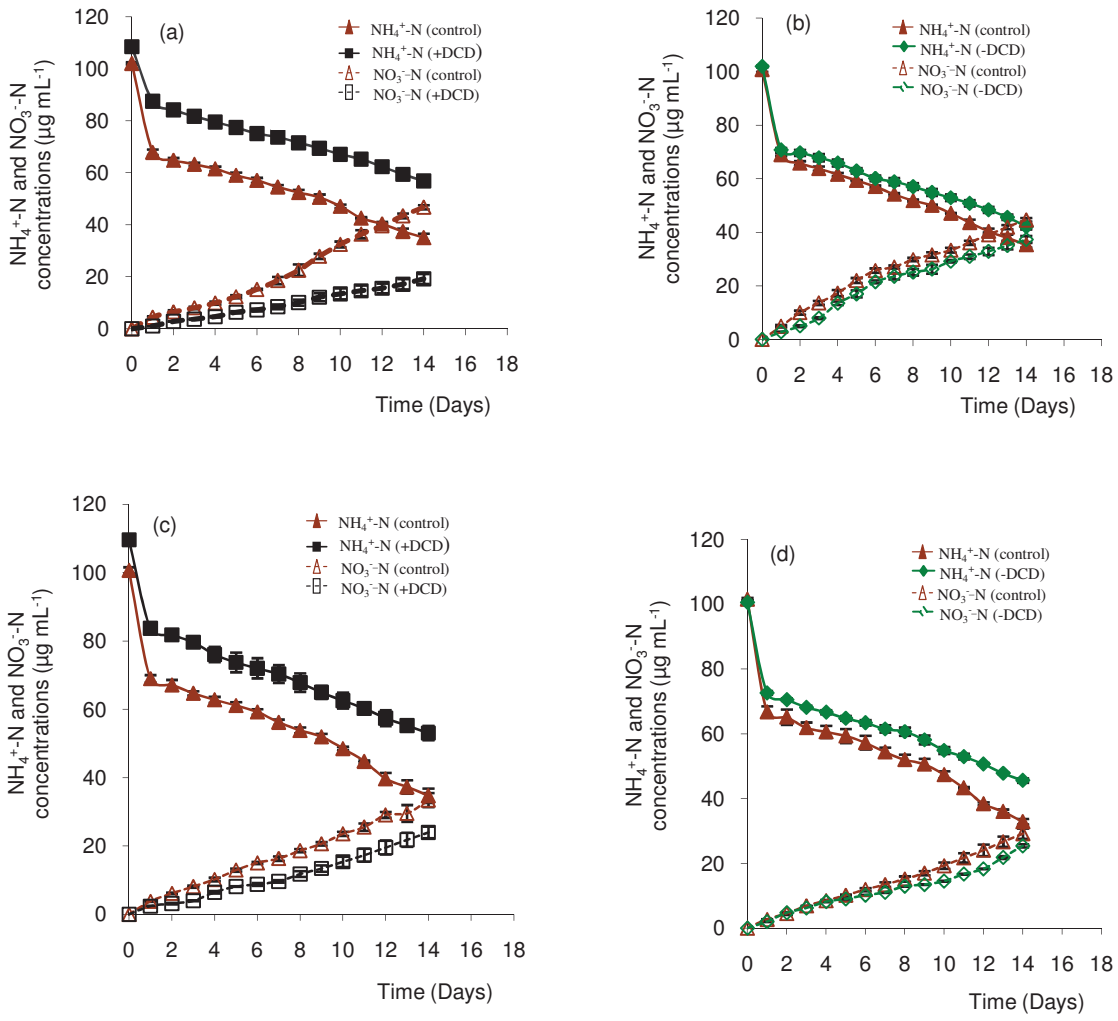


Figure 3.10 Mean concentrations of NH_4^+-N and NO_3^--N in MSL in (a) cycle 1, (b) cycle 2, (c) cycle 3 and (d) cycle 4, during the perfusion experiment. The soils in the perfusion apparatus in cycles 1 and 3 received either $(NH_4)_2SO_4$ solution only (control) or $(NH_4)_2SO_4$ solution with DCD at 20 kg ha^{-1} (+DCD), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only (control and –DCD). Error bars represent the standard errors of the three replicates.

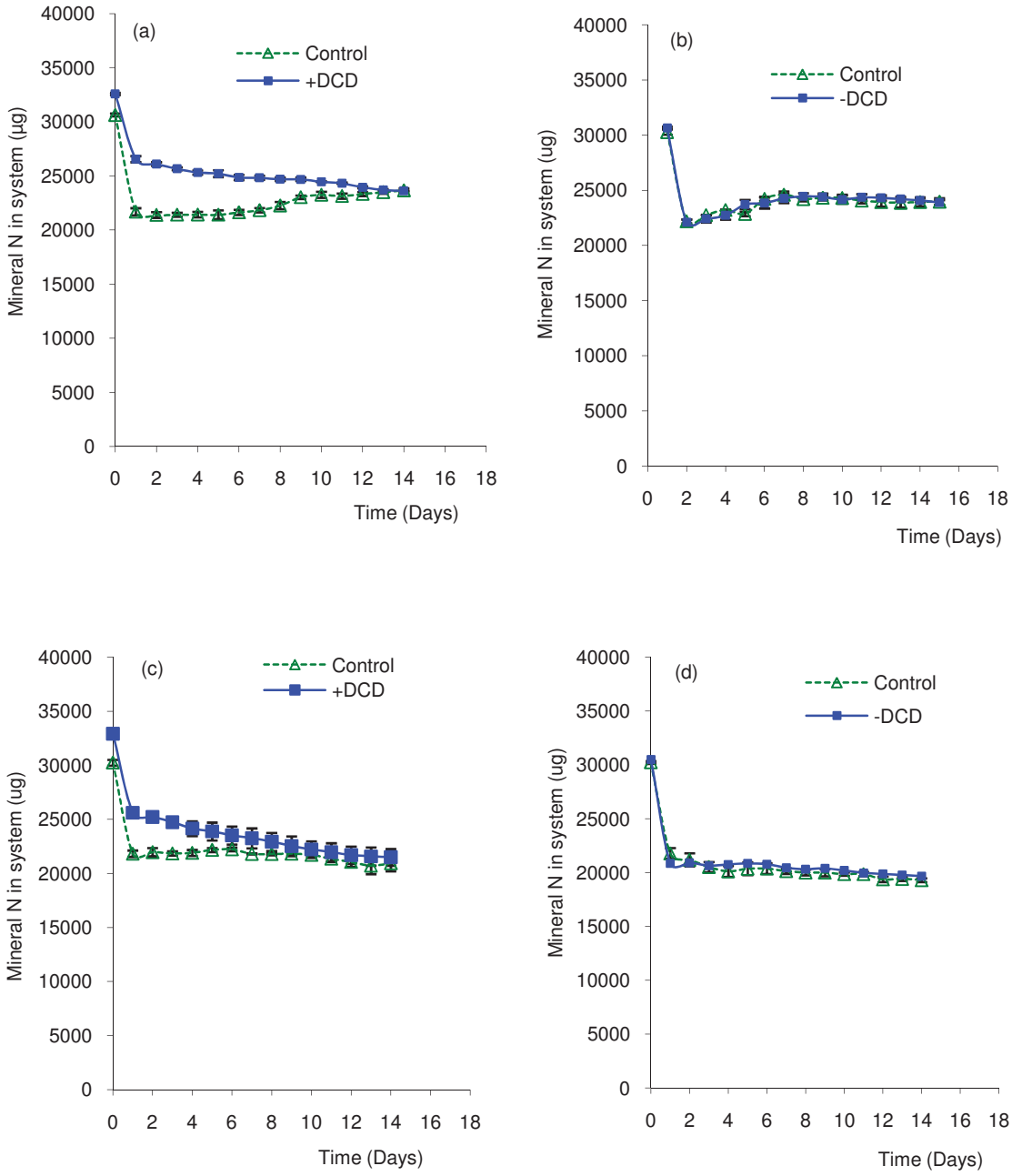


Figure 3.11 Mean total mineral-N in the perfusion system during the experiment with MSL in (a) cycle 1, (b) cycle 2, (c) cycle 3 and (d) cycle 4. The soils in the perfusion apparatus in cycles 1 and 3 received either $(NH_4)_2SO_4$ solution only (control) or $(NH_4)_2SO_4$ solution with DCD at 20 kg ha^{-1} (+DCD), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only (control and –DCD). Error bars represent the standard errors of the three replicates.

The rates of nitrification in the control treatment in all the cycles were reasonably constant over time (Figure 3.12). The addition of DCD in cycles 1 and 3 inhibited the nitrification activity, but it didn't completely halt nitrification. The average rate of NO_3^--N production over the 14 day period in cycle 1 decreased ($P < 0.05$) from 30.4 to 15.8 µg NO_3^--N g^{-1} soil day^{-1} with the addition of DCD (Figure 3.12). The average rate of nitrification in the presence of DCD was therefore only 52% of that in the control treatment.

When the perfusate in cycle 1 was replaced with fresh $(NH_4)_2SO_4$ in cycle 2, the nitrification rate in the soil column that had previously received the DCD treatment increased to reach 82% of the control treatment. The second addition of DCD (cycle 3) again reduced the nitrification rate but not to the same extent as in cycle 1, and this was not statistically significant ($P > 0.05$). The mean nitrification rate was 65% of that in the control soil with an average rate of NO_3^--N production of 18.3 µg NO_3^--N g^{-1} soil day^{-1} in the presence of DCD (Figure 3.12). In cycle 4, the nitrification rate in the soil that had previously received DCD (cycle 3) recovered to be 87% of that in the control soils.

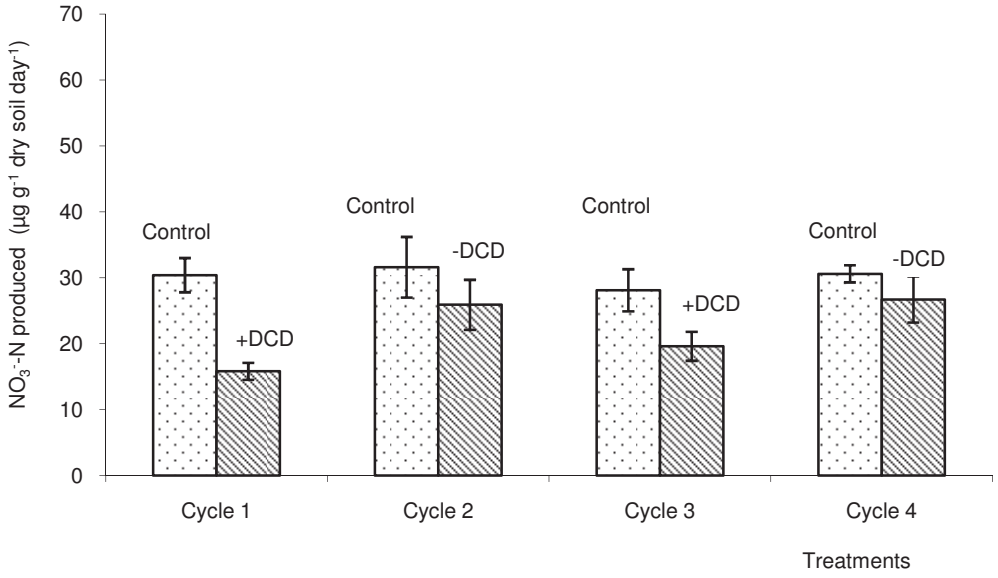


Figure 3.12 Mean nitrification rates in the MSL during the 4 cycles of the experiment. The MSL soils in the perfusion apparatus in cycles 1 and 3 received either $(NH_4)_2SO_4$ solution only (control) or $(NH_4)_2SO_4$ solution with DCD at 20 kg ha^{-1} (+DCD), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only (control and –DCD). Vertical bars are the standard errors for three replicates.

The daily nitrification rates obtained in all the cycles showed considerable variability (Figure 3.13 (i, a) – (iv, a). A clearer indication of the differences in nitrification rates between the treatments and cycles was obtained when the daily graphs were re-plotted as a 4-day moving average (Figure 3.13 (i, b) – (iv, b)). When plotted this way, although nitrification rate varied during each cycle, there was no consistent pattern observed. The addition of DCD in the MSL soil columns reduced the nitrification rates in cycles 1 and 3, while there was recovery in cycles 2 and 4 when the DCD solution was removed from the system and was replaced with the fresh N source.

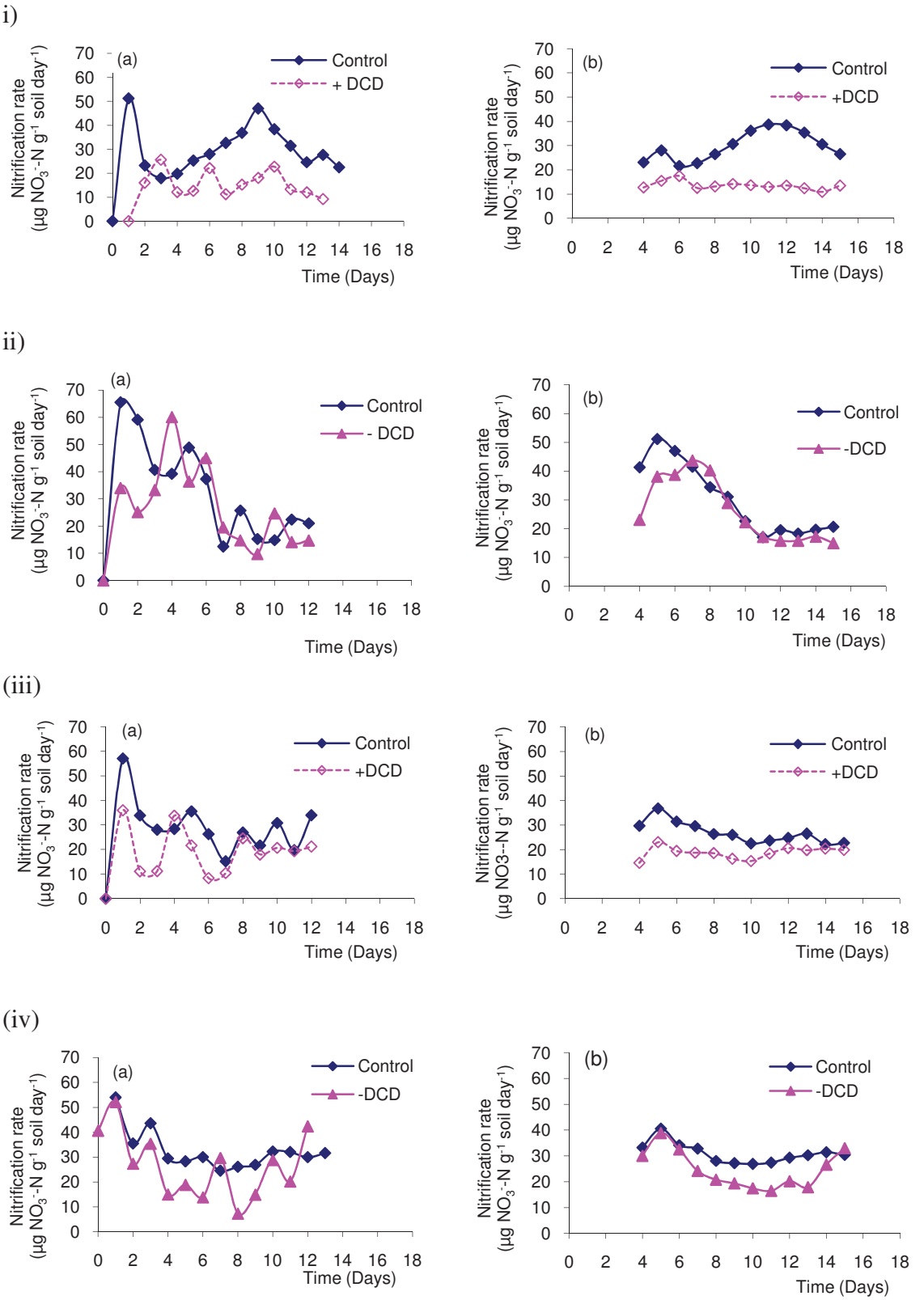


Figure 3.13 Daily (a) and 4-day moving average (b) nitrification rates in MSL in (i) cycle 1; (ii) cycle 2; (iii) cycle 3 and (iv) cycle 4. The soils in the perfusion apparatus in cycles 1 and 3 received either $(NH_4)_2SO_4$ solution only (control) or $(NH_4)_2SO_4$ solution with DCD at 20 kg ha^{-1} (+DCD), while in cycles 2 and 4, all the soils received $(NH_4)_2SO_4$ solution only (control and –DCD).

3.3.3.2 AOB populations

As was the case for the original soils (Figure 3.7), 8 weeks of incubation was sufficient for the viable cells in each of the tubes to multiply and perform detectable nitrification. No further increases in population estimates were obtained when the incubation was extended to 12 weeks (Figure 3.14). The final estimations of AOB numbers in this experiment (Figure 3.15) were therefore based on the 8-week incubation period.

The addition of $(NH_4)_2SO_4$ solution to the control soil columns in all the cycles resulted in a small non-significant ($P > 0.05$) increase in nitrifier populations with AOB numbers in the range 5.1 – 5.9 x 10^5 cells g^{-1} SDW, compared to those obtained in the original MSL (3.8 x 10^5 cells g^{-1} SDW (Figure 3.8). The populations of AOB and the effect of DCD on these populations followed the same patterns as observed in the nitrification rates (Figure 3.15). The numbers of nitrifiers in the control treatments in all cycles were similar (Figure 3.15). However, addition of DCD in cycles 1 and 3 consistently reduced AOB numbers compared to the controls (Figure 3.14 and 3.15). This suppression of AOB numbers was slightly smaller in cycle 3 than in cycle 1 although the difference was not statistically significant ($P > 0.05$). There was a slight recovery in the AOB numbers in cycles 2 and 4 when the DCD solution was removed from the system and was replaced with a fresh N source.

Overall, it appears that DCD inhibits the nitrification activity in MSL, however complete inhibition was not obtained, and there was some evidence of a recovery in the nitrifier populations when the DCD was removed. The second addition of DCD resulted in a similar suppression of nitrification activity to the first addition in the MSL soil.

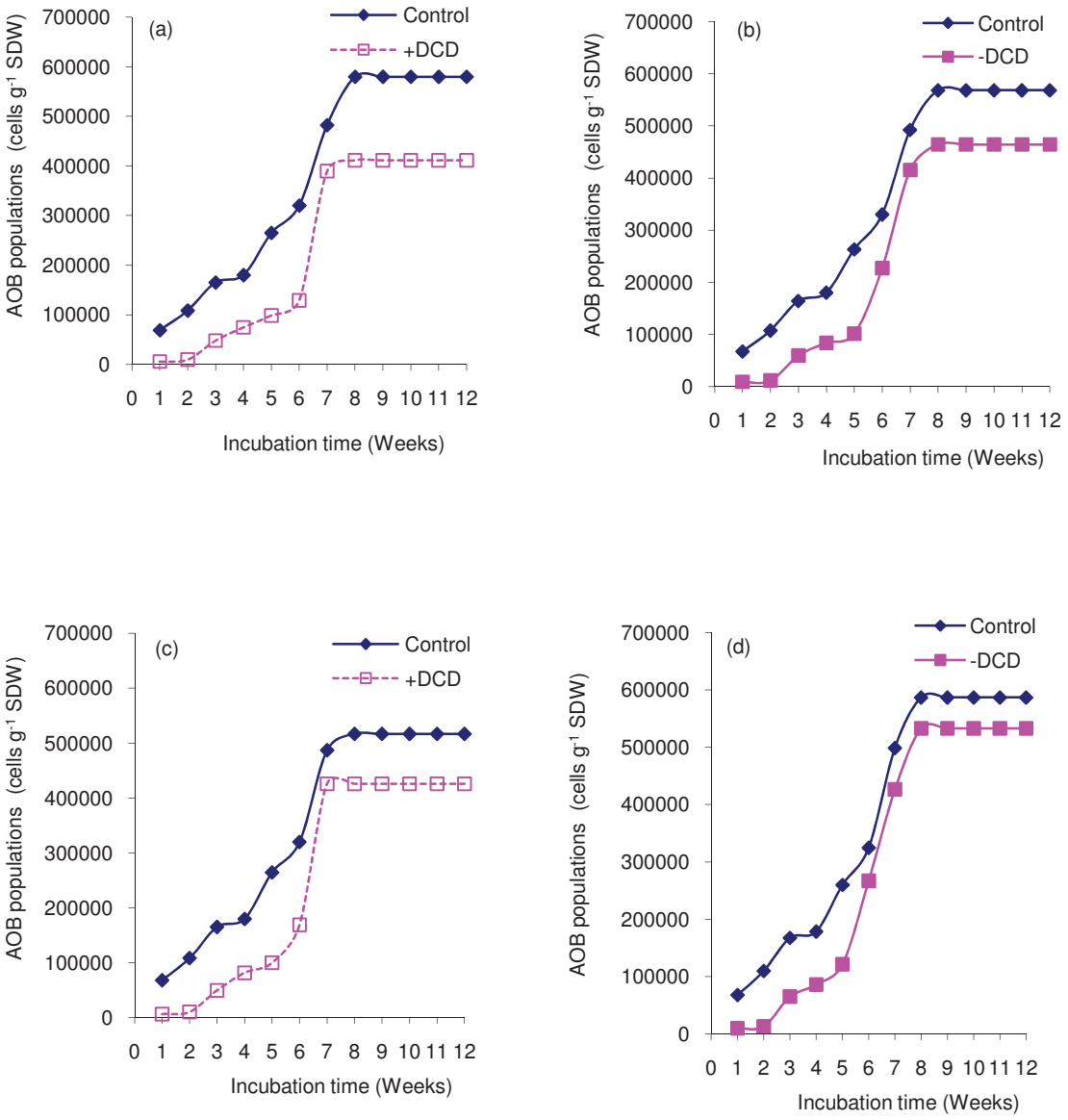


Figure 3.14 Estimates of AOB populations in the MSL with time of incubation in growth media after serial dilution of extracts from soils sampled at the end of (a) cycle 1; (b) cycle 2; (c) cycle 3 and (d) cycle 4. The soils in the perfusion apparatus in cycles 1 and 3 initially received $(NH_4)_2SO_4$ solution only (control) or $(NH_4)_2SO_4$ solution with 20 kg DCD ha^{-1} (+DCD), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only (control and -DCD).

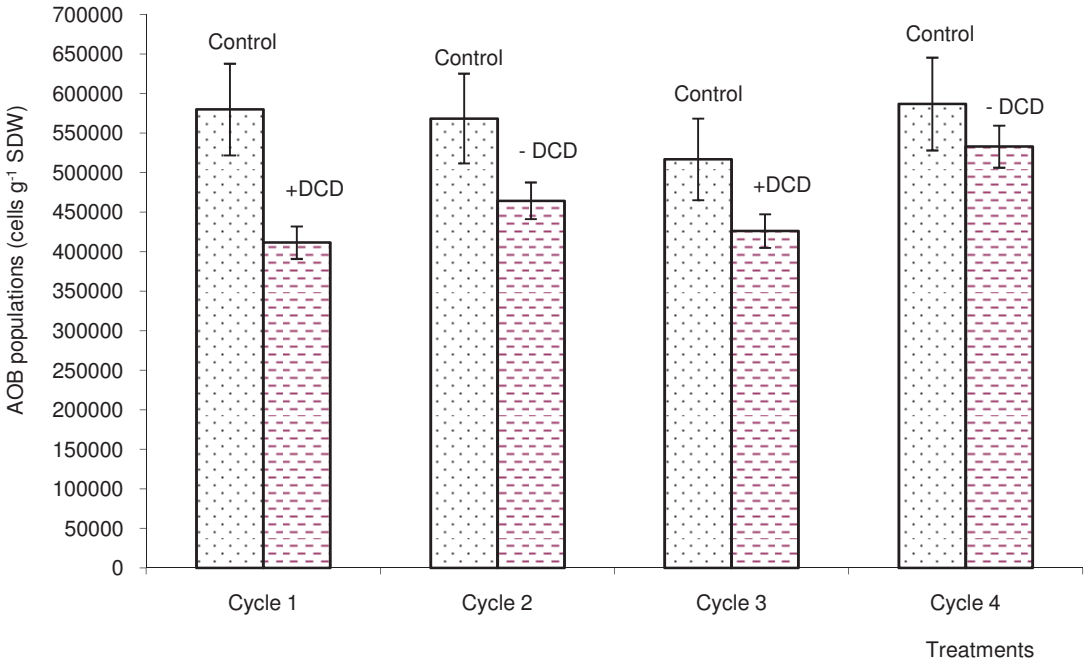


Figure 3.15 AOB populations in the perfusion columns containing MSL soil after each cycle. Control and -DCD ($(NH_4)_2SO_4$ solution only) and + DCD ($(NH_4)_2SO_4$ solution + 20 kg DCD ha^{-1}). Vertical bars in the columns indicate the range of AOB numbers obtained in the 3 replicates in each treatment.

3.3.4 Effect of DCD on soil nitrification activity in MFSL

3.3.4.1 Nitrification activity

The concentrations of NH_4^+-N in the perfusate from the MFSL soil remained higher in the presence of added DCD (at either rate) in cycles 1 and 3 than in the control soils, although the NH_4^+-N concentrations were greater ($P < 0.05$) in the columns with higher rate of DCD (Figure 3.16 (a) and (c)). These higher concentrations of NH_4^+-N in the presence of DCD corresponded with low accumulation rates of NO_3^--N, although there was a slightly larger ($P < 0.05$) build-up of NO_3^--N in cycle 3 than in cycle 1. A greater increase in the concentration of NO_3^--N was obtained in cycles 2 ($P < 0.05$) and 4 after

the perfusate in the soil columns receiving DCD (cycles 1 and 3) was replaced with new $(NH_4)_2SO_4$ solution without added DCD (Figure 3.16 (b) and (d)).

The mass balance for mineral N in the system in each of the cycles (Figure 3.17 (a) – (d)) decreased rapidly on day 1 but this reduction was smaller than in the MSL (Figure 3.11). This decrease in mineral N was caused by a rapid reduction in the concentrations of NH_4^+-N in Figure 3.16 (a) – (d) on day 1, with only a small increase in NO_3^-- N concentrations during this period. As mentioned earlier this is probably due to the adsorption of NH_4^+ onto the MFSL soil particles. The total amounts of mineral-N in each cycle for most of the treatments then continued to decline slightly with time (Figure 3.17) which may be due to volatilisation or denitrification of some of the N in the system. It is interesting to note that throughout the 4 cycles the recovery of total mineral N was slightly lower from the control treatment than from the treatments that been treated with DCD. This could be attributed to the decomposition of DCD, which itself contains N, and would break down over time in the perfusion column, into CO_2 and NH_4^+ (Slangen and Kerhoff 1984).

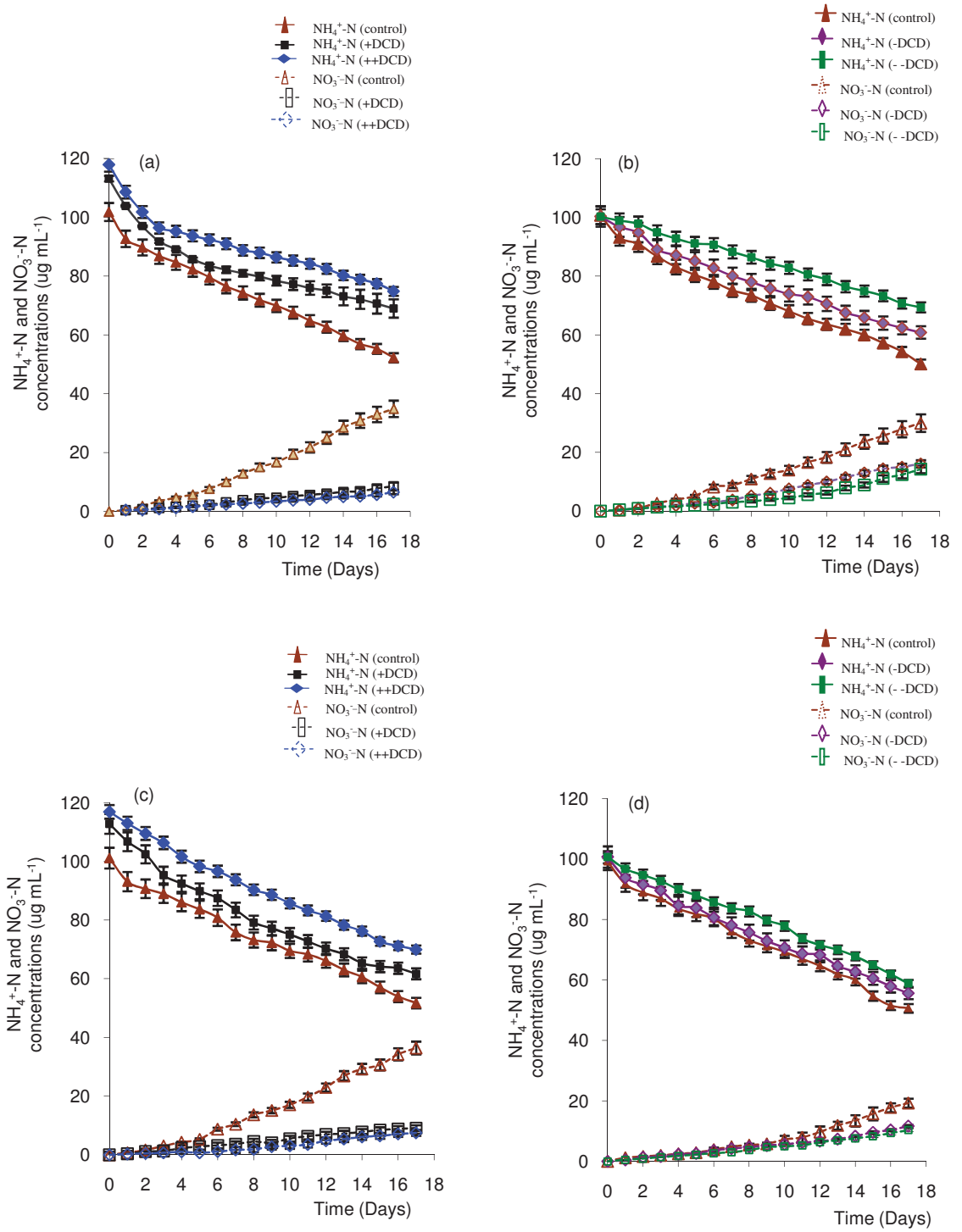


Figure 3.16 Mean concentrations of NH_4^+-N and NO_3^--N in MFSL in (a) cycle 1, (b) cycle 2, (c) cycle 3 and (d) cycle 4, during the perfusion experiment. The soils in the perfusion apparatus in cycles 1 and 3 received $(NH_4)_2SO_4$ solution only (control), $(NH_4)_2SO_4$ solution with 20 kg DCD ha^{-1} (+DCD) and $(NH_4)_2SO_4$ solution with 40 kg DCD ha^{-1} (++DCD), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only (control, –DCD and --DCD). Error bars represent the range of NH_4^+-N and NO_3^--N concentrations in the two replicates.

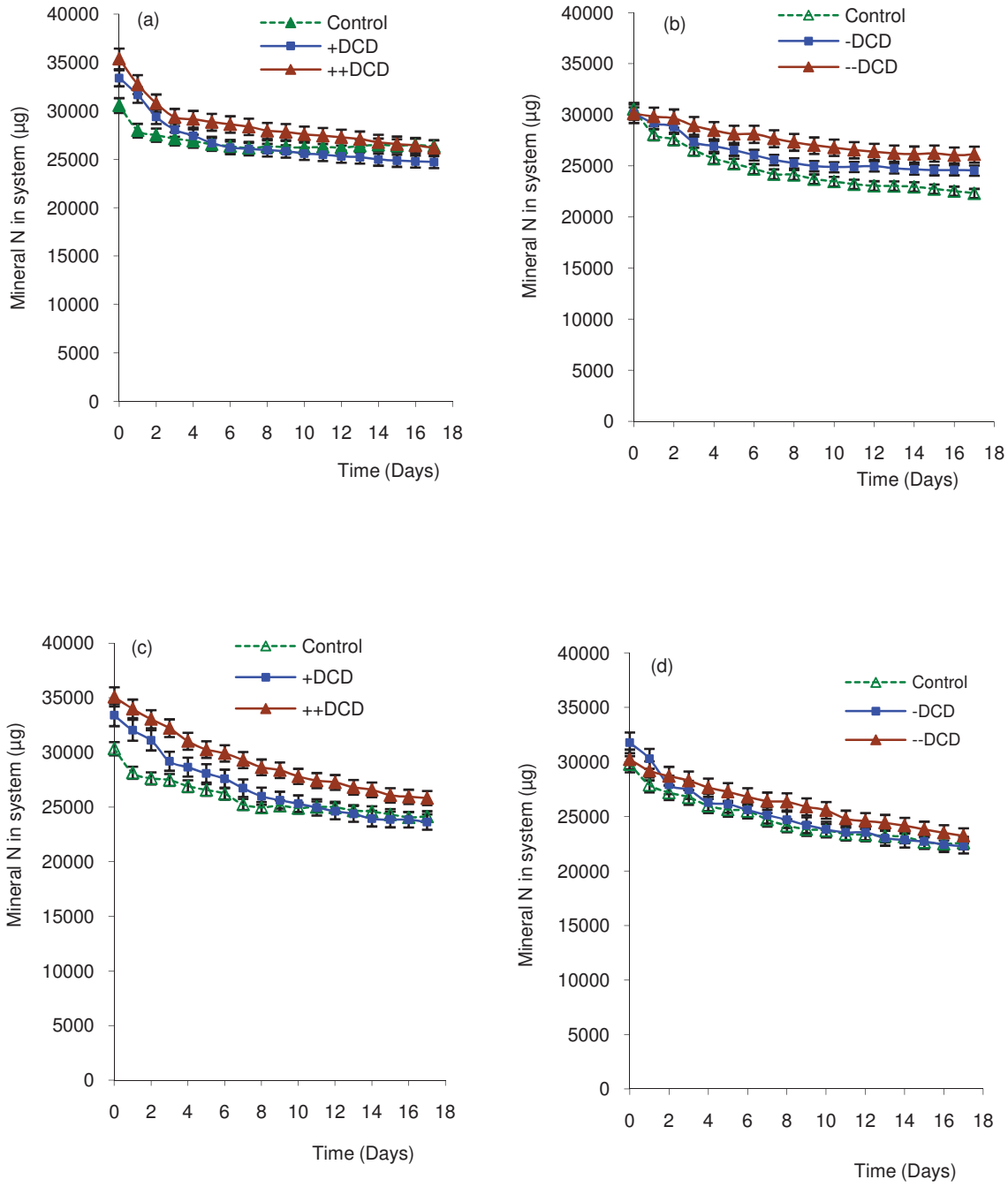


Figure 3.17 Mean mineral-N in the perfusion system during the experiment with MFSL (a) cycle 1, (b) cycle 2, (c) cycle 3 and (d) cycle 4. The soils in the perfusion apparatus in cycles 1 and 3 received $(NH_4)_2SO_4$ solution only (control), $(NH_4)_2SO_4$ solution with 20 kg DCD ha^{-1} (+DCD) and $(NH_4)_2SO_4$ solution with 40 kg DCD ha^{-1} (++DCD), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only (control, –DCD and --DCD). Error bars represent the range of mineral N values obtained in the two replicates.

The rate of nitrification in the control soil columns was reasonably constant between the cycles (Figure 3.18) and was lower than in the MSL soils (Figure 3.12). DCD added at the same rate as to the MSL was much more effective at reducing the rate of nitrification in the MFSL than in the MSL. The nitrification rate in cycle 1 reduced markedly ($P < 0.05$) from 14.2 to 3.6 µg NO_3^--N g^{-1} soil day^{-1}, which was only 25% of that in the control treatment. Doubling the rates of DCD decreased ($P < 0.05$) the nitrification rate still further to 2.7 µg NO_3^--N g^{-1} soil day^{-1}, which was only 19% of that in the control treatment. The second additions of the 2 rates of DCD in cycle 3 (Figure 3.18) caused smaller reductions in the nitrification rate relative to the control than occurred in cycle 1. The nitrification rates in cycle 3 in the low and high DCD treatments were 41 and 31% of those in the control treatment with average nitrification rates of 6.1 and 4.6 µg NO_3^--N g^{-1} soil day^{-1} respectively.

When the perfusates in cycles 1 and 3 were replaced with the fresh $(NH_4)_2SO_4$ solution in cycles 2 and 4 (Figure 3.16 (b) and (d) and Figure 3.18) the nitrification rates in the soil columns that had previously received the low and high rates of DCD increased to 53 and 42% respectively of those in the control treatments in cycle 2, and 64% and 56% in cycle 4. The slightly greater recovery in nitrification rate in cycle 4 than in cycle 2 was similar to that observed in the MSL.

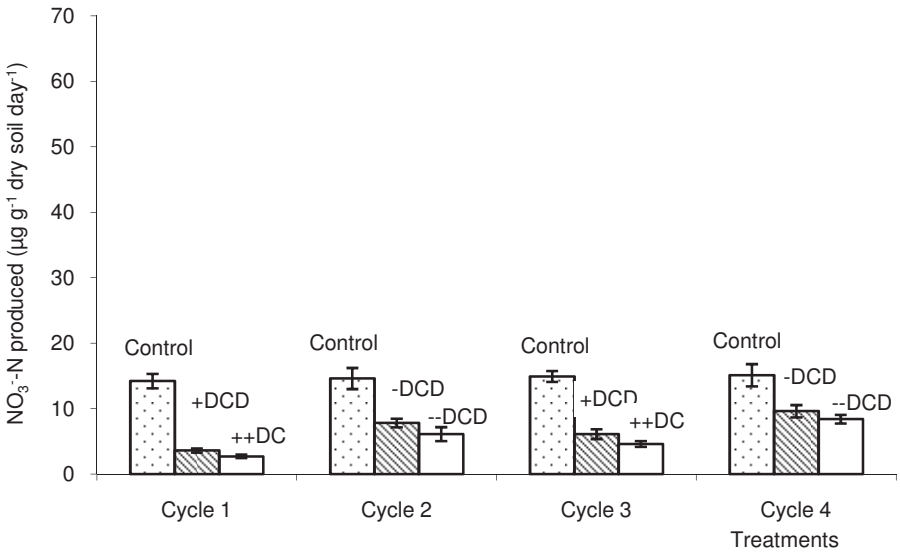


Figure 3.18 Mean nitrification rates in control soil, soil receiving DCD (cycles 1 and 3) and after DCD treatment (cycles 2 and 4) in MFSL. The MFSL soils in the perfusion apparatus in cycles 1 and 3 received $(NH_4)_2SO_4$ solution only (control) and $(NH_4)_2SO_4$ solution with 20 kg DCD ha^{-1} (+DCD and $(NH_4)_2SO_4$ solution with 40 kg DCD ha^{-1} (++DCD), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only (control, –DCD and --DCD). Vertical bars are the range of NO_3^--N obtained in the two replicates.

As was the case with the MSL, the daily nitrification rates in MFSL in each cycle varied considerably (Figure 3.19 (i, a) – (iv, a)), but a clearer picture of the changes in nitrification rates over time was obtained when the daily graphs were re-plotted as a 4-day running average (Figure 3.19 (i, b) – (iv, b)). As was the case with the MSL, when plotted in this way, although the nitrification rate varied during each cycle there was no consistent pattern observed. Greater inhibition in nitrification rates was obtained with the higher rates of DCD applied. The second addition of DCD in cycle 3 was slightly less effective at reducing nitrification rates than the first addition. The inhibited nitrification activity showed some recovery in cycles 2 and 4 and this recovery was greater in cycle 4. Overall, the DCD was more effective in reducing nitrification activity in the MFSL than MSL soils with a greater initial depression of nitrification activity and a lower rate of recovery when DCD was removed.

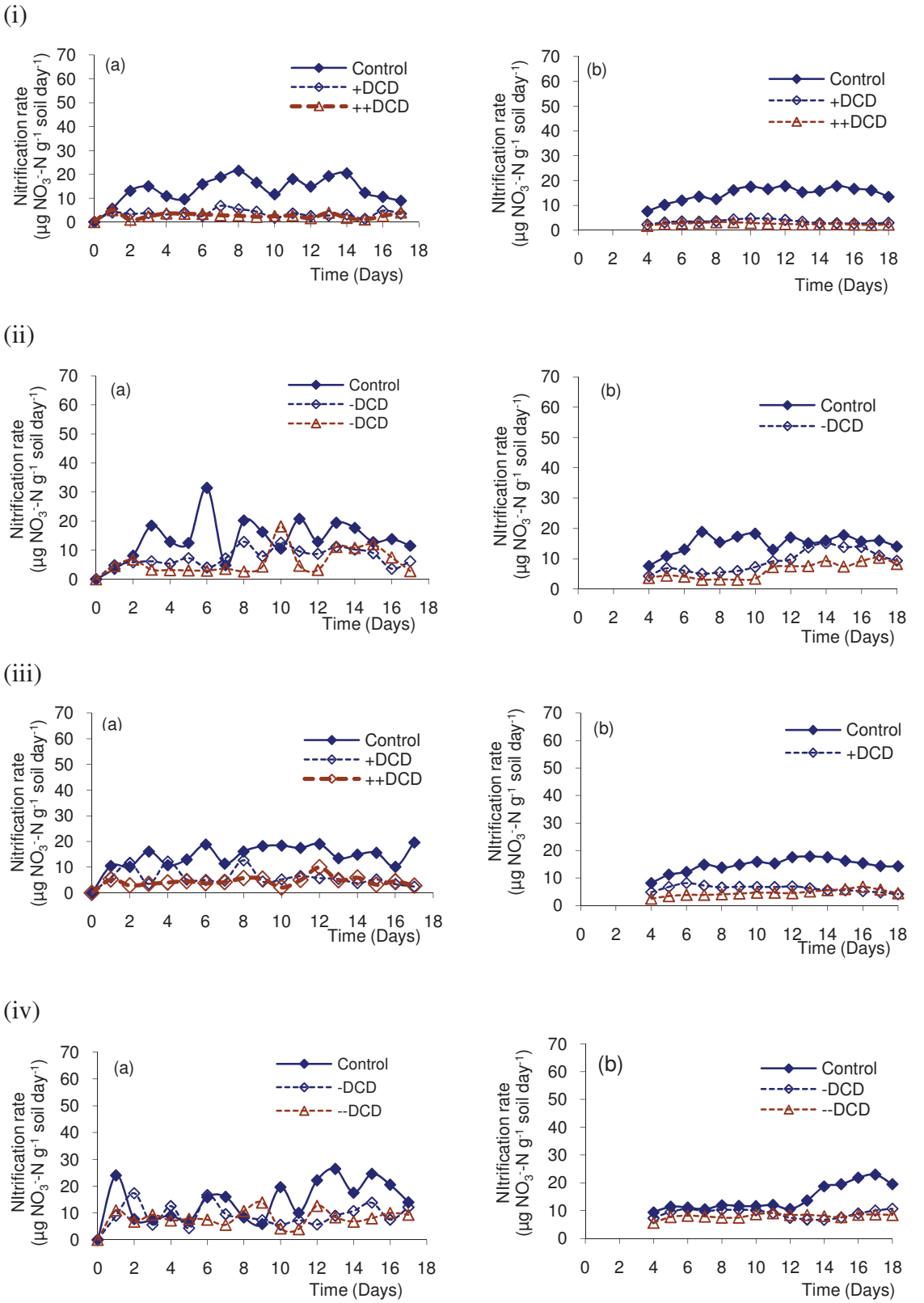


Figure 3.19 Daily (a) and 4-day moving average (b) nitrification rates in MFSL in (i) cycle 1; (ii) cycle 2; (iii) cycle 3 and (iv) cycle 4. The soils in the perfusion apparatus in cycles 1 and 3 received $(NH_4)_2SO_4$ solution only (control) and with DCD at 20 kg ha^{-1} (+DCD) and 40 kg ha^{-1} (++DCD), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only (control and –DCD).

3.3.4.2 AOB populations

The estimates of nitrifier populations in the MFSL soil collected from the perfusion columns (Figure 3.20) followed a similar pattern to that obtained in the original soil (Figure 3.7) and the perfused soil of MSL (Figure 3.14), reaching a maximum after 8 weeks of incubation. The values at week 8 in the MPN procedure were therefore used to calculate the AOB populations in the soils.

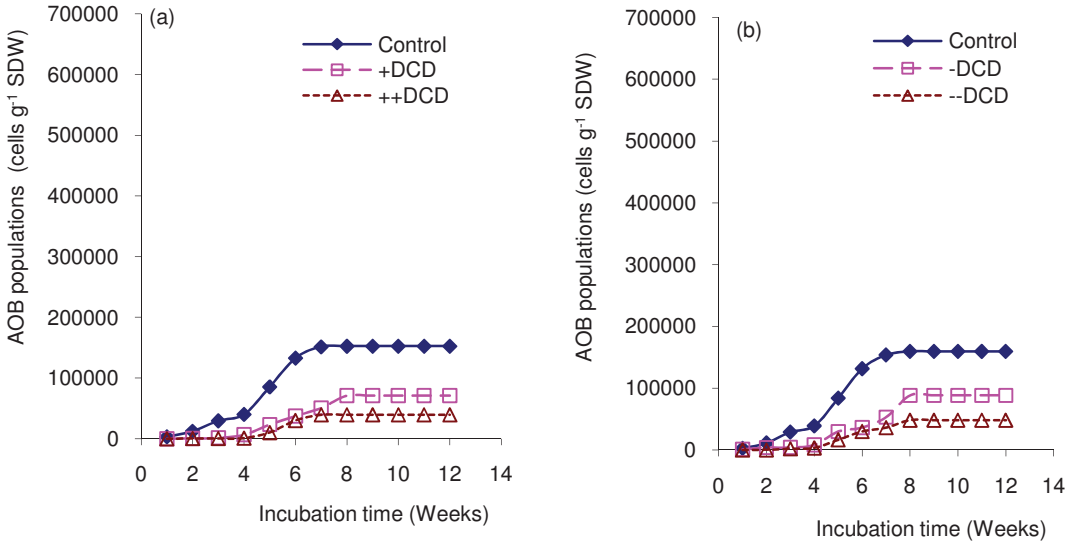
The estimates of AOB populations in the control soils (ranging from 1.5 to 1.6 x 10^5 cells g^{-1} SDW) were very similar in all the cycles (Figure 3.21) indicating that there was no great growth or drop-off of AOB numbers during the experiment. The estimates of AOB populations in the control soils (ranging from 1.5 to 1.6 x 10^5 cells g^{-1} SDW) were very similar in all the cycles (Figure 3.21) indicating that there was no great growth or drop-off of AOB numbers during the experiment. However, addition of an N source to the control soils in the perfusion apparatus resulted in a slight non-significant increase in the numbers of AOB compared to the original MFSL soil, which had an AOB population of 8.7 x 10^4 cells g^{-1} SDW (Figure 3.8).

As observed in the MSL, the addition of DCD had a consistent effect on the AOB populations, which were always lower than those in the control soil columns (Figure 3.20 and Figure 3.21).

In absolute terms, the effect of DCD on the AOB populations was greater in the MSL (a decrease of 168374 cells g^{-1} SDW in cycle 1 and 90679 cells g^{-1} SDW in cycle 3) than in the MFSL (a decrease of 81435 cells g^{-1} SDW in cycle 1 and 76500 cells g^{-1} SDW in cycle 3), when DCD was applied at the same rate (Figure 3.22). However, in percentage

terms DCD was more effective in the MFSL with a percentage reduction of the AOB populations of 53 and 48% in cycles 1 and 3, while only 29 and 18% of the AOB reduction was obtained in the MSL. Applying DCD at twice the rate resulted in further reductions in the AOB populations (Figure 3.21). Although there was some recovery of AOB numbers in cycles 2 and 4 the changes were small and the residual effect of DCD in the MFSL appeared to be greater than in the MSL (Figure 3.15).

In summary, the addition of DCD was effective in reducing the nitrification activity in both soils, but the magnitude of this reduction varied between the two soils and from cycle to cycle. Overall, the inhibition of nitrification in relative terms was more effective in the MFSL, while the DCD was more effective in suppressing the nitrification activity in the MSL in absolute terms.



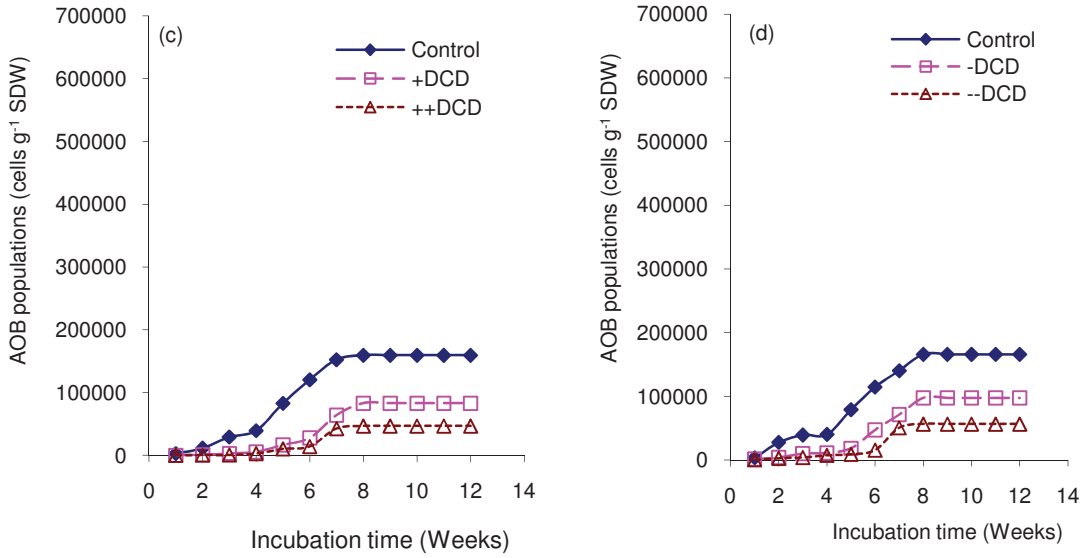


Figure 3.20 Estimates of AOB populations in the MFSL soils with time of incubation in the growth medium after serial dilution (a) cycle 1; (b) cycle 2; (c) cycle 3 and (d) cycle 4. The soils in the perfusion apparatus in cycles 1 and 3 initially received $(NH_4)_2SO_4$ solution only (control), $(NH_4)_2SO_4$ solution with 20 kg DCD ha^{-1} (+DCD) and $(NH_4)_2SO_4$ solution with 40 kg DCD ha^{-1} (++DCD), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only (control, –DCD and --DCD).

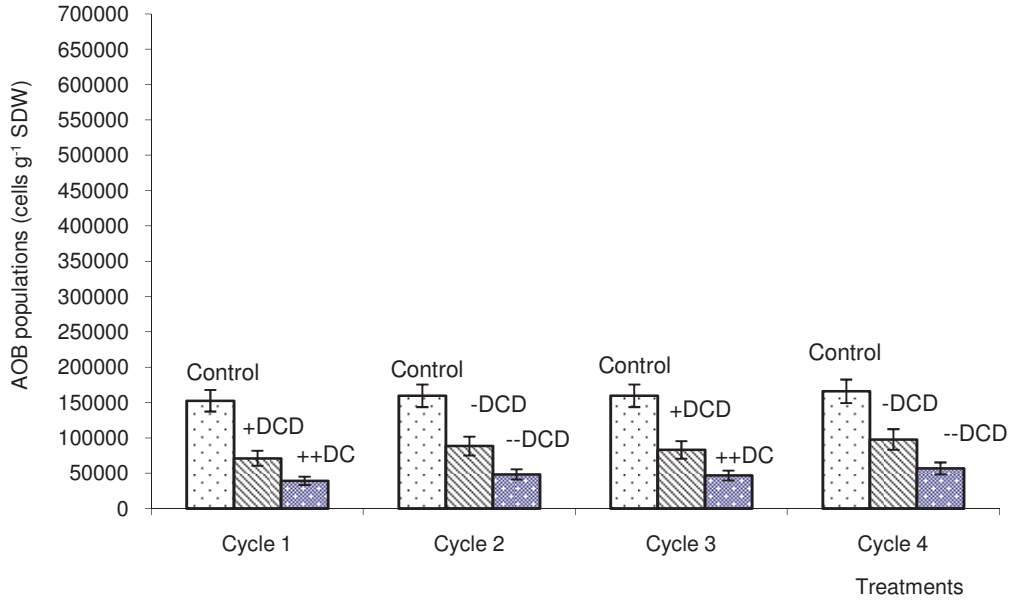


Figure 3.21 AOB populations in the MFSL soils in the perfusion columns after each cycle. The MFSL soils in the perfusion apparatus in cycles 1 and 3 received $(NH_4)_2SO_4$ solution only (control) and $(NH_4)_2SO_4$ solution with 20 kg DCD ha^{-1} (+DCD) and $(NH_4)_2SO_4$ solution with 40 kg DCD ha^{-1} (++DCD), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only (control, –DCD and --DCD). Vertical bars in the columns indicate the range of AOB populations obtained in the 2 replicates in each treatment.

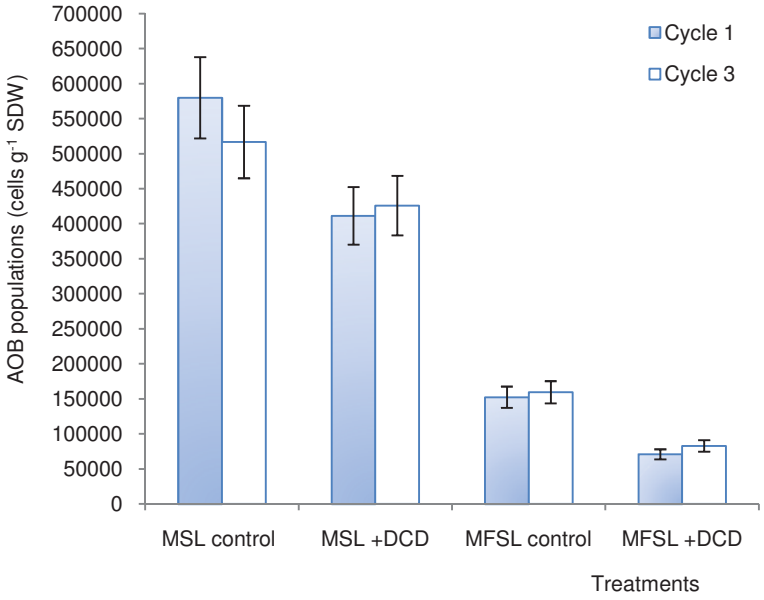


Figure 3.22 The effect of DCD applied at the same rate (20 kg DCD ha^{-1}) in cycles 1 and 3 on the AOB populations in the MSL and MFSL soils. Vertical bars in the columns indicate the range of AOB numbers obtained in the 3 replicates in each treatment for MSL and 2 replicates in each treatment for MFSL.

There appeared to be a relationship between the numbers of AOB and the nitrification rate. Regression analysis gave the following significant linear relationship between the mean nitrification rate and the numbers of AOB (Figure 3.23):

$$y = 4E\text{-}05x + 4.2137$$

$$R^2 = 0.92$$

where y is the mean nitrification rate produced (µg NO_3^--N g^{-1} soil day^{-1}) and x is the AOB numbers (cells g^{-1} SDW).

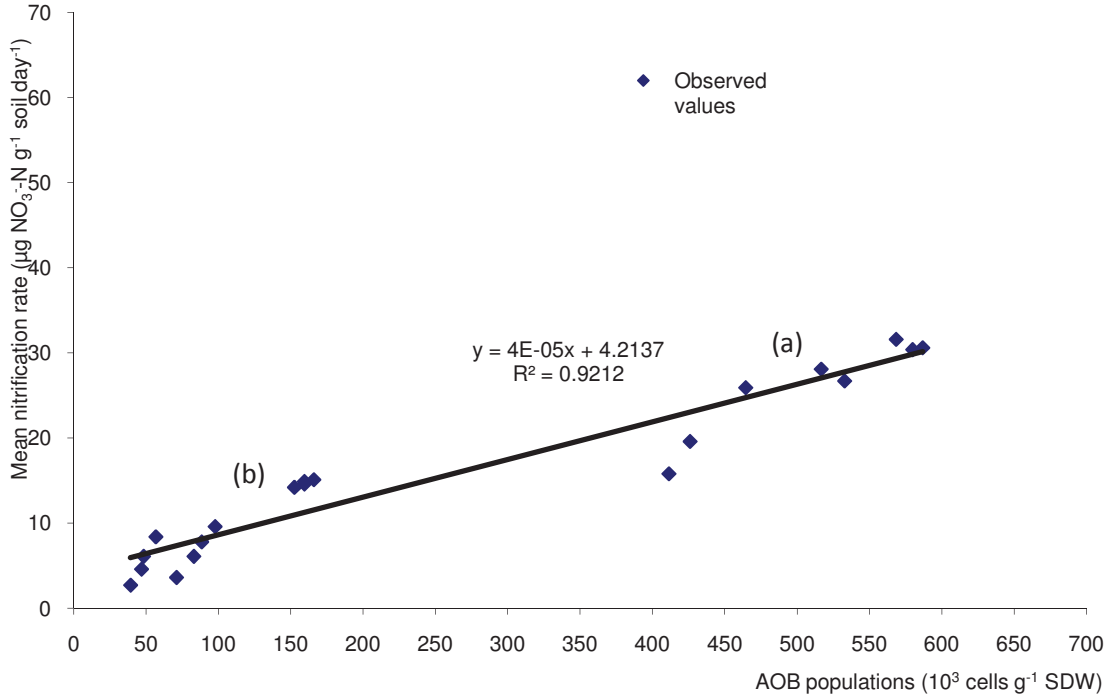


Figure 3.23 Relationship between mean nitrification rate and the estimated numbers of AOB in each cycle in (a) MSL and (b) MFSL.

3.4 General discussion

In this study, the simplified soil perfusion apparatus proved to be useful for monitoring the soil nitrification activity and enabling measurement of the nitrifier populations in the soils. The apparatus offers advantages over other perfusion units such as those described in Section 3.2.3. There was no glass tubing involved in this set-up and the plastic tubing can be easily removed. This combined with the low cost and the short time required for setting-up, is a significant saving. The total unit also uses considerably less bench space than most other types and therefore a greater number can be set up in the same area.

Most importantly, it enabled similar perfusion flow rates to be established for all the soil columns.

As discussed earlier in the Literature Review (Section 2.4), soil nitrification rates vary among soil types as a result of differences in physical, chemical and biological properties. These include pH (Nugroho *et al.* 2007; Rajendra Prasad and Power 1997), aeration (oxygen supply) and soil moisture (Havlin *et al.* 2005), temperature (Focht and Verstraete 1977), abundance of substrate (NH_4^+-N) (Mendum *et al.* 1999) and the population and diversity of nitrifying organisms (Kowalchuck and Stephen 2001; Martikainen 1985; Sahrawat 2008). The nitrification rates obtained in this study for the MSL, TSL and MFSL soils were in the range of 14.4 to 30.4 µg NO_3^--N g^{-1} soil day^{-1}, with the highest rates obtained in the MSL (30.4 µg NO_3^--N g^{-1} soil day^{-1}) and lowest in the MFSL (14.4 µg NO_3^--N g^{-1} soil day^{-1}). These results were in accordance with the nitrification rates found during a 15-day incubation of lowland soils (Kairanga silt loam and Karapoti silt loam), collected from a paddock with history of urine applications, and a Manawatu sandy loam soil collected from a dairy farm which had nitrification rates of 18.3, 17.1 and 16.6 µg NO_3^--N g^{-1} soil day^{-1} respectively (Bowatte 2003). In another study on the Short Term Nitrification Activities (SNA) of 9 New Zealand soils Sarathchandra (1978b) showed that the nitrification activities of the 9 soils were widely different, ranging from 6.0 to 79.44 µg NO_3^--N g^{-1} soil day^{-1}. The nitrification rates in the current study are within this range.

The highest nitrification rate was obtained in the MSL and the lowest in the MFSL. A positive relationship between nitrification rates and AOB numbers was obtained both between soils and also between different DCD treatments in each cycle within each soil

(Figure 3.23). In a more recent study carried out by Di *et al*. (2010), higher nitrification rates were obtained in Waikato, Canterbury and Southland topsoils than in the corresponding subsoils after treatment with urine, and these nitrification rates were significantly related to AOB abundance. In this study although only a relatively small range of soil types were used to study the soil nitrification rates with the perfusion technique, the rankings of the nitrification rates and AOB numbers were similar in the three original pasture soils (Figure 3.9).

As noted briefly in the Literature Review (Section 2.4.1.1), Singh and Kashyap (2006) found there was a significant relationship between soil nitrification activity and the numbers of nitrifiers obtained from the MPN technique. They reported that greater N mineralisation, higher nitrification rates and larger populations of free living cells of NH_4^+- and NO_2^-- oxidising bacteria were obtained during the wet period, when there was adequate soil moisture in all the soils of the tropical forest and savanna sites tested. In contrast, nitrifier populations were lowest during the summer season and this corresponded with the lowest rates of nitrification N-mineralisation. Their results also showed that the N mineralisation and nitrification rates and the numbers of nitrifying cells were consistently higher in forest soils compared to savanna sites.

In another forest soil study at nine sites on acid soils under Scot pine, Nugroho *et al*. (2005) investigated the relationship between and high and low nitrification rates and the presence of AOB, determined with a molecular technique. Their results provided evidence that in the five acid soils that had higher nitrification rates, AOB *Nitrosospira* cluster 2 was detected. Conversely, similar AOB communities could not be detected in the four sites on acid forest soils that had low nitrification rates. Differences in the

nitrification rates between the forest soils were correlated to soil C/N ratio and atmospheric N deposition. The five soils with high nitrification rates were characterised by high initial NO_3^--N concentrations, low C/N ratios and high atmospheric N deposition which may probably have been more favourable for the survival of *Nitrosospira* as compared to the sites with low nitrification rates.

Despite the inherent problems with MPN variability and sensitivity as noted earlier in the Literature Review (Section 2.5), the MPN counts obtained in all the three soils used in this study suggested that they contained significant numbers of AOB, in the range of 8.7×10^4 to 3.5×10^5 cells g^{-1} SDW. These results are consistent with other counts of nitrifying populations (estimated using the MPN technique) in agricultural soils that generally fall in the range of 10^5-10^7 g^{-1} (Stienstra *et al*. 1993).

In New Zealand pasture soils, numbers of AOB have been reported ranging from 10^4 to 10^7 g^{-1} soil (Sarathchandra 1978b). In a more recent study, Di *et al*. (2010) reported that AOB were more abundant in the topsoil than in the subsoil for Waikato, Canterbury and Southland soils, with AOB numbers ranging from 2.0×10^4 – 7.7×10^4. In contrast, lower numbers of nitrifiers have been reported in several natural acid soils, ranging from 10^2-10^4 g^{-1} soil (Carnol and Ineson 1999; de Boer *et al.* 1988; Hastings *et al.* 2000; Pennington and Ellis 1993). The effectiveness of DCD in inhibiting nitrification and reducing AOB numbers in the 2 soils is illustrated in Figure 3.24 and Figure 3.25.

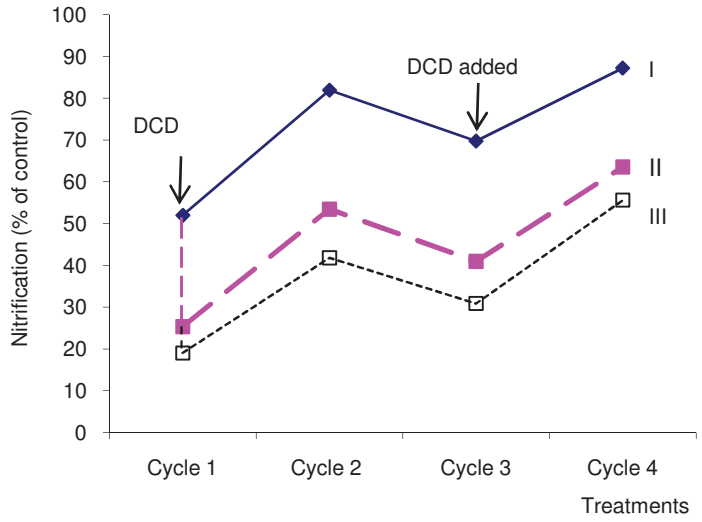


Figure 3.24 The effect of DCD on nitrification rates (expressed as % of control) in the MSL (I) and MFSL (II and III). The soils in the perfusion apparatus in cycles 1 and 3 received $(NH_4)_2SO_4$ solution containing DCD at 20 kg ha^{-1} (I and II) and 40 kg ha^{-1} (III), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only.

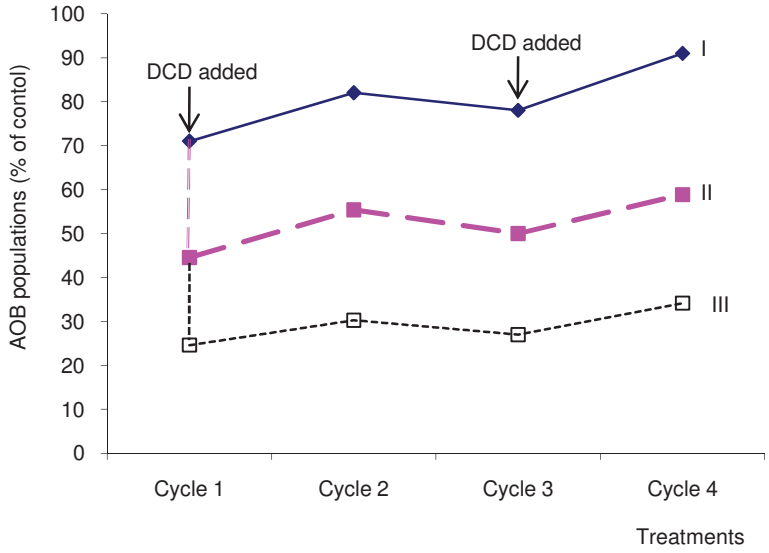


Figure 3.25 The effect of DCD on AOB populations (expressed as % of control) in the MSL (I) and MFSL (II and III). The soils in the perfusion apparatus in cycles 1 and 3 received $(NH_4)_2SO_4$ solution containing DCD at 20 kg ha^{-1} (I and II) and 40 kg ha^{-1} (III), while in cycles 2 and 4, all the soils were treated with $(NH_4)_2SO_4$ solution only.

In both soils, the pattern of DCD inhibition and the subsequent recovery of nitrification activity was similar. However, the effectiveness of DCD varied between the two soils. When expressed as percentage of control, DCD was more effective in suppressing nitrification relative to the control treatment in the MFSL than in the MSL. In absolute terms however, the DCD was more effective in the MSL than in the MFSL. This is illustrated in Table 3.3. As shown in Figure 3.5, the MSL had a higher nitrifying capacity than the MFSL, therefore the addition of DCD resulted in a greater reduction in actual amount of NO_3^--N produced in the MSL than in the MFSL. However in neither soil did DCD completely halt nitrification.

Table 3.3 Effectiveness of DCD (applied at the same rate; 20 kg DCD ha^{-1}; +DCD) in cycles 1 and 3 quantified in percentage (%) and absolute terms in the MSL and MFSL soils.

Effectiveness of DCD	Cycle 1		Cycle 3	
	Soil types			
	MSL	MFSL	MSL	MFSL
i) Nitrification rates expressed as a percentage of control				
Control (%)	100	100	100	100
+DCD (%)	52	25	65	41
ii) Actual nitrification rates				
Control (µg NO_3^--N g^{-1} soil day^{-1})	30.4	14.2	28.1	14.9
+DCD (µg NO_3^--N g^{-1} soil day^{-1})	15.8	3.6	18.3	6.1
Δ (µg NO_3^--N g^{-1} soil day^{-1})	14.6	10.6	9.8	8.8

In the initial stage (Cycle 1) of the perfusion experiment, DCD reduced the nitrification rates in the MFSL to 25 and 19% of the control depending on the rate of application. In contrast DCD only reduced the rate of nitrification to 52% of the control in the MSL. As noted earlier in the Literature Review (Sections 2.9.1) results from a series of experiments carried out by Di and Cameron (2002c) provided evidence that DCD was effective in reducing NO_3^--N leaching with a reduction from 24% to 58% of the control treatments were obtained. The greater nitrification inhibition obtained in the MFSL was probably due to the low organic matter (OM) content and slower rate of DCD degradation in this soil.

As discussed earlier in the Literature Review (Section 2.8.1), soil texture and OM content influence the effectiveness and persistence of DCD. The higher OM content of the MSL may have reduced the effectiveness of DCD due to higher absorption of DCD on the OM, and the DCD may have also degraded more rapidly in the MSL soil. These results are consistent with the laboratory incubation study of McCarthy and Bremner (1989) when DCD was applied at 5 mg kg^{-1} soil to 3 different types of soils, incubated at 25^oC. Their results showed greater nitrification inhibition (41%) was obtained in the sandy soil with the lowest amounts of OM and the lowest inhibition (8%) was obtained in the soil with the highest OM and the least amount of sand. In another laboratory incubation study with three different soils with contrasting OM content, texture and mineralogy.

Singh *et al*. (2008) reported that the greatest reduction in N_2O emissions (both in absolute and percentage terms) was obtained in Tokomaru silt loam with urine applied at 600 mg N kg^{-1} and with added DCD at low (10 mg kg^{-1}) and high (20 mg kg^{-1}) rates.

In percentage terms the second greatest reduction of N_2O emissions was in the Manawatu sandy loam followed by the allophanic Egmont brown loam soil. However in absolute terms the reduction in N_2O emissions due to addition of DCD was greater in the Egmont soil than in the Manawatu sandy soil. The lowest DCD degradation rates for both rates of DCD addition were obtained in the Tokomaru silt loam soil, which had an intermediate OM content, followed by the Manawatu sandy soil and the DCD was degraded rapidly in the brown loam soil. These latter two soils had the lowest and highest OM content respectively and had sand contents of 62.5 and 55%. Overall, the greatest inhibition was in the Tokomaru silt loam with an intermediate OM content, with the lowest sand content and with the slowest rates of DCD degradation.

When fresh $(NH_4)_2SO_4$ solution (not containing DCD) was added in Cycle 2, the nitrification rates did recover somewhat, but did not return to the initial levels in either soil. The nitrification rates in the MFSL in Cycle 2 were 53 and 42% of the control in treatments that had previously received the low and high rate of DCD application respectively. The recovery of nitrification activity in Cycle 2 was somewhat greater in the MSL and reached 82% of the activity in the control treatment.

In Cycle 3, the second additions of DCD again reduced the nitrification rates, although this reduction appeared to be slightly less than in Cycle 1. In the MFSL the nitrification rates were 41 and 31% of the control rate for the low and high rates of DCD respectively, and in the MSL the nitrification rates were 65% of that in the control. These results may suggest preliminary evidence for the adaptation of the nitrifiers to repeated addition of DCD. Russell (1973) stated that

"*after the introduction of inhibitors to the soil, nitrification activity will reduce or cease and will not begin again for a period of days, weeks or months. Eventually however, it will increase again to its original value. In addition, the nitrifiers can be trained to become tolerant to the inhibitors when the inhibitors are applied repeatedly either with the same concentration or applied at higher concentration than the first application, in which the repeatedly addition of inhibitors may be more short-lived or there may even be no effect to the soil nitrification activity as compared with the first application"*.

In more recent work by de Klein *et al.* (2011) on the repeated annual use of DCD (DCD was applied for 4 or 5 consecutive year) in the two New Zealand pasture soils, applied at twice yearly (one application in autumn and spring) at 10 kg ha^{-1} at each application for the free-draining Canterbury soil (Templeton fine sandy loam) and the DCD was applied two or three times for the poor draining Southland soil, showed that the long term use of DCD didn't alter its impact on suppressing the N_2O emissions as compared with the first time when the DCD was applied. They did mentioned that *"although the application of DCD has a temporary effect on nitrification and the N cycle of pastoral soils, however they is a possibility that the nitrifiers could adjust to DCD, especially when if used repeatedly"*.

In another laboratory incubation study Rodgers (1986) studied the effect of repeated field application of DCD, NP and etridiazole to soil. His results demonstrated that inhibition of nitrification in soils amended with DCD in the laboratory was similar in all four soils, irrespective of the soil's pre-treatment in the field. However, amendment with NP or etridiazole inhibited nitrification most in soils that had been field pretreated with these two inhibitors. He concluded that the efficacy of DCD was not affected by

previous field application with any of the three inhibitors tested, whereas previous field application of any of the three inhibitors sensitised the AOB to NP or etridiazole.

At the end of the experiment (Cycle 4), both soils showed recovery of nitrification activity to 64 and 59% of that control activity in the MFSL at low and high rates of DCD addition respectively, and 87% in the MSL. The populations of AOB (Figure 3.25) followed a similar pattern to the nitrification activities (Figure 3.24) with an inhibition of nitrifer populations in Cycles 1 and 3 and the recovery of the temporarily suppressed nitrifier populations in Cycles 2 and 4.

Overall, the mode of action of DCD obtained in the two contrasting soils in this present study was similar to that obtained by Hendrickson and Keeney (1979). Hendrickson and Keeney (1979) illustrated their findings on the effectiveness of NP schematically as in Figure 3.26.

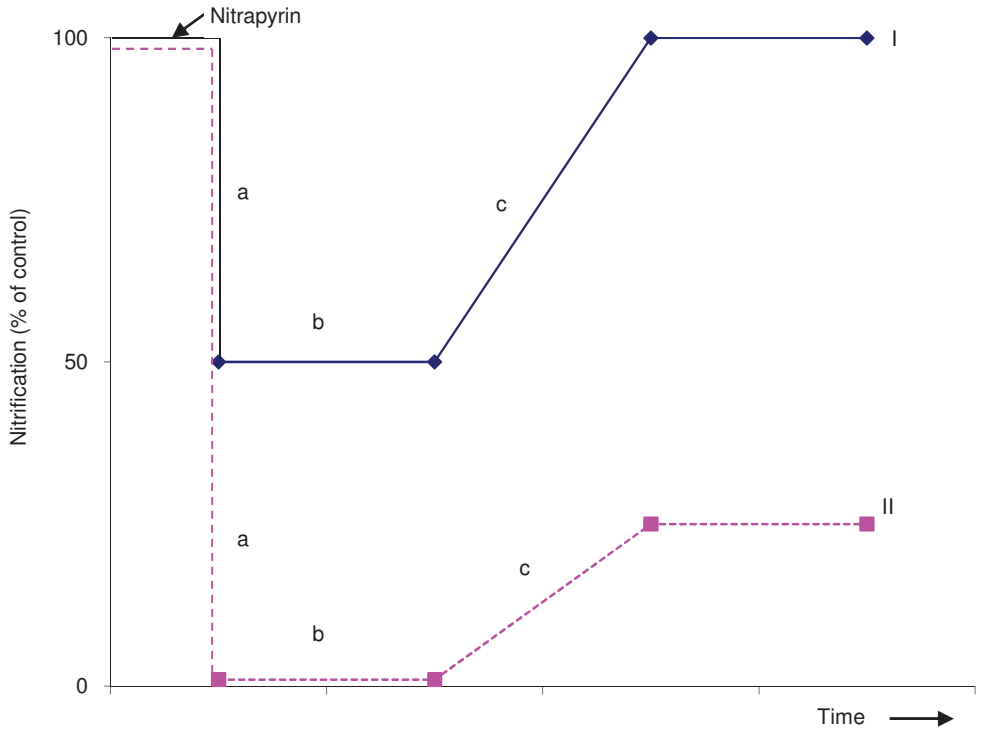


Figure 3.26 Hypothetical curves indicating effect of nitrapyrin (NP) on nitrification rate in a less susceptible soil (I) and a more susceptible soil (II), showing an initial drop in nitrification (a), a lag phase (b) and a recovery phase (c). Adapted from Hendrickson and Keeney (1979).

They developed a test involving pre-incubation of two different types of soils (with contrasting OM and sand contents) with $(NH_4)_2PO_4$ solution to establish a consistent population of nitrifiers. Once all the NH_4^+-N had been nitrified, aqueous NP or water (control) was added. An additional quantity of $(NH_4)_2PO_4$ solution was added to each treatment to maintain a constant rate of nitrification. Two modes of action of the added NP were observed. The first curve (I) illustrates a direct but incomplete effect of NP (a), the rate remains at fixed level for a period of time (b) after which it increases at a rate related to the nitrifier population (c) up to the original level. The second pattern (curve II) has a similar shape, but the soil was more susceptible to the added NP with the initial inhibition being nearly complete (a and b). This was followed by a relatively slow recovery of nitrification rate to a level well below the starting rate and the population

never recovered to the control activity (c). In the study reported in this Chapter, the effect of DCD in the MSL was similar to that in curve I and in the MFSL was more similar to that in curve II.

As discussed earlier in the Literature Review (Section 2.7.1), DCD has been reported to have a bacteriostatic action on nitrifiers, in which it temporarily suppressed the nitrification activity but did not kill the nitrifiers (Rodgers and Ashworth 1982; Zacherl and Amberger 1990). In this present study it seems as if DCD had a primarily bacteriostatic effect on the nitrifiers in the MSL. When a fresh source of N was percolated through the soil column after the addition of DCD, the rate of nitrification and the population of AOB increased almost to the control level. In contrast, the DCD appeared to have more of a bacteriocidal effect in the MFSL with the nitrification rates and numbers of AOB not recovering to anywhere near the levels in the control.

3.5 Conclusions

From the results and discussion, the following conclusions can be made:

1. The simplified perfusion technique was able to monitor the nitrification activity in New Zealand pasture soils and the nitrification rates measured with this technique were in the range of measured nitrification rates observed by others with soil incubation studies.
2. The highest nitrification activity was obtained in the MSL, followed by the TSL and then the MFSL. This corresponds with the numbers of AOB present in those soils.
3. The perfusion technique was also able to monitor the immediate and residual effect of DCD on the soil nitrification activity in the two contrasting soils

4. The modes of actions of DCD in both soils were similar. However, the effect of DCD was more apparent in the soil with lower organic matter (MFSL) than in the MSL.
5. The nitrification rates and the numbers of AOB recovered in both soils when DCD was withdrawn but didn't return to the control levels. This ongoing suppression was however more obvious in the MFSL than in the MSL.

Chapter 4

Decomposition of dicyandiamide (DCD) and the effect on nitrification rates – laboratory incubation study

4.1 Introduction

The review of literature revealed the importance of nitrification as one of the N transformation processes responsible for the conversion of immobile NH_4^+-N into NO_3^--N. The NO_3^--N produced during this process may be lost through leaching and denitrification and is of significant environmental concern.

As already mentioned in Chapter 2, there has been increasing interest in New Zealand and internationally in the use of nitrification inhibitors such as DCD to minimise losses of N by slowing down the nitrification process. Most of the published work involving DCD in New Zealand, as detailed in the literature review, focused on quantifying the effects of DCD on NO_3^--N leaching and N_2O emission from urea fertiliser (Zaman *et al.* 2007), urine deposition on legume-based pasture (Di and Cameron 2003) and dairy shed effluent (Williamson and Jarvis 1997). In Section 2.7.1 of the Literature Review, it was mentioned that soil properties are among the factors that contribute to the effectiveness of DCD. One such property is soil organic matter (OM), with high levels of OM associated with reduced effectiveness of DCD in reducing nitrification activity.

More recently, work carried out by Singh *et al*. (2008) showed that the time taken by DCD to reduce to half of the initial concentration was shortest in an Egmont brown

loam allophonic soil (Typic Orthic Allophonic) and longest in a non-allophanic Tokomaru silt loam (Argillic-fragic Perch-gley Pallic Soil). These results were related to the effectiveness of DCD in reducing emissions of N_2O with DCD being less effective in the allophonic soil than in the non-allophanic soil. Although much research has been carried out on DCD, there is still little information on the persistence of DCD in soil and its consequent effectiveness in reducing in nitrification in soils.

The first perfusion experiment described in Chapter 3 found that DCD was more effective in reducing both the nitrification rates and the population of AOB in the MFSL than the MSL soil. In this chapter a laboratory incubation study was carried out to follow-up some of the findings in Chapter 3 and investigate further the link between the presence of DCD and the nitrification rate in the two different types of soils. In particular, the study aimed to explore whether the variable effectiveness of DCD between soils and over time was due solely to the disappearance of DCD from the soil through microbial breakdown, or whether some other factors, such as adsorption on soil organic matter, were affecting the effectiveness of DCD.

The objectives of this study were therefore to: i) quantify the rate of DCD degradation in Manwatau silt loam (MSL) and Manawatu fine sandy loam (MFSL); (ii) to determine the recovery of nitrification in both soils after the application of DCD; and (iii) to quantify the effect of two different rates of DCD addition on soil nitrate formation.

4.2 Materials and Method

4.2.1 Soil sampling and preparation

Samples of two different pasture soils - Manawatu silt loam (MSL) and Manawatu fine sandy soil (MFSL), both classified as weathered fluvial recent soils (Hewitt 1998) - were used in this study. These soils were the same as those used in Chapter 3. Bulk samples (0-10 cm depth) of each soil were collected from random locations paddocks within Dairy Farm No. 1 at Massey University, Palmerston North, New Zealand. Herbage, stones, roots and other debris were removed from the soils. The field-moist soils were passed through a 2-mm sieve, homogenised by hand and then air-dried for a day. Sub-samples of each of the partially air dried soil were used to determine the soil field capacity using a pressure plate extractor at (1/10 bar) and gravimetric moisture content by drying at 105^{o}c for 16 hours. The soil properties were presented in Table 3.1.

4.2.2 Bulk soils incubation procedure

Samples of field moist soil, equivalent to 2.5 kg of dry soil, were placed in six 3-L plastic containers for each soil type. The three main treatments used in this study were DCD at two rates (20 and 40 mg DCD kg^{-1} soil which were equivalent to 10 and 20 kg DCD ha^{-1} assuming the DCD was distributed through the top 5 cm of soil) and no DCD (control). There were two replicates of each of these main treatments. The DCD was thoroughly mixed with the soil. The application rates of DCD were calculated by assuming the soil bulk density of 1 Mg m^{-3}. The soil moisture content was adjusted and maintained at field capacity throughout the study by monitoring the weight changes of each soil sample in the container and adding deionised water as necessary. These soil samples were incubated at 20^{o}C. The container cover was partially lifted open to allow the exchange of air.

4.2.3 Degradation of DCD and DCD analysis

Two 10-g sub-samples of moist soil were taken from each of the incubated main treatments on day 1, followed by weekly sampling until weeks 10 and 11 for the low and high DCD additions respectively in the MFSL, and weeks 18 and 21 for the low and high DCD additions respectively in the MSL. The 10-g soil samples were mixed with deionised water (1:1 soil to water ratio), shaken for 1 hour, centrifuged at 8000 rpm for 3 minutes and filtered through "Whatman 41" filter paper. Five mL of the supernatant was transferred to a 10-mL centrifuge tube, 0.2 mL of 0.66M H_2SO_4 added and the mixture left for 5 minutes. The extracted DCD was then re-centrifuged at 4300 rpm for 10 minutes. The final supernatant was kept in a 30-mL plastic bottle at room temperature until analysis for DCD (Schwarzer and Haselwandter, 1996). The DCD analysis was performed on a HPLC (Alliance, Waters 2690, Separation module) with a multi-wavelength UV detector at 210nm (Schwarzer and Haselwandter, 1996). The separation of DCD was carried out using a micro-guard column packed with Aminex Resin, heated at 60^{o}C with a flow rate of 0.9 mL/min of 0.025 M H_2SO_4 as the mobile phase. Recording and computing of data was carried out with Millenium^{32} Waters software. A sub-sample of soil was used for moisture determination at each sampling time by drying at 105^{o}C for 16 hours.

4.2.4 Calculation of DCD half-life

The first-order decay rate equation as described in Saggar and Hedley (2001) was used to calculate the rate of DCD degradation in both soils. The decrease in DCD concentrations in both soils resembled first order reaction kinetics and the data were fitted to the exponential function $N = N_0 e^{-kt}$ where, N is the amount of DCD remaining in the soil at time (t), N_0 is the amount of DCD recovered at the beginning of the

experiment in the soil and *k* is the decay constant. The DCD half life is the time taken to reduce DCD concentration to half of the initial values, and was calculated as $t_{1/2} = 0.693/k$.

4.2.5 Nitrification assay and analysis of mineral N

At each of the times that the samples described in Section 4.2.3 were collected from the incubating main treatments and analysed for DCD, two further sub-samples, equivalent to 35 g of dry soil, were taken from each container of the incubating soils and were placed in each of 24 plastic cups, for each soil type. Twelve of the plastic cups of each soil type were treated with 0.5 mL of urea solution (~ 200 µg N/g dry soil), while the other 12 plastic cups of each soil types had 0.5 mL of water added. These cups acted as a control. Soil in the cups was mixed with a spatula after the addition of urea and water and the cups were covered with aluminium foil tops with pin holes. All of these 48 plastic cups were incubated aerobically at 26 ^{o}C in the dark for 2 weeks (Rodgers 1986). Moisture lost during the incubation period was maintained by adding deionised water to bring the soils up to the original weight. At the end of the 2-week incubation time, a 5 g sub-sample of soil from each cup was extracted with 2 M KCl (1:5 soil:extractant ratio) and the extract was analysed colorimetrically for mineral N by an auto analyser (Blakemore *et al.* 1987). Soil sub-samples from each cup were also taken for moisture determination by drying at 105 ^{o}C for 16 hours. In this study the nitrification rates were calculated as the difference between the amount of NO_3^--N produced in the soil treated with urea and the amount of NO_3^--N produced in the soil treated with water only, divided by 14, which was the duration in days of the incubation.

In this Chapter (and Chapters 5 and 6), soil amended with urea was incubated as a way to assess potential nitrification activity. Urea was chosen to provide the NH_4^+ substrate

for nitrification activity because most N added to the soil in fertiliser and animal urine is in the form of urea. In most soils urea is hydrolysed rapidly to NH_4^+ (e.g. Apthorp *et al.* 1987) and this was also the case in this experiment. An additional advantage of using urea was that hydrolysis of urea is accompanied by an increase in soil pH and this partially offset the decrease in pH from nitrification. This reduced the chance that high nitrification potentials would be masked by extremely acidic conditions developing in the incubating soils.

4.3 Results

4.3.1 Degradation of DCD

The concentrations of DCD in the two incubating soils decreased with time (Figure 4.1 (a) and (b)). Higher concentrations of DCD were recorded in both soils at the higher DCD application rate. The DCD appeared to be broken down more quickly in the MSL (Figure 4.1 (a)) than in the MFSL (Figure 4.1 (b)). In the MSL, the DCD concentrations decreased rapidly with time and reached negligible values after 70 and 77 days, for the low and high application rates respectively. In the MFSL the DCD concentrations became negligible after 126 days and 147 days for the low and high DCD application rates respectively.

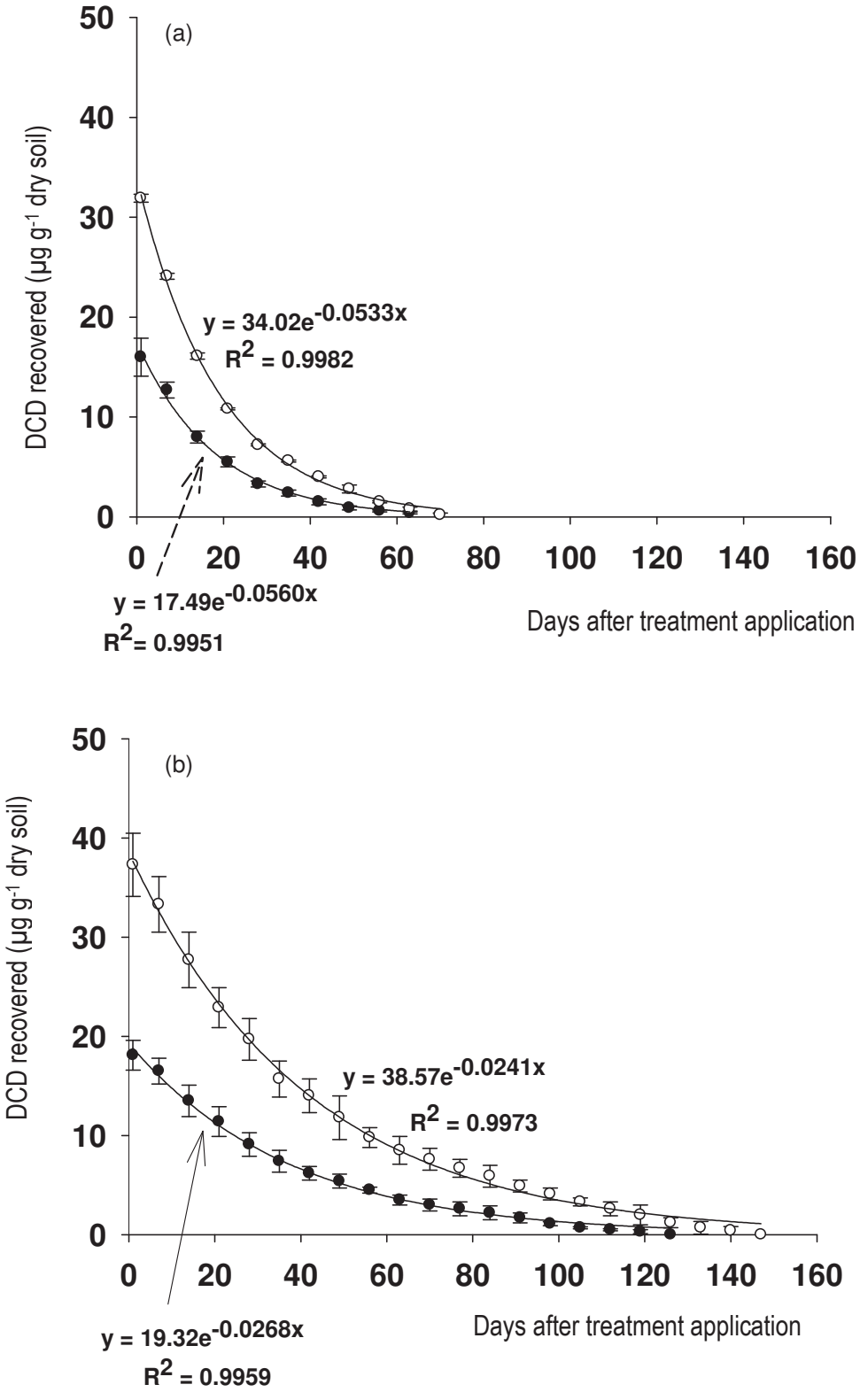


Figure 4.1 Decomposition of DCD with time in (a) MSL and (b) MFSL after application of DCD at 10 (+DCD; –●–) and 20 kg ha^{-1} (++DCD; –⊖–). Each point represents the mean of two replicate analyses of each of two separate subsamples of the incubated bulk soils. Error bars denote standard errors of the four analytical results for each point.

The decomposition of DCD in both soils resembled that of a first-order reaction (Figure 4.1). The MSL soil had a higher degradation rate constant than the MFSL (Table 4.1). The half-life of DCD in the MSL was 12 and 13 days for the low and high DCD application rates respectively. In contrast, the half-life of DCD in the MFSL was 26 days for the low rate of DCD addition and 29 days for the higher rate of DCD application. The rate of DCD addition in both soils did not appear to affect greatly the half life.

Table 4.1 First order DCD half-life and degradation rate constant in the two soils following the application of 2 rates of DCD.

Soil types	Application rates of DCD (mg kg^{-1} of soil)	Degradation rate constant (day^{-1})	Half-life (days)
MSL	10	0.056	12
	20	0.053	13
MFSL	10	0.027	26
	20	0.024	29

4.3.2 Nitrification

4.3.2.1 Nitrate concentrations in bulk soils

The NO_3^--N concentrations in the bulk soils (main treatment) for MSL and MFSL soils that had been incubated without DCD addition, increased slightly over time, but the concentrations were reasonably constant in both soils (Figure 4.2 (a) and (b)). Higher concentrations of NO_3^--N were observed in the MSL than in the MFSL. Addition of DCD at both rates resulted in lower NO_3^--N concentrations in both soils during the initial incubation of the bulk soils (Figure 4.2 (a) and (b)). Once again however, higher

concentrations of NO_3^--N were observed in the MSL (Figure 4.2 (a)) than those observed in the MFSL soil (Figure 4.2 (b)).

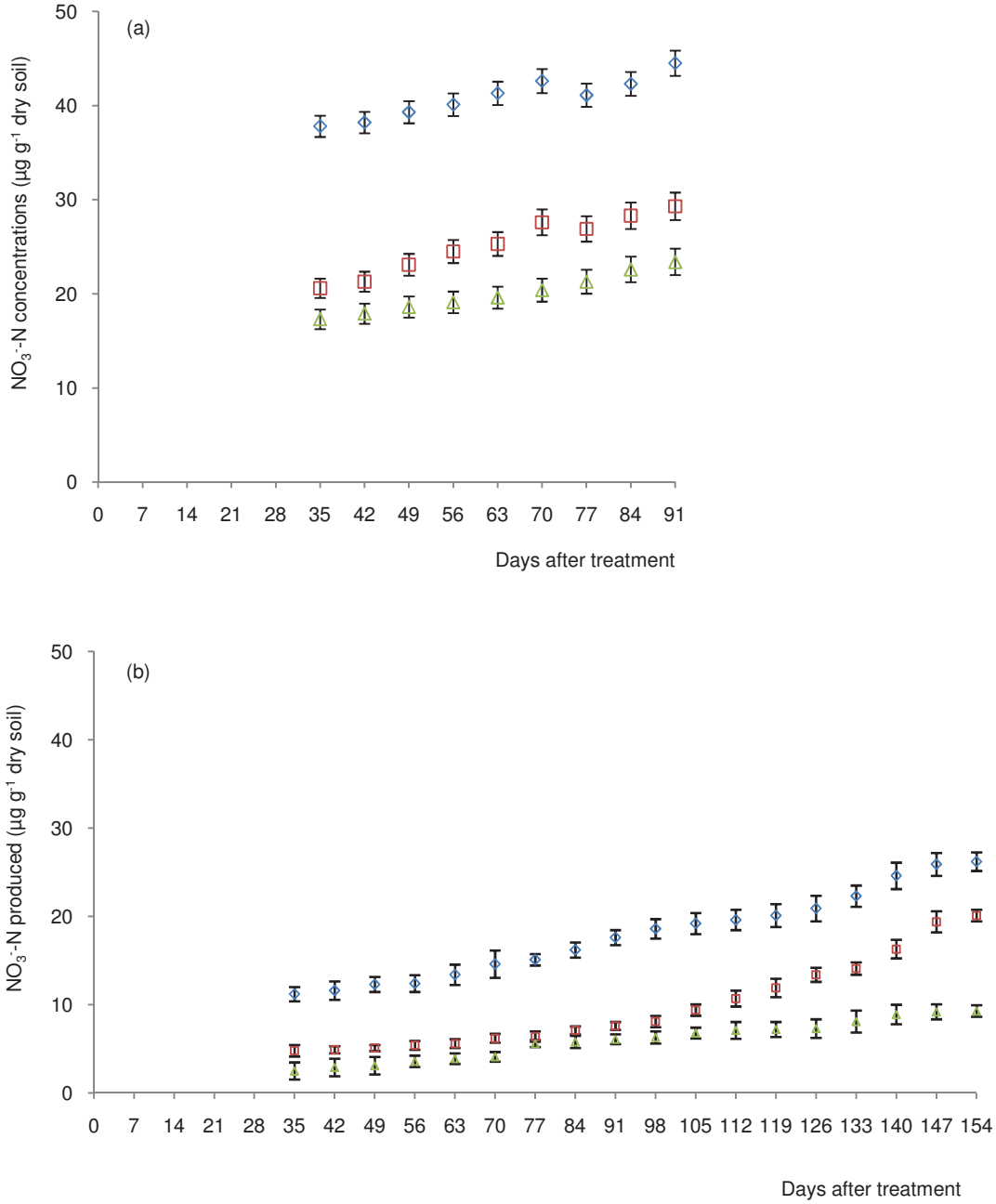


Figure 4.2 Nitrate levels in (a) MSL and (b) MFSL during the initial bulk soil incubation without addition of DCD (◇) and with addition of DCD at low (□; +DCD) and high (△; ++DCD) rates. No NO_3^--N analyses were performed on the bulk incubating soils from from day 1 to day 28. Each point represents the mean of two replicate analyses of each of two separate subsamples of the incubated bulk soils. Error bars denote the standard errors of the four analytical results for each point.

4.3.2.2 Nitrification assays in MSL and MFSL

The concentrations of NO_3^--N in the nitrification assays conducted without added urea did not change greatly from the values initially present in the soils at the time of subsampling and are therefore not discussed further here. When the nitrification capacity of the incubating bulk soils was assayed by adding urea to subsamples and incubating for 14 days, the average nitrification activities in assays in both the MSL and the MFSL soils in the absence of DCD were reasonably constant and averaged 14.5 µg NO_3^--N g^{-1} soil day^{-1} in the MSL (Figure 4.3 (a)) and 8.5 µg NO_3^--N g^{-1} soil day^{-1} in the MFSL (Figure 4.3 (b)) over the duration of the bulk soil incubation.

In Chapter 3, the addition of DCD immediately reduced the nitrification rates in the two soils tested and the reduction was greater in the MFSL than in the MSL. A similar trend was obtained in this incubation study. In the MSL soil at day 1, addition of DCD reduced the nitrification rate during the assay from 14.0 to 5.1 µg NO_3^--N g^{-1} soil day^{-1} at the low rate of DCD addition, and to 3.9 µg NO_3^--N g^{-1} soil day^{-1} when DCD was added at the higher rate (Figure 4.3 (a)). These nitrification rates were 36 and 28% of the rate in the control treatment for the low and high rates of DCD addition respectively, (Figure 4.4 (a)) and equated to inhibitions of 64 and 72%.

In the MFSL addition of DCD reduced the assayed nitrification activity from 8.5 to 0.5 µg NO_3^--N g^{-1} and 0.3 µg NO_3^--N g^{-1} (Figure 4.3 (b)) for the low and high rates of DCD addition, respectively. These nitrification activities were only 6 and 4 % of the control treatment (Figure 4.4 (b)) - equating to inhibitions of 94 and 96%. In this experiment, although a further reduction in the nitrification rate was obtained when the

rate of DCD was doubled, the differences in inhibition of nitrification between these two DCD rates were not large.

Overall, the nitrification activities in both soils treated with low and high rates of DCD increased gradually with time after the addition of DCD (Figure 4.4 (a) and (b) and Figure 4.4 (a) and (b)). In the MSL (Figure 4.4 (a)), 91 days after the addition of DCD the nitrification activities had increased to be 81 and 73% of the control treatment for the high and low rates of DCD addition respectively. In contrast, in the MFSL 154 days after application of DCD the nitrification activities for the low and high addition of DCD were still only 63 and 53% of the control treatment respectively (Figure 4.4 (b)). The nitrification activities in the MSL had therefore appeared to recover more completely than in the MFSL, despite the fact that by the end of the experiment no DCD could be recovered from either soil.

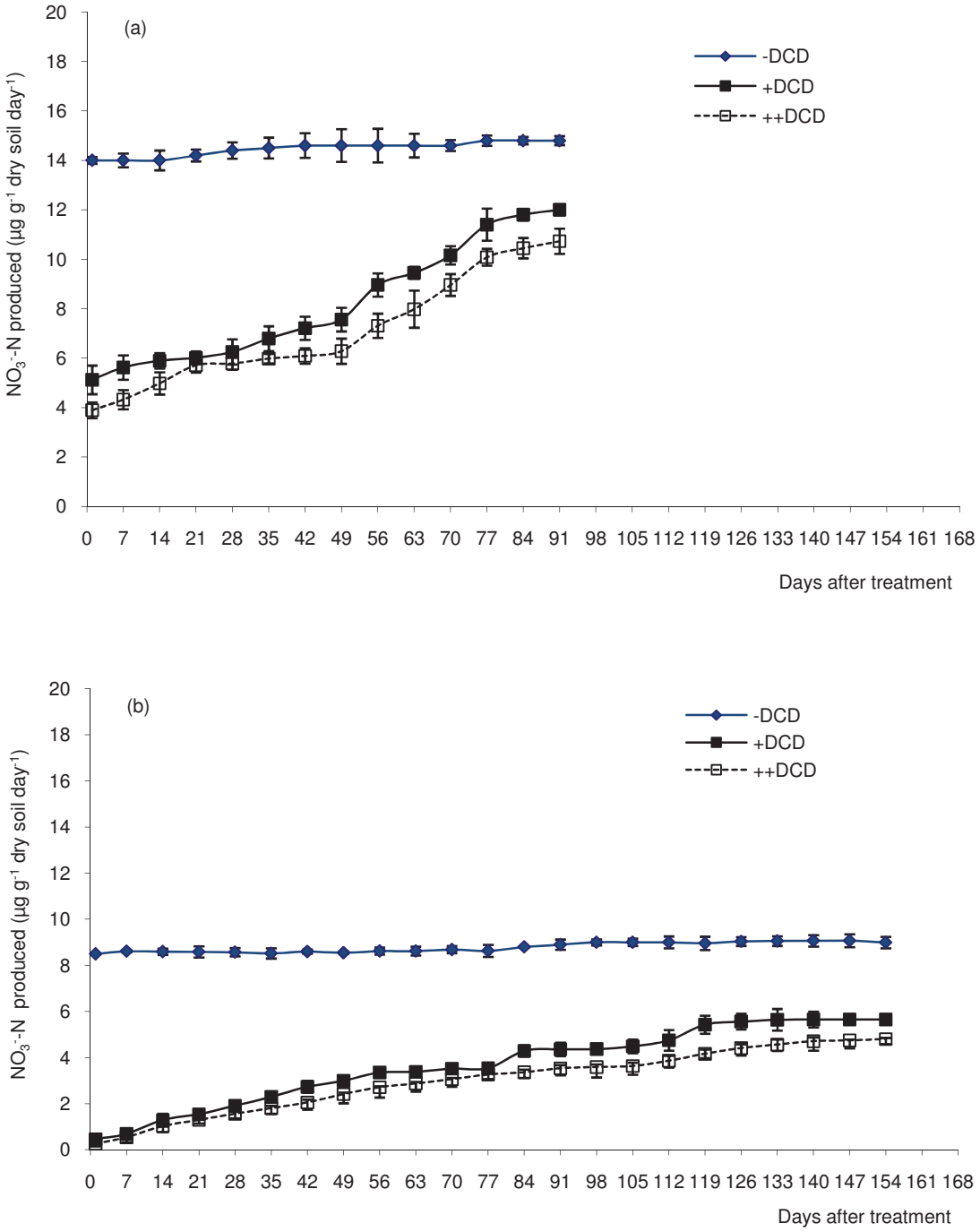


Figure 4.3 Assayed nitrification rates in (a) MSL and (b) MFSL after the addition of urea as affected by time after DCD application at 10 kg ha^{-1} (+DCD) and 20 kg ha^{-1} (++DCD). Each point was the mean of two replicates analyses of the two separate subsamples of the incubated bulk soils. Error bars represents standard error of the four analytical results for each point.

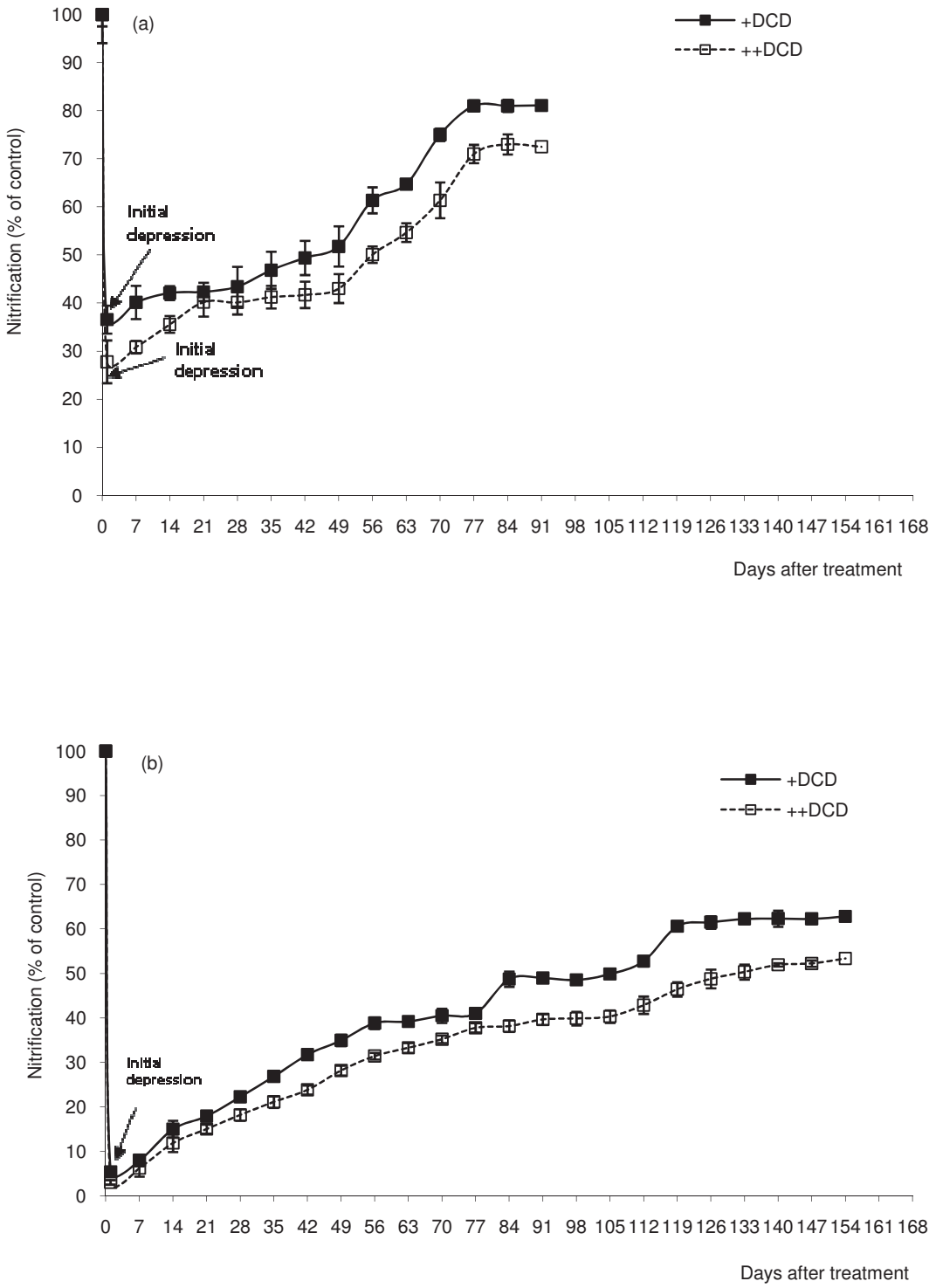


Figure 4.4 Assayed nitrification rates after the addition of urea, expressed as a percentage of control in (a) MSL and (b) MFSL with time after the addition of DCD at 10 kg ha^{-1} (+DCD) and 20 kg ha^{-1} (++DCD). Each point was the mean of two replicates analyses of the two separate subsamples of the incubated bulk soils. Error bars represents standard error of the four analytical results for each point.

4.3.2.3 The effect of DCD concentration on soil nitrate formation

In each of the soils there was a very close relationship between the amount of DCD present in the soil and the rate of nitrification (Figure 4.5 (a)). This relationship did not appear to be affected by the rate of DCD addition, but it did differ between soils. As the DCD was broken down in both soils the quantities of NO_3^--N produced during the assays increased. When the differences in the actual nitrification rates between the control and the DCD treatments in both soils (i.e. the amount of inhibition due to DCD) were plotted against the DCD remaining in both soils (Figure 4.5 (b)), there was again a close relationship between the amount of inhibition and the concentration of DCD. The relationship was not linear and the extent of inhibition appeared to drop slowly as the concentration of DCD decreased until the concentration of DCD was below 5 $\mu g\ g^{-1}$ soil and then decreased more rapidly. For a given concentration of DCD in the soil the extent of inhibition of nitrification was much greater in the MSL than in the MFSL.

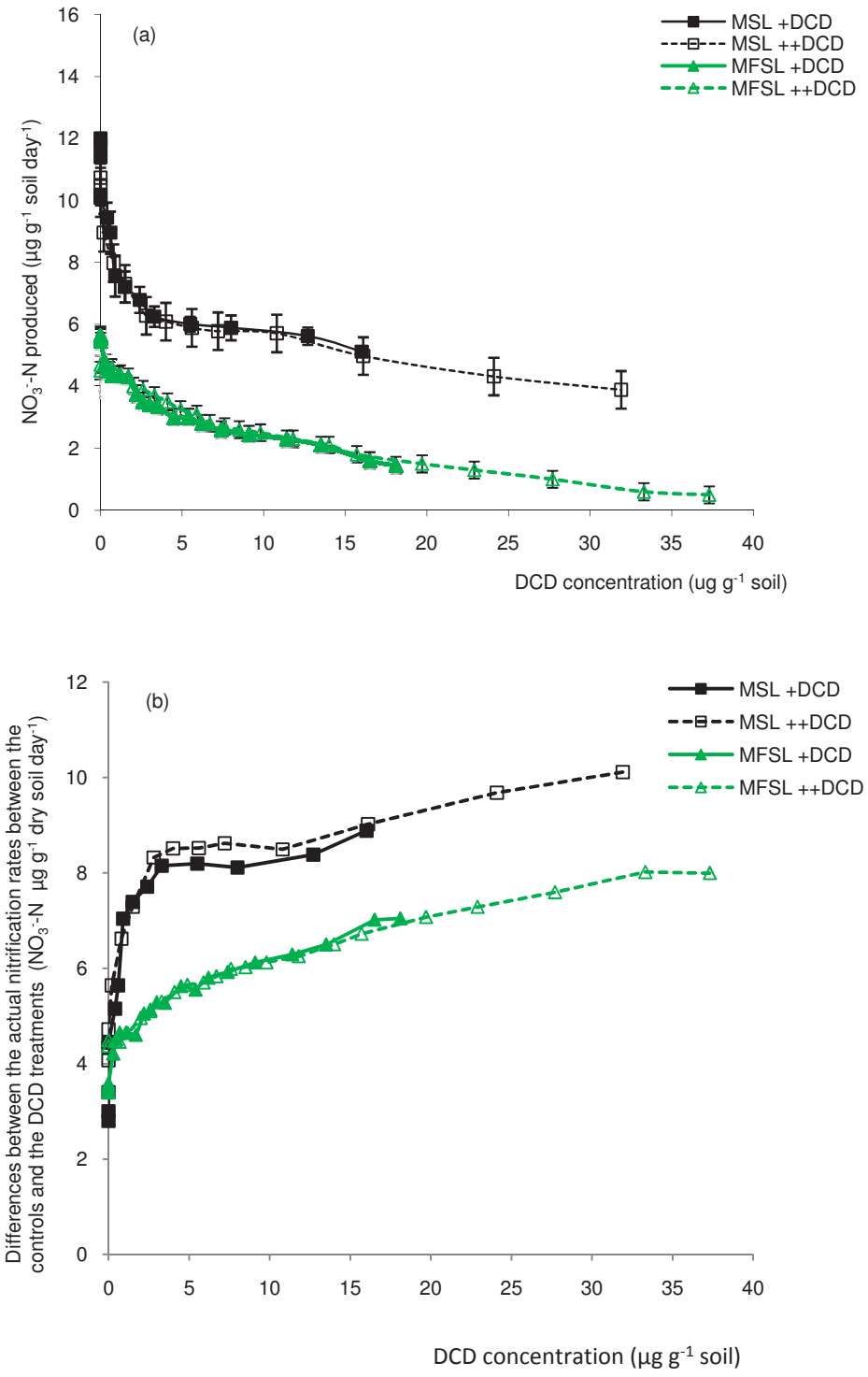


Figure 4.5 Relationship between DCD concentration in the two soils and (a) the rate of nitrification of added urea and (b) the reduction in nitrification rates from those in the control soil without added DCD. DCD was added at 10 kg ha^{-1}; +DCD and 20 kg ha^{-1}; ++DCD. Each point was the mean of two replicates analyses of the two separate subsamples of the incubated bulk soils. Error bars represents standard errors of the four analytical results for each point.

4.4 General Discussion

In the absence of DCD the assayed rate of nitrification of added urea was lower in the MFSL (8.5 µg NO_3^--N g^{-1} soil day^{-1}) than in the MSL (14.5 µg NO_3^--N g^{-1} soil day^{-1}). This pattern was similar to that obtained in Chapter 3 and once again the nitrification rates fall within the range of previously reported nitrification rates in New Zealand pasture soils (6.0 – 79.44 µg NO_3^--N g^{-1} soil day^{-1}) (Sarathchandra 1978b).

The amount of DCD recovered from the two soils at day 1 of the experiment varied between 80 and 93% with 80% being recovered in the MSL at both low and high application rates of DCD while in the MFSL soil the recoveries were 91 and 93% at the low and high rates of DCD addition respectively. These relatively high recovery rates on Day 1 give confidence that the extraction and analytical techniques used in this study are capable of quantifying most of the DCD present in the soil. The lower recovery of DCD obtained in the MSL than in the MFSL was probably due to high OM content and CEC in the MSL (Table 3.1), which contributed to higher DCD sorption. This observation is supported by studies conducted by Singh *et al.* (2008) and Zhang *et al.* (2004) who showed that soils with high OM content and higher CEC sorbed more DCD than soils with lower OM and CEC.

The rate of breakdown of DCD differed between the two soils used in this study. In the MFSL soil, the DCD degraded slower than in the MSL and the time taken by the DCD to reduce to half of its initial values in the MFSL soil was almost double that in the MSL. The rapid degradation of DCD in the MSL was likely due to the higher organic matter (OM) content in that soil (Table 2.2). The results obtained in this present study were in agreement with previous laboratory incubation studies on the effects of soil

types and temperature on the decomposition of nitrification inhibitors (NIs) including DCD (Bronson *et al.* 1989; Di and Cameron 2004a; McCarty and Bremner 1989; Puttanna *et al.* 1999; Singh *et al.* 2008). In those studies the researchers concluded that the NIs were degraded more rapidly in soils with higher OM, and were therefore less effective in inhibiting nitrification in those soils. The breakdown of these inhibitors was also accelerated with increasing temperature.

In this study, the half lives of DCD were similar for both the low and high rates of DCD application in both soils. A similar result was reported in Di and Cameron (2004a), who found that the rate of DCD application did not have a major effect on the DCD half-life, when DCD was applied at two rates equivalent to 7.5 and 15 kg ha^{-1} applied with urea (25 kg N ha^{-1}) and urine (1000 kg N ha^{-1}) in a Lismore silt loam. However, Singh *et al.* (2008) observed that the application of DCD at 20 mg kg^{-1} resulted in significantly higher DCD half-lives in all the three different types of soils tested as compared to those obtained in the soils treated with 10 mg kg^{-1} DCD.

The addition of DCD at low and high rates reduced the nitrification rates in both soils. The inhibition was greater in the MFSL than in the MSL when expressed as a percentage of the control, but the reverse was the case when the actual nitrification rates were compared (Table 4.2) and as shown earlier in Figure 4.5 (b). This was because of the very much higher nitrification rates in the MSL control soil in the absence of any added DCD.

Table 4.2 Effectiveness of DCD (applied at low; 20 kg DCD ha^{-1} (+DCD) and high rates; 40 kg DCD ha^{-1} (++DCD)) quantified in percentage (%) and absolute terms in the MSL and MFSL soils.

Effectiveness of DCD	Soil types	
	MSL	MFSL
i) Nitrification rates expressed as a percentage of control		
Control (%)	100	100
+DCD (%)	36	6
++DCD (%)	28	4
ii) Actual nitrification rates		
Control (µg NO_3^--N g^{-1} soil day^{-1})	14.5	8.5
+DCD (µg NO_3^--N g^{-1} soil day^{-1})	5.1	0.5
Δ (µg NO_3^--N g^{-1} soil day^{-1})	9.4	8.0
++DCD (µg NO_3^--N g^{-1} soil day^{-1})	3.9	0.3
Δ (µg NO_3^--N g^{-1} soil day^{-1})	10.6	8.2

These results highlight the difficulty in comparing the effectiveness of DCD between soils that may differ in their initial nitrification activities. In a recent study carried out by de Klein *et al.* (2011) on the repeated annual use of DCD to reduce N_2O emissions from cow urine, they concluded that it is important to consider the N_2O emission reductions in absolute terms (kg N_2O-N ha^{-1}) rather than relative terms when considering the potential benefits of NI in order to ensures farmers are provided with a value for its C credits in reducing the green house gases emissions. In this case, addition of DCD effectively halted nitrification activity for a period in the MFSL, whereas there was still significant nitrification activity in the MSL. However, if the initial nitrification activity in the MFSL had been similar to that in the MSL we do not know whether the

addition of DCD would still have halted the nitrification. Subbarao *et al*. (2006) mentioned that

"*the nitrifier population may be one of the factors that determine the effectiveness of inhibitors in the soil"*

The amount of DCD recovered in the soils decreased with time and eventually became negligible. This gradual decrease in the concentration of DCD corresponded with a partial recovery in the nitrification rates in the two soils. Good relationships were obtained between the quantity of DCD remaining in the soil and the nitrification rate. These relationships did not appear to be affected by the rate of DCD addition, but they did differ between soils. As a corollary to this, the extent of inhibition of nitrification within a soil was also closely related to the concentration of DCD (Figure 4.5 (b)). Once again this relationship varied between the soils with the same concentration of DCD resulting in greater inhibition in the MSL soil than the MFSL soil. But for the reasons discussed earlier it is difficult to be certain of the reasons for this.

In both soils, although all the DCD eventually disappeared, the nitrification rate did not fully recover to the level of the controls within the time scale of the experiment. This was most evident in the MFSL. If as already discussed in Chapters 2 and 3, DCD has an exclusively bacteriostatic effect on nitrifiers then the nitrification rate would be expected to return to the level in the control once all the DCD had disappeared. The fact that this did not happen may suggest that the DCD has a bacteriocidal effect on some of the most active types of AOB, thus altering permanently the community structure of AOB in the soil. If this was the case it would have important practical implications and therefore more work is required probably using advanced molecular

techniques to investigate the possible shifts in the community structure of AOB following the additions of DCD.

4.5 Conclusions

The breakdown of DCD differed between the two soils and was much quicker in the MSL soil. As there was a close relationship between the concentration of DCD remaining in the soil and the extent of inhibition of nitrification, the longer half life of the DCD in the MFSL would result inhibition of nitrification over a longer time period. The rate of DCD addition did not appear to affect greatly the DCD half life in both soils.

For each soil there was a close relationship between the concentration of DCD remaining in the soil and the nitrification rate. But this relationship was different between soils. Care must be taken when comparing the effectiveness of DCD between soils. In the current study the percentage decrease in nitrification activity in the MFSL was much greater than in the MSL. Indeed, the nitrification activity in the MFSL was effectively halted for a time after the application of DCD. But when expressed in absolute terms there was a greater decrease in nitrification activity, and the production of NO_3-N, in the MSL.

Overall therefore, the effectiveness of DCD in reducing NO_3-N production in grazed pasture systems is a function of both its half life in the soil and also the extent of inhibition of nitrification at a given concentration of DCD in the soil. In this study the two soils had contrasting rankings for these two properties. More work is required on understanding an predicting the likely cost-effectiveness of DCD in contrasting soils.

It appears as if there may have been an ongoing residual effect of DCD in both soils, which resulted in only a partial recovery in the nitrification rates in the two soils. If this was because the DCD had selectively removed the active AOB, then there may be an opportunity to carry out a further work using molecular techniques to determine the possible shifts of AOB community structure following the additions of DCD to the soil.

Chapter 5

Laboratory study on nitrification in hill country and lowland soils amended with urea

5.1 Introduction

Earlier studies (Bowatte 2003) have shown that N transformations in hill country soils are highly variable across different landscapes with contrasting topography in North Island hill country pasture. For example, soils collected from campsite areas at the Ballantrae hill country research station had greater nitrification rates (11.3 µg NO_3^--N/g soil/day) than soils collected from the steep slopes (2.1 µg NO_3^--N/g soil/day). In addition, the nitrification rate after urine application was considerably lower in soils from the flat and steep hill country sites at the summer dry Waipawa experimental farm than on soils from the corresponding sites on the summer wet Ballantrae experimental farm (Bowatte 2003).

From the study of Bowatte (2003) soils from steep slopes in hill country pastures have very low rates of nitrification, and these rates do not increase, even when the soils are incubated in ideal conditions in the laboratory, with non-limiting amounts of ammonium available. The question arises as to whether these soils contain some naturally-occurring inhibiting factor. In his study he concluded that that the history of N application, the C/N ratio of the soil and the soil organic matter quality are the likely contributing factors to the variation in nitrification rates in soils from these. However, it is not well understood whether the low nitrification rates in steep site soils relate to low

numbers of ammonia oxidising bacteria (AOB) as compared to the campsite and lowland soils or whether these soils contain some naturally-occurring inhibiting factor that slows down the nitrification rate in some way. The experiment described in this Chapter explored this possibility a little further.

The specific objectives of this study were to quantify the nitrification rates in soils collected from two contrasting adjacent sites (steep slope (SS) and flat campsites (CS)) in a hill country paddock at Ballantrae AgResearch hill country research station of Grassland Division, Palmerston. Both soils are described as Wilford hill silt loams which are classified as Mottled Argillic Pallic Soils formed from tertiary sediments under an annual rainfall of 1000-1400 mm (Hewitt 1998). These nitrification rates were then compared with the nitrification rates in soil collected from a lowland dairy farm. This soil was a Manawatu silt loam (MSL), a Weathered Fluvial Recent Soil (Hewitt 1998). The numbers of nitrifying AOB in the hill country soils and in the lowland dairy farm soil were also determined.

5.2 Materials and Methods

5.2.1 Sampling site and soil preparation

Three different soils were used in this study. The soils from the SS and CS sites were obtained from AgResearch Ballantrae hill country research station of Grassland Division, 20 km north east of Palmerston North in the foothills of the Southern Ruaahine Range. The MSL was obtained from Dairy Farm No. 1 Massey University, Palmerston North. Approximately 10 kg of field moist topsoil (0-10 cm) was collected from multiple sampling locations at each of these sites. The stones, roots and other debris were removed from the soils which were then partially air dried for a day before

sieving. The partially air dried soils were passed through a 2-mm sieve in the laboratory, homogenised by hand and again air dried for a day. A sub-sample of each of the sieved soils was used to determine the gravimetric moisture content by drying at 105 ^{o}C for 16 hours. Other soil sub-samples were used to determine the soil water holding capacity through a pressure plate extractor at (1/10 bar) followed by drying at 105^{o}c for 16 hours.

5.2.2 Soil incubation - N transformation in soils amended with urea

Based on the measured initial soil moisture content, a weight of field moist soil equivalent to 12 g of dry of soil was placed in sixteen 50 mL of beakers. Eight of the beakers of each soil were treated with 1.0 mL of urea solution (~ 200 µg N/g dry soil), while the remaining eight beakers were used as controls (without urea addition). The moisture content of each soil was brought to its field capacity by adding deionised water. After the addition of urea, the soil in the beaker was mixed with a spatula and the beaker was then covered with aluminium foil, with pin holes in it. All of these 48 beakers were incubated aerobically, at 26 ^{o}C in the dark for 0, 5, 10 and 15 days. The incubation time for the 0 days was for carried out for 4 hours. There were two replicates for each treatment of each soil at each sampling time. Each beaker was weighed every 3 days and the weight loss was replaced with deionised water to keep the moisture content constant throughout the incubation period.

At the end of each incubation time, 12 beakers were selected and a sub-sample of soil from each beaker was taken for determination of the moisture content by drying at 105^{o}C for 16 hours. A second sub-sample was extracted with 2 M KCl solution by shaking for 30 minutes (1:5 soil:extractant ratio). The soil extract was analysed

colorimetrically for mineral N by an auto analyser (Blakemore *et al.* 1987). The concentrations of extractable NH_4^+-N and NO_3^--N in the soil at time zero (before incubation) and after the 15 days of incubation were used to calculate the net nitrification rate (µg NO_3^--N g^{-1} dry soil day^{-1}). All N transformations were expressed on a dry weight basis.

5.2.3 Estimation of AOB population

A sub-sample from the control (without urea) at Day 0 and at the end of the 15 days of incubation with urea was taken from each of the SS, CS and MSL soils for the MPN assays. The AOB population was determined by MPN method previously described in Chapter 3 (Section 3.2.6).

5.3 Results

5.3.1 Chemical properties of the three soils

The three soils varied in their chemical properties as shown in Table 5.1.

Table 5.1 Chemical properties of the three soils studied.

Soils	pH	NH_4^+-N (µg g^{-1})	NO_3^--N (µg g^{-1})	Total C (%)	Total N (%)	C:N ratio
Steep slope (SS) soil	5.51	2.2	0.7	5.6	0.4	14
Flat campsite (CS) soil	5.39	8.3	9.3	5.3	0.5	11
Lowland soil (Manawatu silt loam)	5.25	10.2	12.1	4.8	0.5	10

5.3.2 Mineral N changes and nitrification in the presence and absence of added urea

In the CS and MSL control treatments there were slight reductions in the NH_4^+-N concentrations with time (Figure 5.1 (i)). Over the same time period the concentrations of NO_3^--N in the CS and MSL soils increased. The increases in the concentration of NO_3^--N were much greater than the decreases in the concentration of NH_4^+-N over the time, and thus in these two soils there was an overall increase in the total mineral N during the incubation – probably as a result of mineralisation of organic N. There were no obvious changes in the mineral N fractions in the SS soil during the incubation (Figure 5.1 (i)).

The addition of urea led to an immediate increase and high concentration of NH_4^+-N in all three soils (Figure 5.1 (ii)). It is interesting to note that in all three soils, very rapid ammonification was observed within a short period of time. This presumably reflects high urease enzyme activity in all the three soils. The rapid hydrolysis of urea also caused an increase in pH in all three soils immediately after the addition of urea (Figure 5.2). Since there was a rapid hydrolysis of urea to NH_4^+-N in this study, the nitrification process was not an example of urea-linked ammonia oxidation, in which urea enters the cells by diffusion, and is hydrolysed intracellularly to NH_3 prior to nitrification (Allison and Proser 1991; Burton and Prosser 200; de Boer *et al*. 1989a).

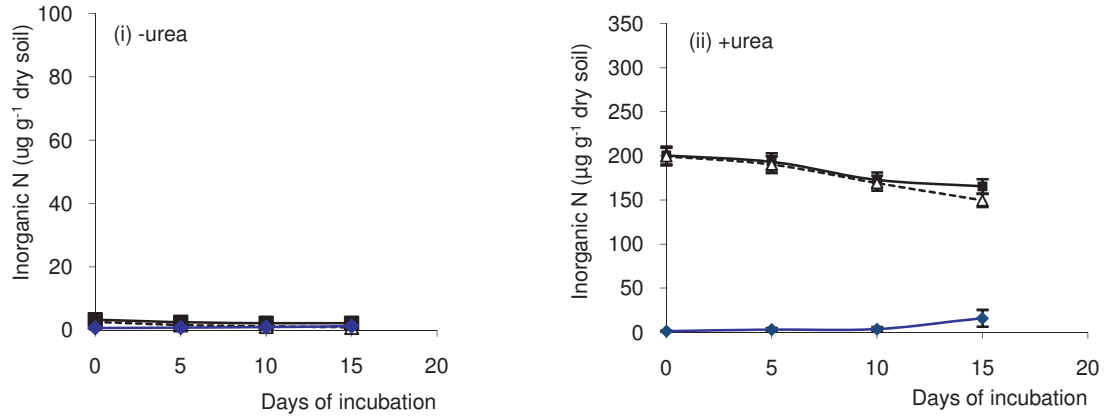
The addition of urea led to an immediate increase the concentration of NH_4^+-N in all three soils (Figure 5.1 (ii)). In the SS soil the concentration of NH_4^+-N then decreased slowly (from 200 to 166 $\mu g\ g^{-1}$ soil) over the 15-day incubation period. At the same time there was a small increase (from 1.2 to 15.9 $\mu g\ g^{-1}$ soil) in the concentration of

NO_3^--N. The increase in NO_3^--N was not as great as the decrease in NH_4^+-N and as a consequence, the concentration of total mineral N decreased slightly during the incubation. In contrast, the concentration of NH_4^+-N in the CS and MSL soils decreased rapidly reaching 31.2 and 24.3 µg g^{-1} soil after 15 days in the CS and MSL soils respectively. The NO_3^--N concentrations increased rapidly over the same time in both the CS and MSL soils and peaked at 139.6 and 259.3 µg g^{-1} soil respectively after the 15 days of incubation.

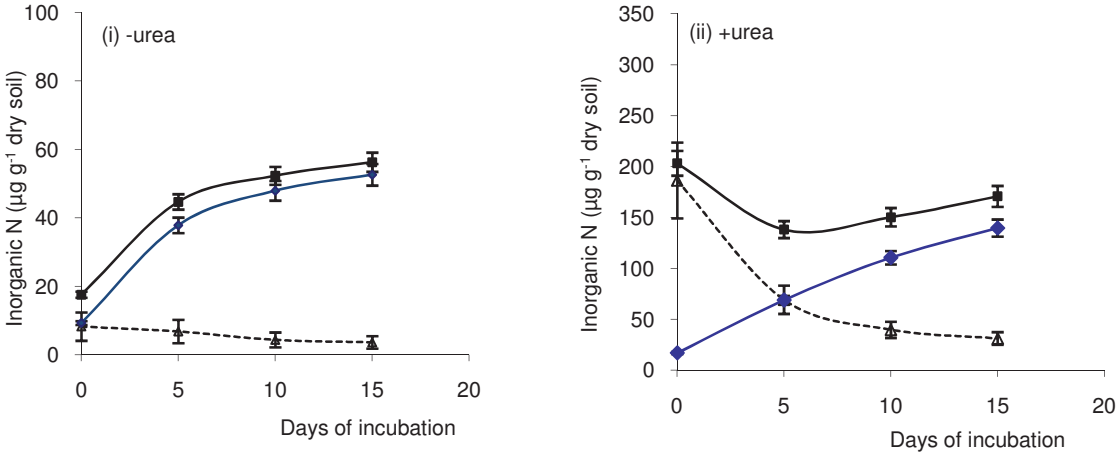
The total mineral N concentrations in the CS and MSL soils following the addition of urea decreased slightly in the first 5 days, but increased thereafter until day 15. In the CS soil the total mineral N concentration at the end of the incubation was similar to that immediately after the addition of urea on day 1. In the soil from the lowland dairy farm however, the total mineral N concentration at the end of the incubation was considerably (283.6 µg g^{-1} soil) higher than immediately after the addition of urea. This presumably results from mineralisation of organic N in this soil during the incubation

The initial pHs of the three soils in the control treatment were in the range 5.25-5.51 (Figure 5.2 (a)). There was a small decrease in the pH of all the control soils over time during the incubation, however these difference were not significant. Following the addition of urea the pH increased in all the soils as a consequence of urea hydrolysis. The highest pH (6.12) was recorded in the SS soil (Figure 5.2 (b)). These elevated pH levels then decreased as the incubation proceeded and nitrification took place. This decrease in pH was small in the SS soil but was very large in the other two soils. By the end of the incubation the pHs in the CS and MSL soils were 4.68 and 4.41 respectively.

Steep slope soil



Flat campsite soil



Lowland soil

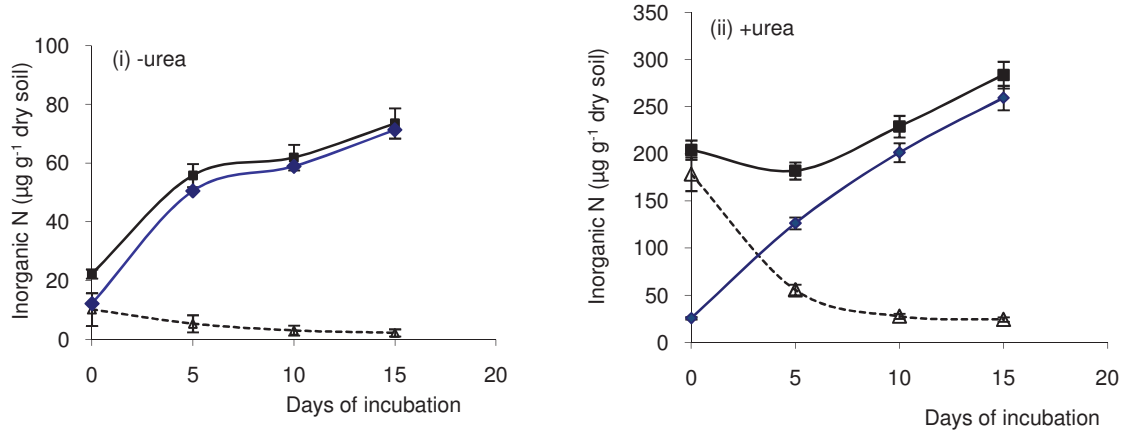


Figure 5.1 Total mineral N (-■-), NH_4^+-N (-△-) and NO_3^--N (-◆-) concentrations during incubation of control (-urea) and urea treated (+urea) soils. Error bars represent the range of inorganic N concentrations obtained between the two replicates. Note that the concentration scale in the control graphs differs from that in the + urea treatment.

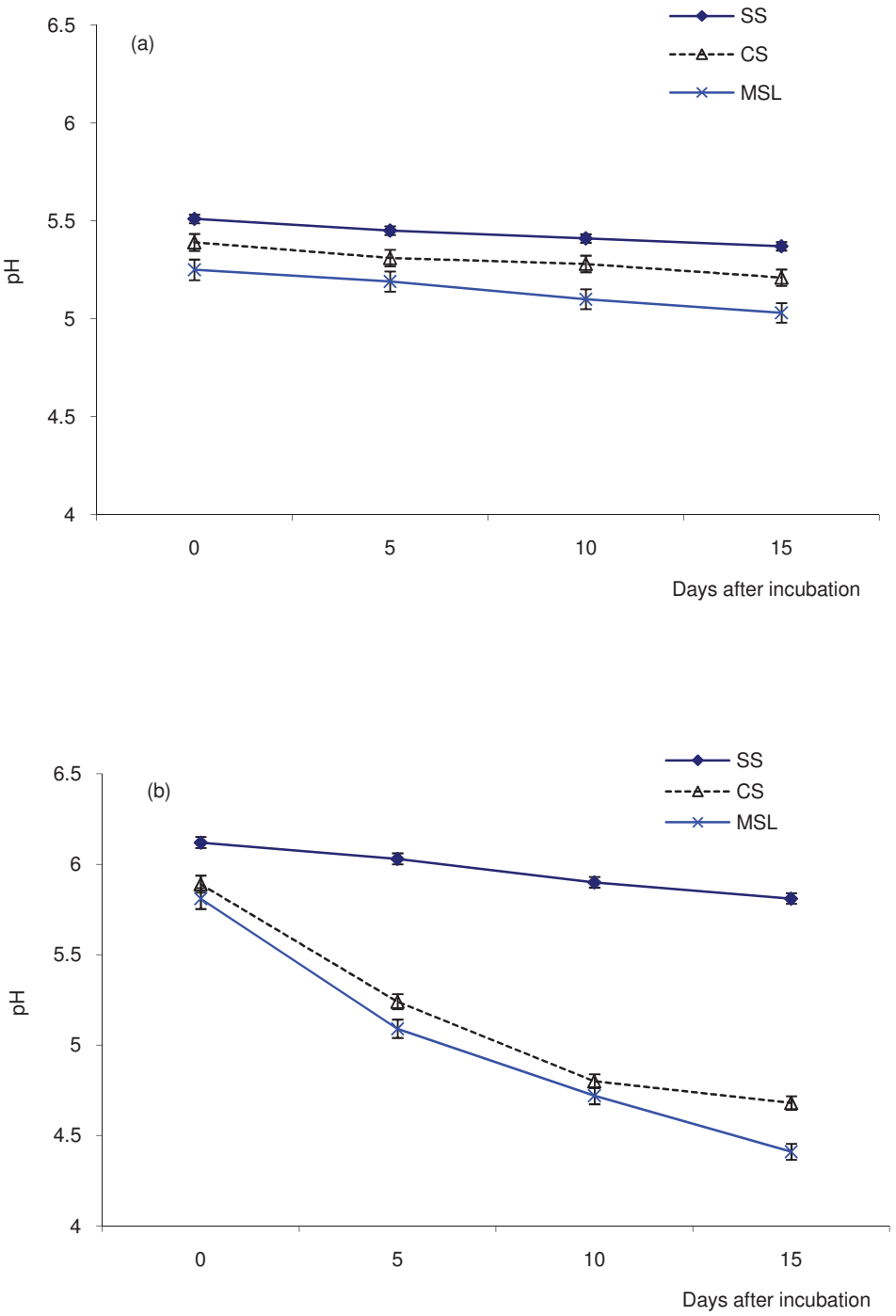


Figure 5.2 Changes in pH with time of incubation in (a) control soils and (b) soils with urea addition. Vertical bars denote the range of pH obtained between the two replicates.

5.3.3 Nitrification rates in the control and urea treated soils

The average nitrification rates in the control soils over the 15 days of incubation were 0.04, 2.9 and 3.6 µg g^{-1} soil day^{-1} in the SS, CS and MSL soils respectively (Figure 5.3 (a)). Following the addition of urea to the CS and MSL soils, the rates of nitrification in these two soils (8.2 and 15.6 µg g^{-1} soil day^{-1} respectively) were substantially higher than in the control soils. In contrast, addition of urea resulted in only small increase in the nitrification rate in SS to 1.0 µg g^{-1} soil day^{-1}.

When the nitrification rates in the control and urea added soils were calculated over successive 5-day intervals (Figure 5.3 (b) and (c)), the nitrification rates in the control SS soil was almost negligible throughout the incubation period, while they decreased over time in the CS and MSL soils. Following urea addition to the CS and MSL soils (Figure 5.3 (b)) the nitrification rates in both soils increased markedly over the control in the first 5-day period and then declined over time from 5 to 15 days. In the SS soil, the addition of urea had little effect on the nitrification rate for the first 10 days, but from days 10 to 15 there was a small increase in nitrification rate. However the nitrification rate in the SS soil was always much lower than in the other two soils.

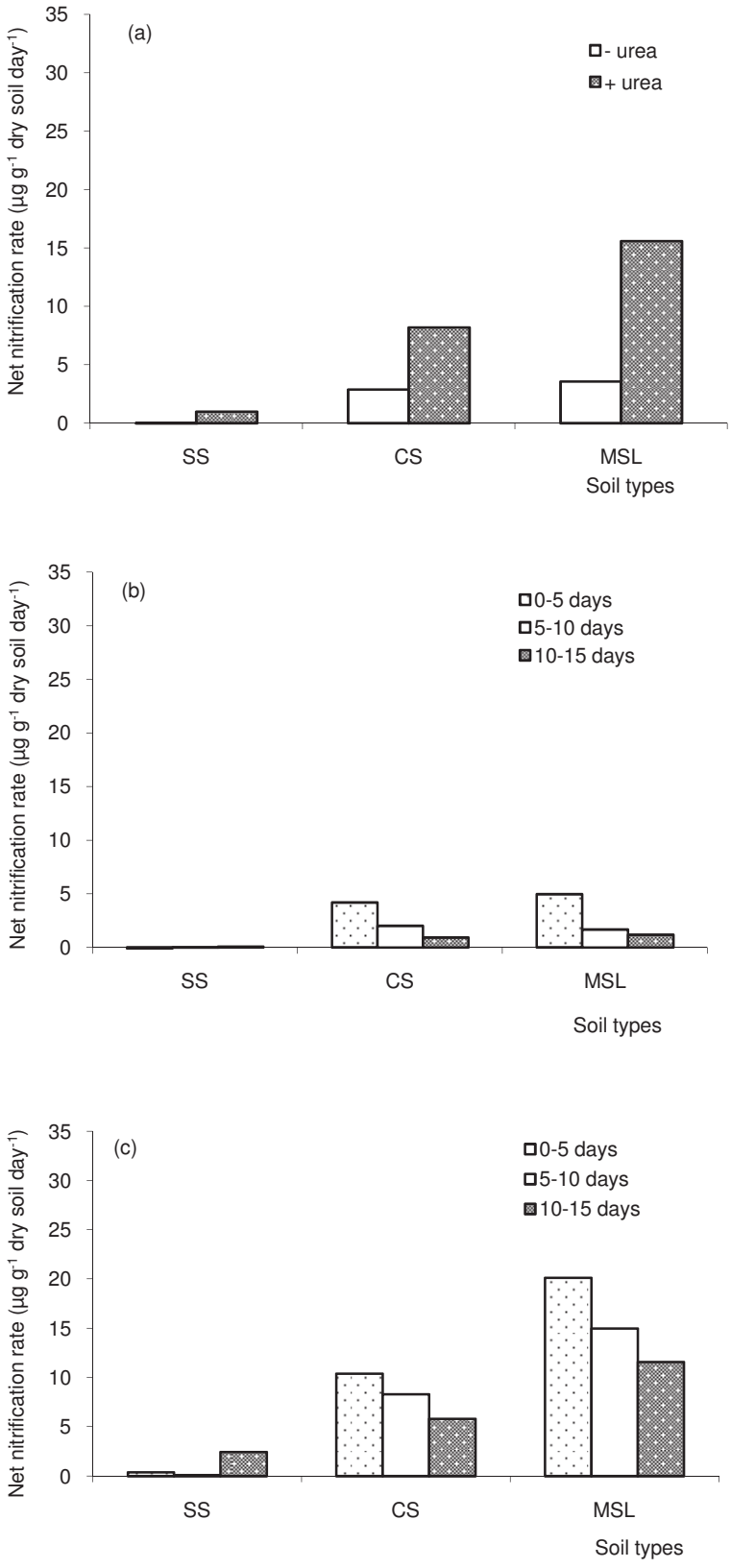


Figure 5.3 Nitrification rates in the three soils; (a) during the 15-day incubation period, with and without urea addition, (b) in successive 5-day periods in the control soils and (c) successive 5-day periods in the soils with urea added.

5.3.4 Estimation of AOB populations

In without the urea treatment (-urea), the soil collected from the SS has a low AOB population (9530 cells g^{-1} SDW) from the AOB populations measured in the CS (55476 cells g^{-1} SDW) and MSL (375000 cells g^{-1} SDW) soils (Figure 5.4). After the addition of urea (+urea) (Figure 5.4), the numbers of AOB increased to 27634, 221325 and 789123 cells g^{-1} SDW in the SS, CS and MSL soils respectively.

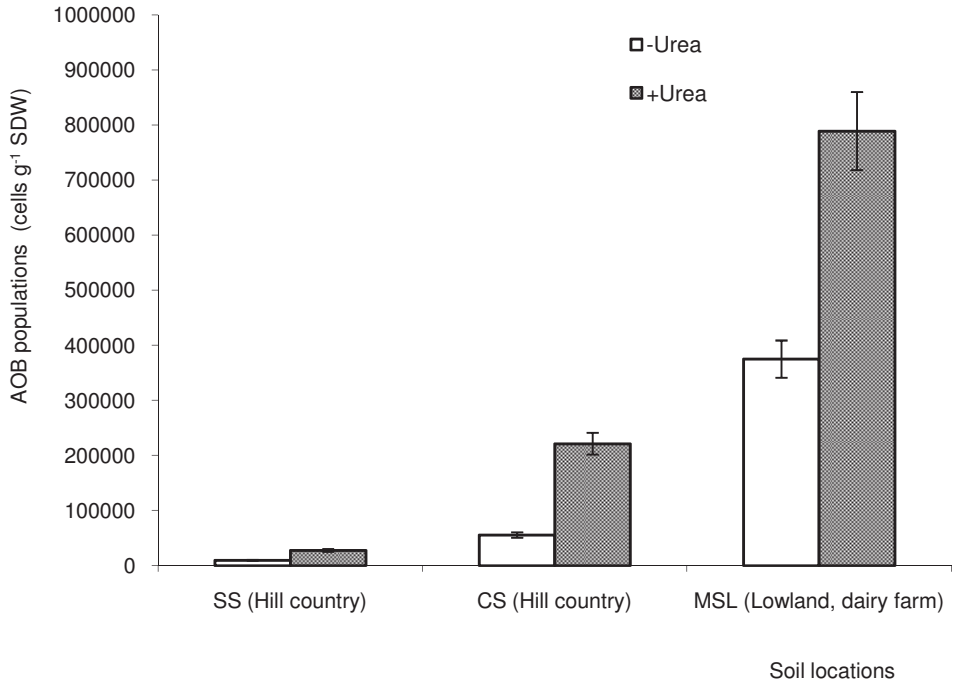


Figure 5.4 Viable population size of AOB in steep slope, SS; camp site, CS and (c) Manawatu silt loam, MSL. Vertical bars in the columns indicate range of AOB populations obtained in the 2 replicates in each treatment.

5.4 General Discussion

The nitrification rates obtained in this present study confirmed the earlier published work by Bowatte (2003). In his study he found that the soils collected from a lowland dairy farm had higher nitrification rates than hill country soils. On the other hand, in the hill country, soils collected from a steep slope (SS) had a considerably lower rate of nitrification than the soil collected from an adjacent flat camp site (CS). The author concluded that the history of N application, C/N ratio of the soil and quality of soil organic matter (SOM) were likely factors to have influenced the nitrification activities in the soils tested. In addition, earlier work in hill country suggested that the stock tend to camp on flat areas of land and significant quantities of nutrients are transported to these areas from off the steeper slopes where the sheep graze (Gillingham & During 1973; Rowarth & Gillingham 1990) and that these may have alter the nutrient cycling and soil fertility in that area. Furthermore, soils collected from flat CS have low C/N ratios resulting in higher mineralisation of organic N than in the soil collected from SS, which normally have high C/N ratios (Ledgard *et al*. 1982), thus therefore contributing factor in the higher nitrification rates observed in CS than SS sites.

In this present study, another factor that is likely to have contributed to the low nitrification rates in the SS soil was the lower numbers of nitrifying AOB present in this soil than in the CS and MSL soils. In acid Scot pine forest soils it has been demonstrated that the low nitrification rates obtained in the four soils tested was associated with the absence of AOB, *Nitrosospira* cluster 2, while in the five soils which had higher nitrification rates, *Nitrosospira* cluster 2 was detected (Nugroho *et al.* 2005). They also showed that the differences in nitrification rates between the forest soils were correlated to soil C/N ratio and atmospheric N deposition. In other studies

Laverman *et al*. (2000, 2001) attributed the spatial and temporal variation in NO_3^- production in acid forest soils in the Netherlands to biotic and abiotic factors affecting the numbers and/or activity of AOB and apparently not due to differences in ammonia oxidiser diversity or community structure. In agreement with these findings, a number of studies have shown that the numbers of AOB present in the soil correlate with the nitrification rates and are influenced by site topography, vegetation cover, fertiliser and OM inputs and soil moisture status (Jha *et al.* 1996a; Jha *et al.* 1996b; Singh and Kashyap 2006).

As discussed earlier in the Literature Review (Chapter 2), substrate availability either from the ammonification of OM or from fertilisers containing urea or ammonium is the sole source of energy for nitrifiers and is crucial to the nitrifying process - providing that other environmental factors are not limiting. In this present study it was observed that there was a considerable increase in the rates of nitrification and the AOB populations in the CS and MSL after the addition of urea, while there was only a slight increase in the SS soil.

A study on the effects of different fertiliser treatments on nitrification in two coniferous soils in southern Finland showed that net nitrification was stimulated by urea but to a lesser degree by wood ash and not by ammonium nitrate or nitroform (Martikainen 1984). In a more recent study, He *et al.* (2007) showed that the population sizes of AOB and AOA changed greatly in response to different fertilisation treatments in an experimental plot that had received 16 years continuous fertilisation treatments – the highest population of AOB and AOA being found in the NPK + OM treatment while the

lowest numbers of AOB and AOA were recorded in the N treatment alone. Their results further demonstrated that the highest potential nitrification rates were recorded in the NPK + OM treatment and these were were 5-10 times higher than those in mineral fertiliser treatments.

In this present study the addition of urea in the SS soil did not greatly increase the nitrification rate and the AOB numbers in absolute terms, compared to the CS and MSL soils, even with a plentiful supply of NH_4^+ substrate from the urea and the associated higher pH. There were three possible reasons that might have contributed to this. Firstly, it could be due to the presence of some inhibiting factors in the soil that inhibited the growth of AOB. Secondly, it could be due to the dominance of AOA over AOB in these soils. In a recent study of six grazed grassland soils sampled across New Zealand, Di *et al*. (2009) reported that AOA were present in large numbers in these soils, but neither their abundance nor their activity increased with the application of urine as an NH_3 substrate, suggesting that their abundance was not related to the rate of nitrification. In contrast, the numbers of AOB and the rate of nitrification increased in response to the urine addition, suggesting a significant relationship between the abundance of AOB and the rate of nitrification. Finally, it could be due to abundance of heterotrophic bacteria than the AOB present in that SS soil, but more work is required to evaluate and to confirm the role of heterotrophic nitrifiers in this low fertility SS soil.

The possibility of there being some type of inhibiting factor present in the soil was explored further by a more detailed analysis of the AOB populations and nitrification rates. Although the absolute increase in AOB numbers in the SS soil over the 15-day incubation was small, but it was comparable to the increases in the other two soils

(Table 5.2), when calculated as AOB population ratio. This might suggest that the small increases in absolute AOB numbers in the SS soil resulted mainly from the initially small population size rather than an inhibiting factor in soil preventing AOB growth. This is in contrast to the finding of Bowatte (2003) that in SS soils there was very little increase in nitrification rate even after incubation for three months. But Bowatte (2003) did not measure the AOB populations in his samples.

When the "activity" of individual AOB in the 3 soils was assessed, the nitrification rate per AOB (Table 5.2) was roughly similar in all the soils. Certainly, the activity of AOB in the SS soil was not orders of magnitude less than in the other 2 soils – once again suggesting that the AOB that were present were not inhibited greatly in their activity.

Table 5.2 AOB populations prior to and following incubation of soils for 15 days with added urea, and the average daily nitrification rate per AOB over the 15-day incubation period calculated using the average of the AOB populations at Day 0 and Day 15.

Soils	AOB numbers at day 0 (cells g^{-1} SDW)	AOB numbers at day 15 (cells g^{-1} SDW)	AOB population ratio (Day 15/Day 0)	Nitrification rate per AOB (μg NO_3^--N day^{-1} AOB^{-1})
Steep slope soil	9530	27634	290	5.3×10^{-5}
Flat campsite soil	55476	221325	400	5.9×10^{-5}
Lowland soil (Manawatu silt loam)	375000	789123	210	2.7×10^{-5}

5.5 Conclusions

This study showed that nitrification rates in the SS soils from hill country are much lower than in the CS soils and a lowland MSL from a dairy farm and that these low nitrification rates were associated with similarly low populations of AOB in the SS soil. Although there were very much differences in the nitrification activity observed in the three soils, it was interesting to note that the ammonification through the urease enzyme activity were seems to be high in the three soils. However, at this point, it is not known how rapid is the ammonification had occurred in each soil. The urease activity may be very rapid in the high nitrifying soils; CS and MSL (i.e occurred in few hours time), while more slower in the SS soil.

The fertiliser N history and continuous high N inputs through greater return of excreta in the CS and lowland MSL soils may have encouraged larger AOB populations and thereby have stimulated higher nitrification activity than in the SS soil. The AOB that were present in the SS soils however did appear to reproduce at a rate, and to have a nitrification activity that were similar to the AOB in the other two soils. This appeared to be different than the finding of Bowatte (2003), and this was investigated further in the following experiment.

Chapter 6

The effect of mixing the low and high nitrifying soils on nitrification activity

6.1 Introduction

This investigation follows on from the measurement of nitrification activity in the hill country soils carried out earlier in Chapter 5 and aims to provide additional evidence as to whether there is a possibility of inhibition or a "poisoning effect" on nitrifiers in the soil collected from steep sites in hill country. In Chapter 5, it was found that soil collected from steep slopes had low nitrification rates and a low population of nitrifiers. It was not however totally clear whether the low initial population of AOB in soils from steep slopes resulted from low inputs of ammonium substrate over many years, or whether in addition there was an inhibitory effect that may have prevented a build-up of the nitrifiers.

As already mentioned earlier in the Literature Review, several researchers have suggested that allelopathy, or the release of inhibitory compounds from plant roots such as tannins, phenolic acids and phenolic glycosides, can suppress the nitrifier activity in mature grassland ecosystems (Lata *et al.* 1999, 2004; Munro 1966; Rice and Pancholy 1973).

Earlier work by Bowatte (2003) demonstrated that even after incubating soils from steep slopes with urine for up to three months there was little evidence of an increase in

nitrification**,** which led him to suggest that perhaps an inhibitory effect was operating. Sanches (2009) in her study on proximal sensing the botanical composition of New Zealand dairy and sheep pastures showed that perennial ryegrass and white clover were the most major component of most lowland swards. On the other hand, on steep hill country farms where the soil fertility is lower, browntop was the dominant grass species.

The work reported in this chapter investigated the effect of mixing soils with initially low nitrification activity (collected from steep slopes) with soils with initially high nitrification activity (collected from camp sites) on the subsequent nitrification activity in the resulting soil mixtures. The nitrification activity was assessed with and without urea amendment under laboratory conditions over a 15-day period. Measurements were made were after 0, 5, 10 and 15 days of incubation. In this study, it was hypothesized that if the soil collected from the steep slope had some nitrification inhibition characteristics, then mixing that soil with another soil that initially had high nitrification activity would result in a suppression of nitrification activity in the soil mixture.

6.2 Materials and Methods

6.2.1 Sampling sites and soil preparation

Approximately 10 kg samples of field moist topsoil (0-10 cm) were collected from two different landscapes (flat camp site; CS and steep slopes; (SS) of the Ballantrae AgResearch hill country research station of Grassland Division, 20 km north east of Palmerston North in the foothills of the Southern Rauahine Range. Both soils are described as Wilford hill silt loams which are classified as Mottled Argillic Pallic Soils formed from tertiary sediments under an annual rainfall of 1000-1400 mm (Hewitt

1998). Stones, roots and other debris were removed from the soils which were then partially air dried for a day before sieving. The partially dried soils were then passed through a 2-mm sieve in the laboratory and homogenised by hand. The sieved soils were then again air-dried for two days.

A sub-sample of each of the sieved soils was used to determine the gravimetric moisture content by drying at 105^{o}c for 16 hours. Seven mixtures of the two soils were then prepared. The ratios (CS:SS) of the two soils (calculated on an oven-dry weight basis) in the mixtures were as follows: 100:0, 80:20, 60:40, 50:50, 40:60, 20:80 and 0:100. Once these soil mixtures had been prepared, the soil water holding capacity of each was determined with a pressure plate at 1/10 bar followed by drying at 105^{o}c for 16 hours.

6.2.2 Soil incubation study and soil analysis

Based on the measured initial soil moisture content of each of the soil mixtures, a weight of field moist soil equivalent to 20 g dry weight of the soil was placed in each of 32 plastic cups. This procedure was repeated for each soil mixture. Sixteen of the plastic cups were incubated without urea (control) and the remaining 16 plastic cups were incubated after addition of a urea solution - each cup receiving 200 µg N g^{-1} dry soil. There were four replicates for each treatment for each soil mixture. The moisture content for each soil mixture was brought up to its field capacity by adding deionised water. Soil in the cups was mixed with a spatula after the addition of urea solution and deionised water, and the cups were covered with aluminium foil, with pin holes in it. The 224 cups were incubated aerobically at 26^{o}C in the dark for either 0, 5, 10 or 15 days. The incubation time for the 0 days was for carried out for 4 hours.

Each cup was weighed every 3 days and the weight loss was replaced with deionised water to keep the moisture content constant throughout the incubation period. At the end of each of the incubation times, 8 cups from each of the soil mixtures (4 cups treated with urea solution and 4 cups acting as a control) were selected randomly and a soil sub-sample from each cup was collected for moisture content determination by drying at 105^{o}C for 16 hours. A second sub-sample was extracted with 2 M KCl solution by shaking for 30 minutes (1:5 soil:extractant ratio). The soil extract was analysed colorimetrically for mineral N by a Technicon auto analyser (Blakemore *et al.* 1987).

Net nitrification rate (µg NO_3^--N g^{-1} dry soil day^{-1}) was calculated to be the concentration of NO_3^--N in the laboratory-incubated soil minus the concentration in the initial sample. All N transformations were expressed on a soil dry weight basis. The total carbon (C) and nitrogen (N) concentrations in the CS and SS soils were analysed with a Leco FP-2000 CNS (LECO Corp., St Joseph, MI, USA) and the total C and N concentrations in the other soil mixtures were calculated from these values.

6.3 Results

6.3.1 Chemical properties of the two soils

The initial soil pHs for the CS and SS soils were 5.35 and 5.45 respectively. The predominant component of 2M KCl-extractable mineral N in the CS soil was NO_3^--N (25.1 µg g^{-1} soil), while the NH_4^+-N concentration was 8.3 µg g^{-1} soil. In the SS soil, a lower concentration of NH_4^+-N was measured (2.5 µg g^{-1} soil), and the concentration of NO_3^--N was almost negligible (0.62 µg g^{-1} soil). The CS soil had higher total C (5.61%)

and N (0.50%) than the SS soil which had 4.66% and 0.32% of total C and N respectively. The C/N ratio of the SS soil (15:1) was higher than the CS (11:1) soil.

6.3.2 Changes in mineral-N in the mixed soils with and without addition of urea

There was a slight decrease in the concentration of NH_4^+-N over time in the control treatments of all the soil mixtures, except for the SS soil (Figure 6.1 a (i) - f (i)). At the same time the concentrations of NO_3^--N increased. The increases in the concentrations of NO_3^--N in the control soils were slightly greater than the decreases in NH_4^+-N, and as a result the concentrations of total mineral N increased slightly over time. This suggests that there was some mineralisation of organic N occurring in the soil, as well as nitrification of the NH_4^+-N. In the 100% SS soil, there were no changes in the concentrations of NH_4^+-N, NO_3^--N and total mineral N in the absence of added urea (Figure 6.1 g (i)).

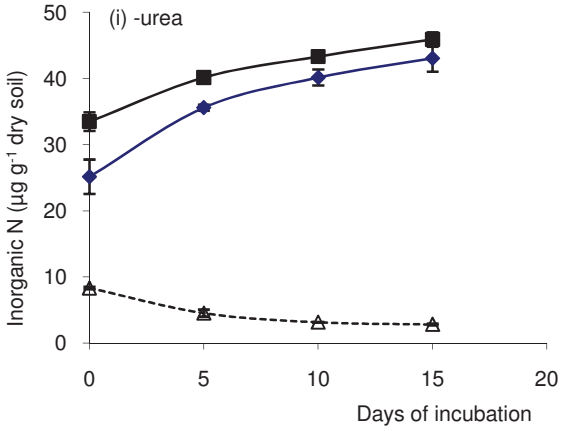
The concentration of NH_4^+-N in all the soil mixtures increased immediately after the addition of urea. As observed in Chapter 5, high concentrations of NH_4^+-N were obtained within a short period of time (day zero) and were presumably due to high levels of urease enzyme activity which caused the rapid hydrolysis of urea. This was also supported by the increase in pH at day zero in all the soil mixtures after the additions of urea (Figure 6.2).

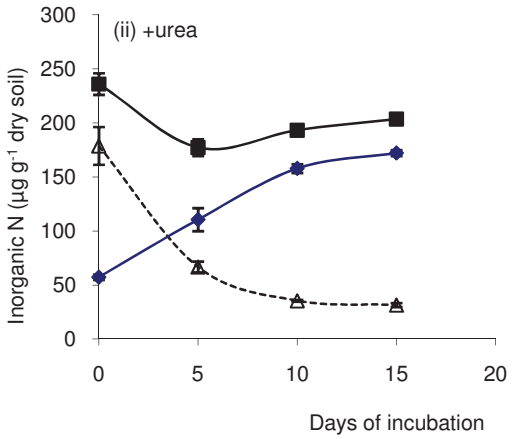
In the 100% CS soil treated with urea, the concentration of NH_4^+-N then decreased rapidly from 178.8 µg g^{-1} soil at 0 days to 31.5 µg g^{-1} soil at the end of the 15 days of incubation (Figure 6.1 (a, ii)). In contrast, in the 100% SS soil (Figure 6.1 (g, ii)), the

NH_4^+-N nitrified only slowly and the NH_4^+-N concentration was still 164.5 µg g^{-1} soil at the end of the 15 days of incubation. The concentration of NO_3^--N in the SS soil increased very slowly from 1.55 at 0 days to 5.61 µg g^{-1} at 15 days, while the amount of NO_3^- -N produced in the 100% CS soil increased rapidly, reaching 172.1 µg g^{-1} soil at the end of the 15 days of the experiment. The higher the ratio of CS to SS in the soil mixture that received urea, the greater the amount of NH_4^+-N converted to NO_3^-–N (Figure 6.1 (a, ii) – (d, ii)). Following the addition of urea to most of the soil mixtures, the total inorganic N decreased sharply in the first 5 days and this was followed by a small increase until day 15. In the 100% SS soil (0:100), the total inorganic N slowly decreased from day 0 until the end of the 15-day experiment.

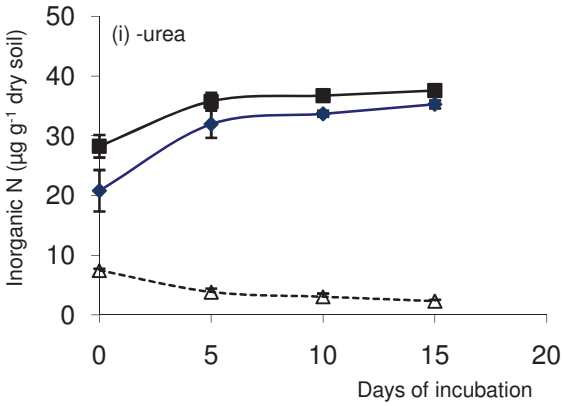
The initial pHs of the CS and SS soils were 5.35 and 5.45 respectively, and the initial pH values of the soil mixtures were within this range. There was some evidence of a decrease in pH with time of incubation in the control soils, particularly in the mixtures with a high proportion of the CS soil but none of the differences in pH was statistically significant (Figure 6.2 (a)). The addition of urea increased the soil pH in all the soil mixtures with a maximum pH of 6.0 being obtained in the 100% SS soil at day 0 (Figure 6.2 (b)). In the soil mixtures with higher proportions of CS soil (CS:SS; 100:0; 80:20; 60:40; 50:50) the pH decreased rapidly in the first 5 days and then declined more slowly to finish below 5.0 after 15 days. In contrast, in the 100% SS soil the pH decreased only slowly from day 0 and was still at 5.8 at the end of the incubation time.

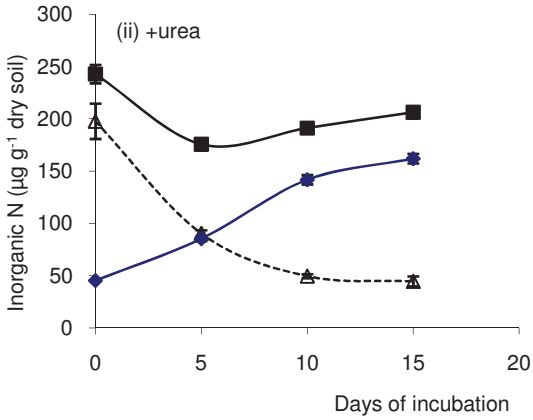
a) 100:0



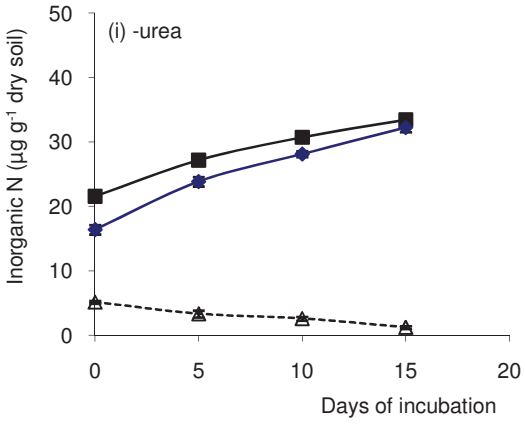


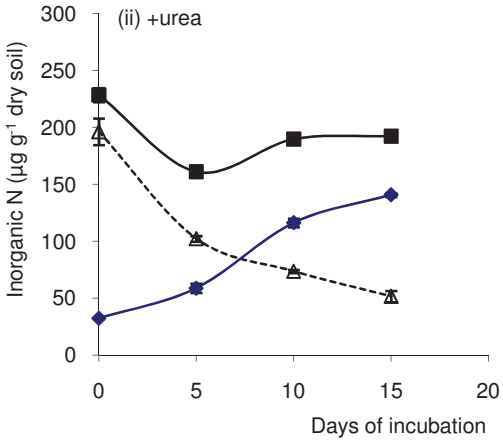
b) 80:20



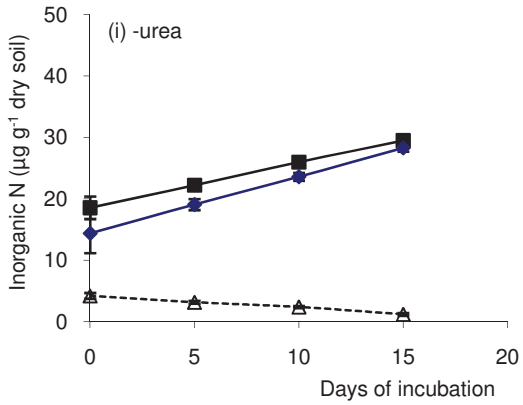


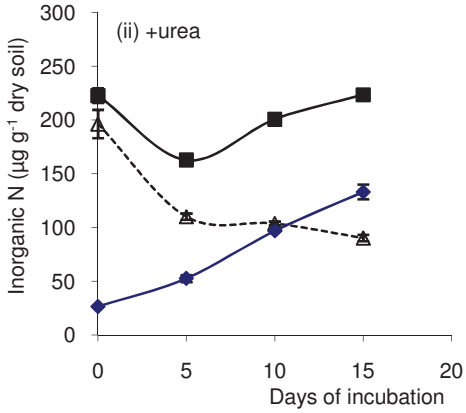
c) 60:40



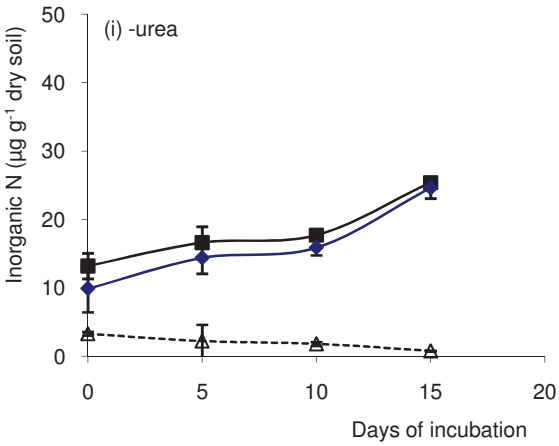


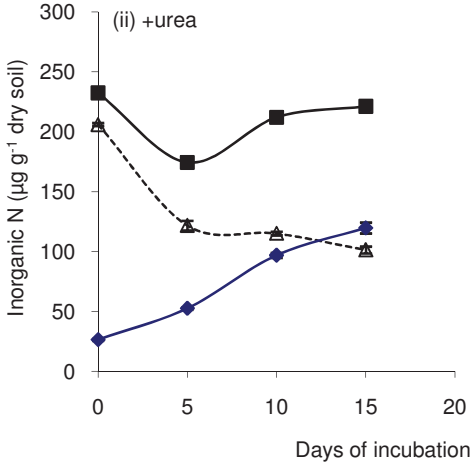
d) 50:50



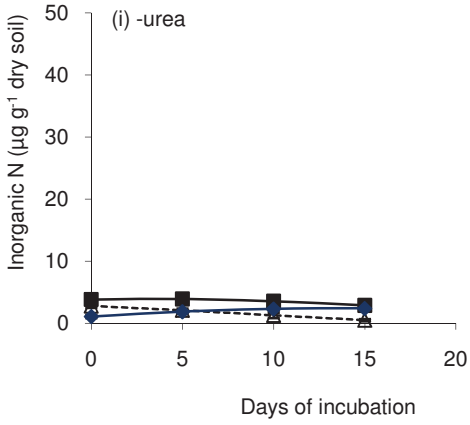


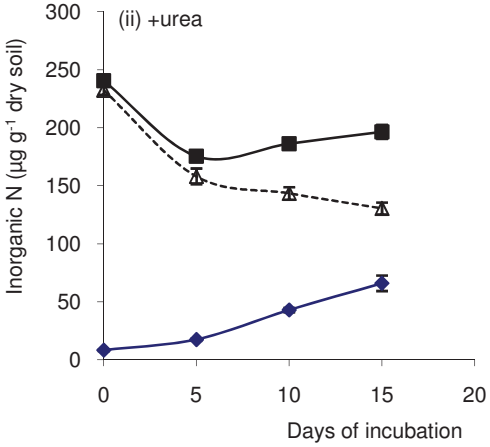
e) 40:60





f) 20:80





g) 0:100

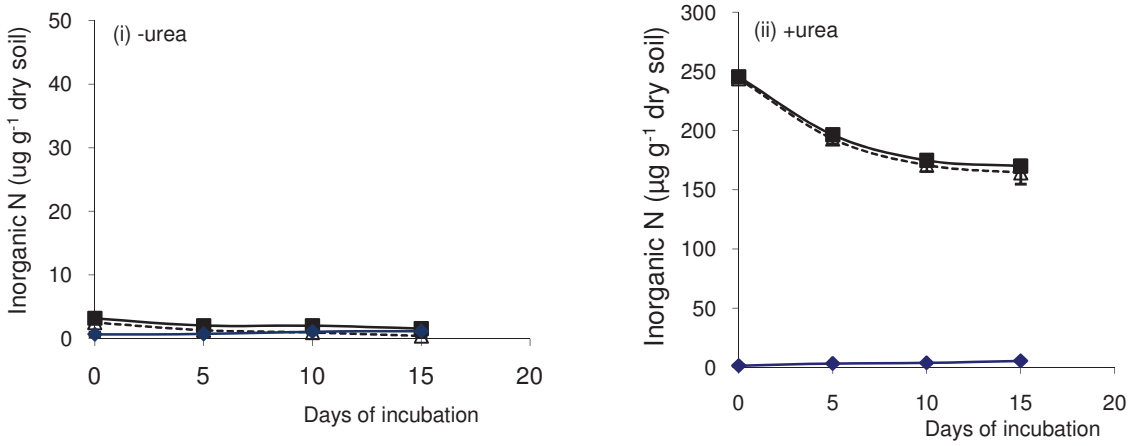
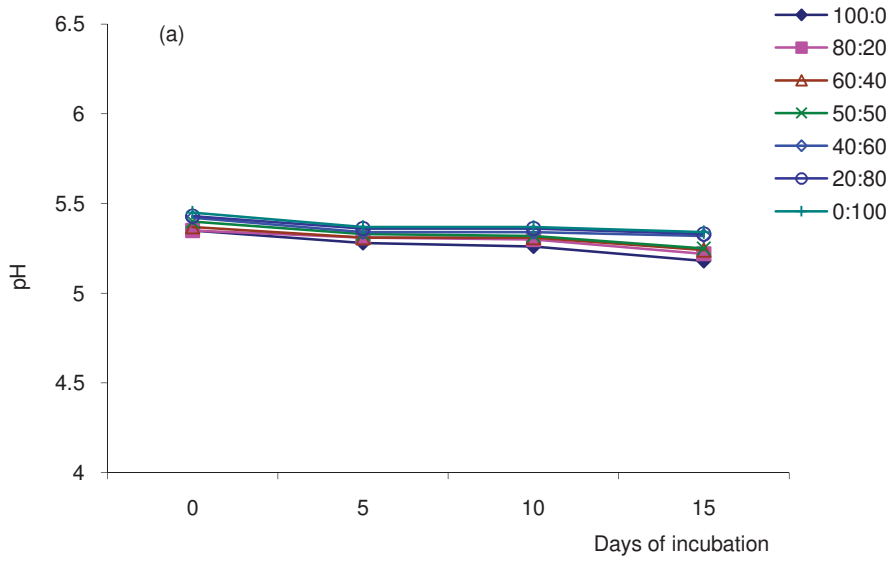


Figure 6.1 Concentrations of mineral N fractions in mixtures of camp and steep soils (ratio indicated represents proportions of CS:SS) following the addition of urea ((ii)+ urea) and without urea ((i)-urea; control) and subsequent incubation. Error bars represent the standard deviation of the four replicates. Note that the concentration scale in the control (- urea) graphs differs from that in the + urea treatment. Symbols in both (i) and (ii) represent total mineral N (-■-), NH_4^+-N (-△-) and NO_3^--N (-◆-) concentrations.



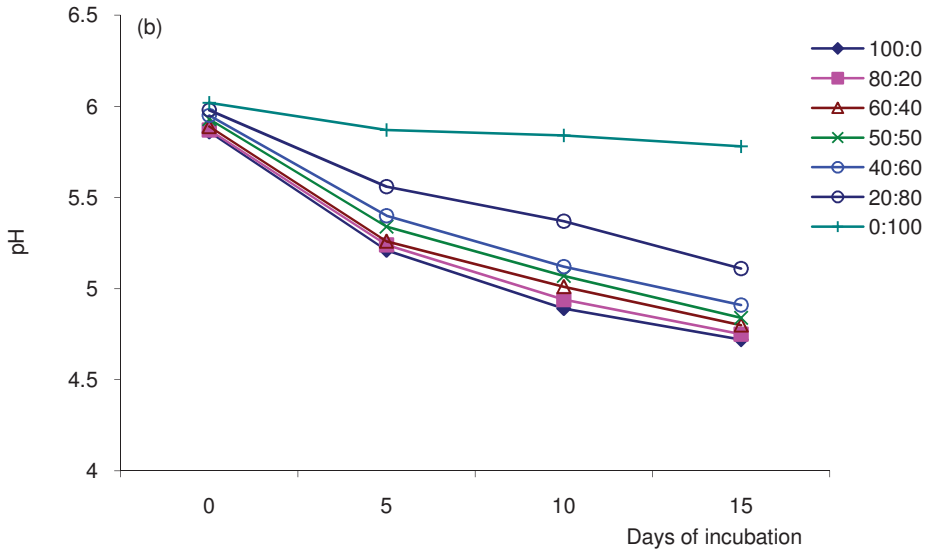


Figure 6.2 Changes in pH with time of incubation in (a) control soils and (b) soils with urea applications (ratio indicated represent proportions of CS:SS). Vertical bars denote the standard deviation of four replicates.

6.3.3 Nitrification rates in the control soil and soil with added urea

The nitrification rates in soils with added urea (Figure 6.3 (b)) were substantially greater than in the control soils (Figure 6.3 (a)). In the control soils, the nitrification rates decreased over time in the soil mixtures with a high proportion of CS soil. However, as the proportion of CS in the soil mixtures decreased below 50%, the changes in nitrification rate became more variable. The nitrification rates in the mixtures containing 80% and 100% of the SS soil were almost negligible.

When urea was added to the soil mixtures, three patterns of nitrification were observed. In the high nitrifying soil (100% CS) the nitrification rate declined over time, from 0 to 15 days of the experiment. However, in most of the other soil mixtures, the soil nitrification rates were lower in the first 5 days and then increased from day 5 to day 10, before declining again until the end of experiment. In the low nitrifying soil (100% SS)

there was little change in the soil nitrification rate, which remained very low throughout the experiment.

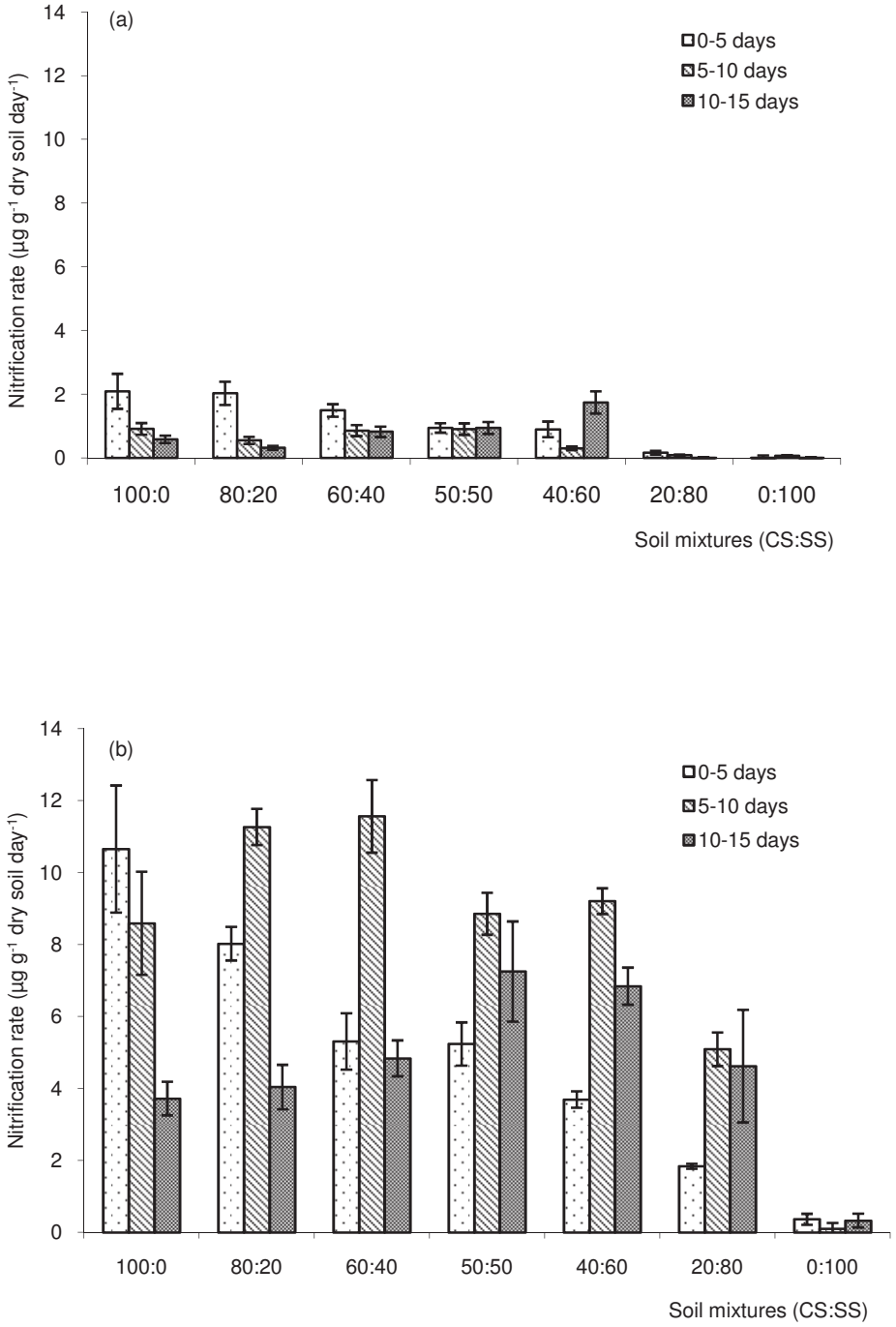


Figure 6.3 Nitrification rates in the mixtures of camp and steep sites soils (CS:SS) in (a) the control treatment and (b) the treatment with added urea, during the 15-day incubation. Vertical bars denote the standard deviation of four replicates.

6.4 General Discussion

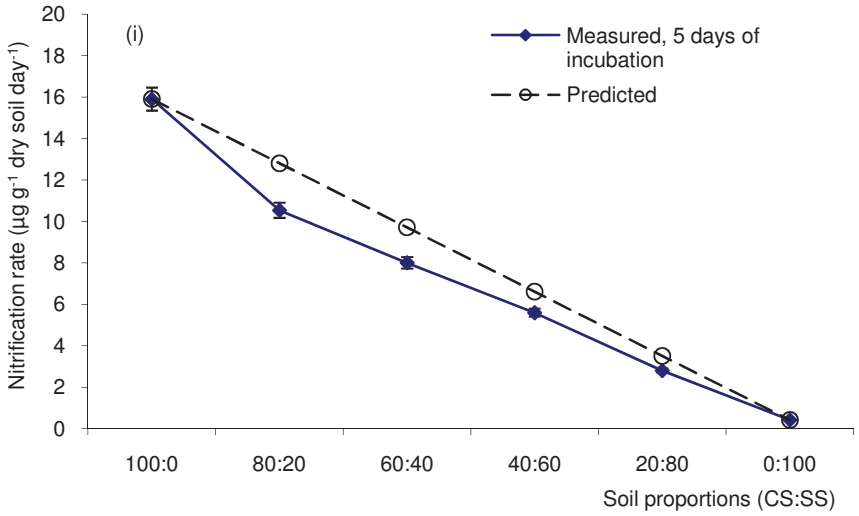
To investigate whether mixing the SS soil with the CS soil was having an inhibitory effect on the nitrification activity, the measured nitrification rate in each of the soil mixtures containing the CS soil was compared with the "predicted" nitrification rate, based on the proportion of the CS soil in the mixture (Figure 6.4).

Two phases were apparent. At day 5 the amounts of NO_3^--N produced in each of the mixtures that contained both soils were lower than those predicted, based on the proportions of the CS soil in the mixture (Figure 6.4 (i)). This suggests that the addition of the SS soil may be having a slight inhibitory effect on the nitrification activity in the CS soil. By days 10 and 15 (Figure 6.4 (ii) and (iii)) however, the amounts of NO_3^--N being produced in all of the soil mixtures containing both CS and SS soil were higher than those predicted based on the proportion of CS soil in the mixture.

These observations could be explained as follows. In the soil mixtures containing the CS soil there were two opposing processes operating. The first was a probable growth in the population of nitrifiers following the addition of urea. This suggestion is supported by the data presented in Figure 6.3 (b) whereby the nitrification rates over days 5-10 were greater than over days 0-5 in all the soil mixtures containing both CS and SS soils. However, as the population of nitrifiers increased and nitrification proceeded, the supply of available NH_4^+-N decreased (Figure 6.1) and the pH decreased (Figure 6.2 (b)). Both of these processes served to slow down the rate of nitrification. As the proportion of CS soil in the mixtures increased, this sequence of events became more advanced. Thus by day 5 in the 100% CS soil much of the NH_4^+-N had already been consumed and the pH had dropped. This caused reduced nitrification rates in both

days 5-10 and 10-15. In the soil mixtures containing between 40 and 80 % of the campsite soil the nitrification rate increased until day 10 and then started to drop away. In the soil mixture containing 20% CS soil there was little evidence of a decrease in the rate of nitrification by day 15 because this soil still had some available NH_4^+-N present and the pH had not dropped greatly.

Because nitrification in the 100% CS soil was inhibited from day 5 to a greater extent than in the other soil mixtures (Figure 6.3 (b)), predicting the nitrification rate in the mixtures on the basis of their relative proportions of the two soils resulted in an under-prediction of the measured nitrification rate (Figure 6.4 (b) and (c)). Interestingly, even with the supply of plentiful NH_4^+-N substrate there was no evidence of an increase in nitrification activity over time in the SS soil (Figure 6.3 (b)).



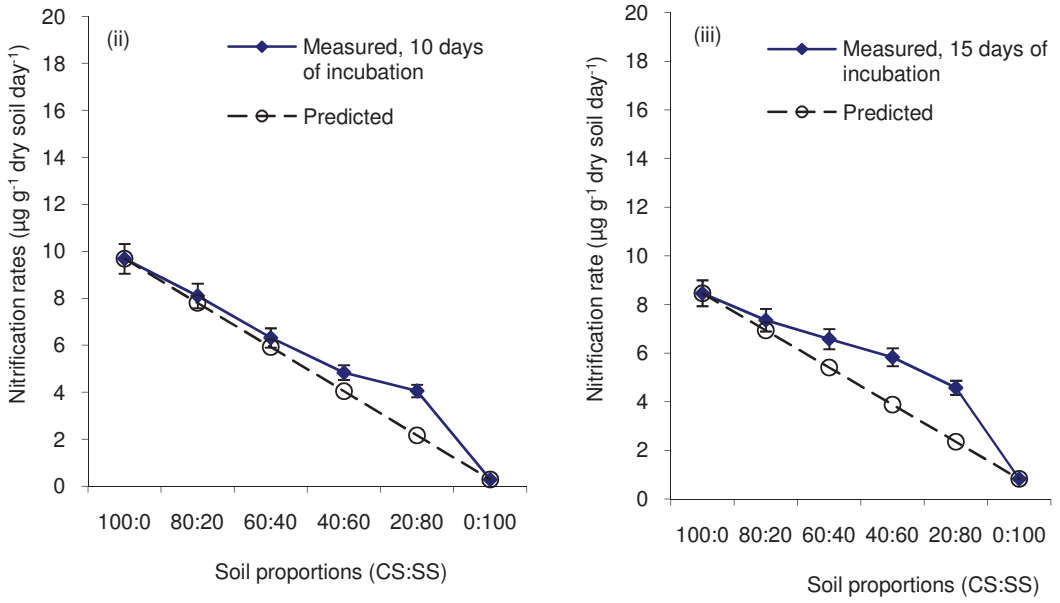


Figure 6.4 Measured and predicted nitrification rates in soil mixtures after the addition of urea and incubation for (i) 5 days, (ii) 10 days and (iii) 15 days. Vertical bars represent the standard deviation of four replicates.

6.5 Conclusions

The main conclusions that can be drawn from this study are as follows:

1. There was perhaps some evidence of an inhibition effect of the low nitrifying (SS) soil on the nitrification activity in the mixed soils, but any toxic effect was not sufficient to totally stop nitrification activity in the soil mixtures.

2. That the addition of urea in the 100% SS soil didn't greatly increase the nitrification rates could possibly be attributed to the presence of more heterotrophic nitrifiers than AOB, as discussed in Section 5.4.

3. Although the experimental design would have detected a major inhibition of nitrification activity, it turned out to be less well suited for detecting partial inhibition. This was because the nitrification capacity of the CS soil was so high that nitrification in that soil was very quickly inhibited by a lack of substrate and a low pH. It therefore

did not provide a stable “control rate” of nitrification against which the nitrification in the soil mixtures could be confirmed.

Chapter 7

Overall summary and future work

This chapter summarises the major research findings reported in this thesis related to nitrification in soils and in particular, the reasons for the great variation in the nitrification activity between pastoral soils and also the variation in the effectiveness of artificial nitrification inhibitors, such as DCD. It concludes with some recommendations for future research.

7.1 Overall summary

In many countries around the globe, including New Zealand, increasing inputs of N fertiliser, increasing application of waste effluents and continued return of animal urine and dung to agricultural land has increased the interest in nitrification. Nitrification is the microbial oxidation of NH_4^+ to NO_3^- via NO_2^- and a vital soil N transformation that can lead to significant N losses from ecosystems through leaching and denitrification of potentially mobile NO_3^-.

The Literature Review identified that nitrification occurred in wide range of environments. These include agricultural soils, grasslands, forest soils, treated sewage, waste-water, freshwater lakes and sea-water. In most systems, autotrophic AOB contribute more to nitrification than do heterotrophic microorganisms. Autotrophic AOB belong to a monophyletic group within the β-subclass *Proteobacteria* and are comprised of two genera, *Nitrosomonas* and *Nitrosospira*. Conventional and molecular

analyses have indicated a dominance of *Nitrosospira* among the AOB inhabiting most terrestrial environments, and a grouping of *Nitrosospira* clusters 1, 2 and 4 are more prevalent in soils that are either acidic, retired from agricultural use or that have never been cultivated and/or received applications of N fertiliser. *Nitrosospira* cluster 3 appears to be widely distributed in agricultural soils with high N availability. In addition, *Nitrosomonas* species have been detected in swine-manure fertilised plots, enrichment cultures and wastewater treatment systems, supporting the view of their preference for high ammonia and high pH environments.

The Literature Review showed that nitrification rates vary widely in soils and are thought to be controlled principally by NH_4^+ availability, temperature, moisture and oxygen. In New Zealand soils, a wide variation in nitrification rates within landscapes has been observed and the reasons for this variation are not completely understood. Considerable progress has been made by other researchers, either in New Zealand or elsewhere on mitigation strategies to minimise the losses of N via NO_3^- via leaching or N_2O emissions. These have included adding nitrification inhibitors (NIs), for example DCD, to the soil. DCD slows down the rates of nitrification by inhibiting or temporary suppressing the activity and population of autotrophic AOB, thereby reducing the fraction of NH_4^+ converted to NO_3^- and decreasing N losses via NO_3^- leaching or denitrification. However, over time DCD is broken down by soil microorganisms and the nitrification rate increases again. A further addition of DCD will again slow down the rates of nitrification. In New Zealand most researchers have concentrated their studies on quantifying the effects of DCD on N losses from urea fertilisers and urine deposition in legume based-pasture. The majority of the research has indicated that DCD offers potential benefits in reducing NO_3^- leaching and losses of N_2O to the

atmosphere, as well as enabling greater NUE, which will result in increased pasture yield. Similar positive results, relating to the usage of DCD and other NIs have also been reported in other countries. In contrast however, a few other researchers have found little or no benefit from the use of NIs, in terms of decreasing NO_3^- leaching, reducing the losses of N_2O and increasing plant yield.

According to published literature, the effectiveness of NIs varies between soils and this depends in part on its half-life in soils, which is controlled by its rate of degradation by soil microorganism. Soil factors that have been found to be important in affecting DCD performance in soils include the soil type, temperature, pH and soil moisture. Therefore, the current study reported in this thesis explores the reasons for both the natural variation in nitrification rates within the landscape and also the variable effectiveness of DCD.

The first objective (Chapter 3) of this thesis was to develop a perfusion system that could be used to monitor the nitrification rates of New Zealand pasture soils over time and to further use this technique to quantify the immediate and residual effects of one or more additions of DCD on the soil nitrification rates and the population of autotrophic AOB. The second objective (Chapter 4) was to determine whether the variable effectiveness of DCD between soils and over time was due to the disappearance of DCD from the soil through degradation by soil microorganisms or whether in some way DCD is less effective in some soils than others. This work was carried out to follow-up the findings obtained from Chapter 3.

The Literature Review showed that N transformations in hill country soils are highly variable across different landscapes. It was concluded that the history of N application, the C/N ratio of the soil and the SOM quality are the factors that are likely to contribute to this variation. However, it is not known whether the populations of autotrophic AOB may contribute to this variability or whether these soils contain some naturally-occurring inhibiting factor that slows down the nitrification rate in some way. A small study was therefore undertaken to investigate this issue a little further (Chapter 5). This incubation study investigated the nitrification activity in two soils collected from the same hill country paddock. One sample was collected from relatively flat camp site areas in the paddock and the second sample was collected from adjacent steeply sloping areas. The final chapter (Chapter 6) followed up the findings in Chapter 5 and used a series of laboratory incubations to investigate whether the low-nitrifying steep slope soil contained some inhibiting agent that could also inhibit nitrification when the soil was mixed with the high-nitrifying camp site soil.

The first objective of developing the perfusion technique to monitor the changes over time of nitrification rates in three types of New Zealand pasture soil was achieved. This study demonstrated that a simplified soil perfusion apparatus was useful for monitoring the soil nitrification activity and enabling measurement of the nitrifier populations in the pasture soils. In this study, although only a small range of soil types was used the rankings of the nitrification rates and AOB numbers were similar in the three original pasture soils. Despite the inherent problems with MPN variability and sensitivity as noted earlier in the literature review, the MPN counts obtained in all the three soils used in this study suggested that they contained significant numbers of AOB and the

population sizes were consistent with other counts of nitrifying populations, (estimated using the MPN technique), in agricultural soils.

The perfusion apparatus was then used to study the mode of action of DCD in two contrasting soils in a long term experiment that involved 4 successive cycles of nitrification. In both soils, the addition of DCD in cycles 1 and 3 inhibited the nitrification activity. When the DCD was removed from the perfusion system and with the introduction of a new source of N in cycles 2 and 4, the rates of nitrification then increased to some extent, but did not return to the initial levels in either soil. The effectiveness of DCD varied between the two soils, but in neither soil did DCD completely halt nitrification. In terms of percentage reduction in nitrification rates, DCD was more effective in suppressing nitrification relative to the control treatment in the MFSL than in the MSL. In cycles 1 and 3, when the DCD was added at the same rate (20 kg DCD ha^{-1}) to both soils, the nitrification rates in the MFSL were reduced to 25 and 41% of the control respectively while in the MSL, DCD reduced the nitrification rates to 52 and 65% of the control respectively. However, in absolute terms, the DCD was slightly more effective in the MSL than in the MFSL. In the MSL the reductions in nitrification rate from addition of DCD in cycles 1 and 3 were 14.6 and 9.8 µg NO_3^--N g^{-1} soil day^{-1} respectively while in the MFSL the corresponding reductions in nitrification rate were only 9.8 and 8.8 µg NO_3^--N g^{-1} soil day^{-1} respectively.

The populations of AOB followed a similar pattern to the nitrification activities with an inhibition of nitrifer populations in the presence of DCD in Cycles 1 and 3, and the partial recovery of the temporarily suppressed nitrifier populations in Cycles 2 and 4. The extent of this recovery of AOB populations when DCD was removed differed

between the two soils and raised the possibility of contrasting effects of DCD on the nitrifiers present in the two soils. Firstly, there was a possibility that DCD had a primarily bacteriostatic effect on the nitrifiers in the MSL. When a fresh source of N was percolated through the soil column after the addition of DCD, the rate of nitrification and the population of AOB increased almost to the control level. In contrast, the DCD appeared to have more of a bacteriocidal effect in the MFSL with the nitrification rates and numbers of AOB not recovering to anywhere near the levels in the control.

The experiment reported in Chapter 4 demonstrated that the degradation of DCD in the MFSL soil was much slower than in the MSL, with the half life being almost twice as long as in the MSL. The additions of DCD reduced the nitrification rates in both soils, but a greater effect in terms of percentage reduction was obtained in the MFSL than in the MSL. In the MFSL soil, the addition of DCD at two rates (20 and 40 kg ha^{-1}) reduced the nitrification rates to 6 and 4% of those in the control soil respectively while in the MSL the nitrification rates were reduced to 36 and 28% of the levels in the control. However, when the reduction in nitrification rate was expressed in absolute terms the reductions in nitrification rate in the MSL were 9.4 and 10.6 µg NO_3^--N g^{-1} soil day^{-1} for the two rates of DCD addition, which were slightly greater than the corresponding reductions in the nitrification rates in the MFSL were 8.0 and 8.2 µg NO_3^--N g^{-1} soil day^{-1}. de Klein *et al*. (2011) suggested that absolute rather than relative reduction in N_2O emissions due to the addition of DCD is important when considering incentives to farmers for adopting DCD to reduce total on farm emissions.

For each soil there was a close relationship between the concentration of DCD remaining in the soil and the nitrification rate, but this relationship was different between soils and was not affected by the rate of DCD addition. Thus, as the DCD remaining in the soil gradually decreased there was a corresponding increase in the nitrification rate However, although all the DCD eventually disappeared from both soils, the nitrification rate did not fully recover to the level of the controls within the time scale of the experiment, and this effect was more obvious in the MFSL. As already discussed earlier, the added DCD might have a predominantly bacteriostatic effect in the MSL, and more of a bacteriocidal effect in the MFSL.

The nitrification rates obtained in this present study (Chapter 5) confirmed the earlier published work by Bowatte (2003) in which he found that within the hill country landscape, soils collected from a steep slope (SS) had a considerably lower rate of nitrification than the soil collected from an adjacent flat camp site (CS). Bowatte (2003) concluded that the history of N application, the C/N ratio of the soil and the quality of soil organic matter (SOM) were likely factors to have influenced the nitrification activities in the soils tested. In this present study, another factor that is likely to have contributed to the low nitrification rates in the SS soil was the lower numbers of nitrifying AOB present in this soil than in the CS soil. In contrast with the CS soil and a soil from a flat-land dairy farm (MSL), the addition of urea in the SS soil did not greatly increase the nitrification rate and the AOB numbers in absolute terms over time, even with a plentiful supply of NH_4^+ substrate from the urea and the associated higher pH. However, the AOB that were present in the SS soil appeared individually to reproduce and nitrify at similar rates to the AOB on the other two soils.

The possibility of there being some type of inhibitory agent in the SS soils was explored further in Chapter 6. The results suggested that the SS soil may have had a small effect on the nitrification activity of the high-nitrifying campsite soil when the two soils were mixed together. However, the initial effect was small and unforeseen problems with the experimental design prevented a longer term assessment of this effect.

7.2 Future directions

To bridge the important gaps that arose during this PhD research, future investigations have been recommended as follows:

- Although the MPN technique gave estimates of AOB populations that correlated well with nitrification rates in the soils used in this study, a larger number of soil samples needs to be studied to provide a better understanding of the relationships between the numbers of AOB and the nitrification rates.

- More work, probably using advanced molecular techniques, is required to investigate possible shifts in the community structure of AOB following the additions of DCD. Results of this study suggest that although nitrification rates gradually recover following the addition of DCD they do not reach the levels observed in the control soils to which no DCD had been added. If DCD has selectively removed the most active AOB then this may have important practical implications.

- All the four chapters described in this thesis were based on controlled laboratory experiments. More long term field studies are needed to examine the effects of one or more additions of DCD on the nitrification rates and activity of the nitrifying soils. In Chapter 3 there was some indication of reducing effectiveness of DCD when it was reapplied in the soils.

- In Chapter 5 low populations of AOB were obtained in the SS soil compared to the CS soil and in Chapter 6, even when there was ample NH_3 substrate added to the SS, there was no marked increase the nitrification rates and the population of AOB. Further investigation is required into why nitrification rates in these steep slope soils remain low, even when favourable conditions for nitrification are established. In particular, it would be useful to conduct a glasshouse trial to investigate whether the pasture species commonly found on SS soils have an inhibitory effect on nitrification. Several overseas studies have identified some plant species that have an inhibitory effect. In addition it may be useful to quantify the heterotrophic nitrifiers in this soil to evaluate the possible contribution of these nitrifiers to the nitrification activity.

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