

School of Biosciences

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MANAGING THE IMPACT OF GRASSLAND MANAGEMENT ON NITROUS OXIDE EMISSIONS THROUGH AN IMPROVED UNDERSTANDING OF SOIL MICROBIAL COMMUNITIES

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ABSTRACT

Nitrous oxide (N_2O) plays an important role in the atmospheric radiative balance and in stratospheric ozone chemistry and has a global warming potential 298 times higher than CO_2. Grasslands occupy two thirds of the agricultural land in the UK. have been estimated to emit up to 0.54 – 0.58 Gg of N_2O per year. N_2O is produced as a result of nitrifying and denitrifying soil microbial communties. Both microbial groups are strongly influenced by soil physical factors such as oxygen availability, with decreases in soil oxygen increasing N_2O production from the soil. Grasslands are subject to a range of managment treatments that enhance the production of N_2O, such as cattle traffic and the movement of heavy agricultural machinery, which increases soil bulk density, resulting in a reduction in available air-filled pore space. This in combination with the use of fertilisers, such as urea and cattle slurry and also the deposition of urine, lead to the ideal conditions for N_2O production to proceed. Mitigation techniques, such as the use of nitrification inhibitors, such as Dicyandiamide (DCD) are used to reduce these emissions, however, even with the use of these nitrification inhibitors N_2O emissions still proceed. An improved understanding of the effect of bulk density and soil water-filled pore space (WFPS), as well as the movement of gas through compacted soils is elucidated. The importance of soil heterotrophic nitrification was observed in compacted grassland soils and a crucial pathway of the effect of a commonly used eukaryotic protein synthesis inhibitor (cylcoheximide) used to distinguish between soil bacterial and fungal activity was highlighted and the implication for its use in soil nitrogen cycling studies is discussed. Limitations of DCD were also highlighted, with indications of alternative pathways of microbial N_2O production in grassland soils treated with this nitrification inhibitor.

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LIST OF ABBREVIATIONS

DCD - Dicyandiamide

NH_4^+ - Ammonium

NO_3^- - Nitrate

DOC – Dissolved organic carbon

IC – Inorganic carbon

WFPS – Water-filled pore space

NA – Nitrification activity

NR – Nitrate reductase

C_2H_2 - Acetylene

CHX - Cycloheximide

STM – Streptomycin sulphate

AOB – Ammonia oxidzing bacteria

AOA – Ammonia oxidizing archaea

AMO – Ammonia monooxygenase

N_2OR – Nitrous oxide reductase

N_2O – Nitrous oxide

CO_2 – Carbon dioxide

CHAPTER 1. INTRODUCTION

1.1 Agricultural nitrous oxide (N_2O) emissions

Nitrous oxide (N_2O) plays an important role in the atmospheric radiative balance and in stratospheric ozone chemistry and is currently believed to contribute to a radiative forcing of 0.15 W m^{-2} (Flechard et al. 2007); a global warming potential 298 times higher than CO_2, with a lifetime of approximately 114 years (IPCC. 2007). Production and emission of N_2O in the environment is of major importance for global warming (Dickinson and Cicerone. 1986), as well as for the destruction of the stratospheric ozone layer (Crutzen, 1970).

Two thirds of N_2O emissions come as a result of emissions of natural (unmanaged) soils and oceans (Khalil et al. 2002; Denman et al. 2007). A study by Khalil et al. (2002), based on global measurements over the last several thousand years from polar ice cores, showed that N_2O emissions had risen by 9.4% (27 ppbv) since pre-industrial times and that these emissions had significantly increased between 1900 and 2000. In the 1990's global anthropogenic N_2O emissions were approximated to be in the range of 7 ± 1 Tg N_2O-N yr^{-1} (Kahlil and Rasmussen. 1992). More recent estimates of N_2O fluxes put net anthropogenic N_2O fluxes at around 5.3 Tg N_2O-N yr^{-1} (Davidson and Kanter. 2014). The 2007 report by the IPCC determined that much of the increase in anthropogenic N_2O emissions were attributed to the expansion of agricultural land area and increased subsequent fertiliser use (IPCC. 2007). Earlier estimates of agricultural N_2O emissions put net agricultural fluxes at 6.8 Tg N_2O-N y^{-1} (Moiser et al. 1998), however, these estimates declined in the following decade and were within the range of 4.3 – 5.8 Tg N_2O-N y^{-1}(Denman et al. 2007; Crutzen et al. 2008; Davidson. 2009). Although a more current report by Davidson and Kanter, (2014) estimated that agriculture emits 4.1 Tg N_2O-N y^{-1}).

1.2 *Grassland nitrous oxide emissions*

In 2009 Saggar et al. (2009) stated that managed grasslands, which can be defined as the manipulation of natural vegetative grasslands for a desired production goal, were estimated to account for 25% of global agricultural lands, covering over 3 billion hectares of the 14.9 billion hectares of the worlds land surface (Saggar et al. 2007), with a significant proportion of world milk (27%) and beef (23%) production derived from these grasslands (Sere et al. 1995). Their continued existence and productivity is dependent upon management practices, such as grazing intensity and frequency, cultivation, re-sowing and renovation, recycling of animal wastes and fertiliser application (Saggar et al. 2007). Grasslands cover 4.4 million km^2 (440 million hectares), 20.7% of the land area in the EU-28 (Eurostat. 2018). A study by Flechard et al. (2007), examined annual N_2O emissions from grazed and non-grazed grasslands across 10 European managed grasslands. They compared both fertilised and non-fertilised management practices and identified that fertilised grazed grasslands increased annual emissions by a factor of 4 when compared to non-fertilised grazed grassland (1.77 vs 0.48 kg N_2O-N ha^{-1} y^{-1}) and by a factor of 3 when fertilised un-grazed grasslands were compared to non-fertilised un-grazed grasslands (0.95 vs 0.32 kg N_2O-N ha^{-1} y^{-1}), in addition, grazing stimulated annual N_2O emissions (0.48 kg N_2O-N ha^{-1} y^{-1} vs 0.32 kg N_2O-N ha^{-1} y^{-1}). Rees et al. (2013) later demonstrated that across a range of European agricultural sites total nitrogen addition was the strongest determining factor in relation to prediction of N_2O emissions, accounting for 77% of the variance in managed grassland systems.

In the UK, grasslands occupy about two thirds of the agricultural land area (DEFRA, 2016), with N_2O emissions estimated to emit up to 0.54 and 0.58 Gg (Gigagrams) per year from UK improved grasslands (1990 – 2009, respectively)

(Skiba et al. 2012). Recently UK grasslands have been simulated to emit between 0.5 and 7 kg N_2O – N ha^{-1} y^{-1} (Fitton et al. 2017), with fertiliser input contributing most to the range of N_2O emissions after the use of Monte Carlo simulations based on 10 years of data collection (2001 – 2010). Therefore, N_2O emissions from grasslands remains a substantial source of N_2O from both European and UK agriculture. As plant productivity in many grasslands is N limited (LeBauer & Treseder. 2008; Lee et al. 2010), the use of both inorganic and organic fertiliser is common practice in order to obtain optimum grass yields for livestock. Other studies have demonstrated that the application of urea and slurry increased N_2O emissions from managed grasslands (Clayton et al. 1997; Smith et al. 1997; Luo et al 2007; Burchill et al. 2014), however, it has been noted that the largest sources of N_2O found in grazed pastures derives from animal excreta, which can account for up to 80% of N_2O emissions from these systems (Luo et al. 2010). Therefore, although fertiliser use has been demonstrated to increase grassland soil N_2O emissions, the impact of animal grazing has shown a further strong influence on the variability of N_2O fluxes.

1.3 Sources of grassland nitrous oxide emissions

1.3.1 Nitrification

Although N_2O emissions have been suggested to originate from chemical alterations of nitrogen compounds such as NO_2^-, known as chemodenitrification (Chalk and Smith. 1983), research on the sources of N_2O from soils has indicated that most, if not all, of the N_2O evolved was produced by biological processes, and that little, if any, was produced by chemodenitrification chemical processes (Bremner. 1997). Soil microbial N_2O production occurs via nitrification (ammonia oxidation) and nitrate dissimilation (denitrification and nitrate ammonification); pathways

described in Figure 1.1. The first, the nitrification pathway has two stages; oxidation in the first stage (1) of ammonia/ammonium to hydroxylamine and then to nitrite, and then a second stage (2) where NO_2^- is rapidly oxidized to nitrate ($NH_4^+/NH_3 \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$).

Chemolithotrophs, a name derived from 'lithos' a Greek word meaning stone and indicating the inorganic nature of the nutrients of chemoautotrophs (Oren. 2009). Autotrophic ammonia oxidizers are characterized by their ability to oxidize ammonia under aerobic conditions (Kowalchuk and Stephen. 2001). As chemolithotrophs they use ammonia as the sole source of energy and reducing power to obtain the carbon (C) needed for cellular growth by fixation of inorganic carbon sources such as carbon dioxide (CO_2) (Shaw et al. 2006). It has even been demonstrated that ammonia oxidizing bacteria (AOB) and archaea (AOA) in forest soils only fix 1 µg of carbon to biomass after oxidizing 59.8 µg and 58.2 µg of ammonia-N respectively (Norman et al. 2015). Ammonia oxidation in soil is thought to be mainly due to two types of micro-organisms; AOB and AOA. The process catalysed by both AOB and AOA uses two enzymatic steps in the reduction of ammonia to nitrite. The first mediates the reduction of ammonia to hydroxylamine (NH_2OH) and is found in the cytoplasmic membrane, using ammonia monooxygenase (AMO) enzyme. This step reduces ammonia to hydroxylamine through the following process;

$$NH_3 + 2H^+ + O_2 + 2e^- \rightarrow NH_2OH + H_2O$$

The second step is the oxidation of NH_2OH by the hydroxylamine oxidoreductase (HAO) enzyme to NO_2^- as seen below;

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4\ e^-$$

Although hydroxylamine is produced by AOA after reaction with AMO, however, the gene responsible has to date not yet be characterised. Although, a study by Vajrala et al. (2013) demonstrated that *Nitosopumilus maritimus* can oxidize NH_2OH to NO_2^- and that the formation of NO_2^- was as result of NH_3 oxidation proceeding via NH_2OH formation. This was achieved through the use of $^{15}NH_4Cl$ tracer studies, therefore indicating that a similar process is taking place in AOA when compared to AOB (Vajrala et al. 2013).

The next second stage of the nitrification process, nitrite oxidation is carried out by nitrite oxidizing bacteria. The key enzyme for NO_2^- oxidation by NOB is nitrite oxidoreductase (NXR), which shuttles two electrons per oxidized NO_2^- into the electron transport chain, as facultative chemolithoautotrophs (Nitrobacter spp.) oxidize NO_2^- to NO_3^- and fix carbon dioxide (CO_2) via the Calvin-Benson-Basham pathway (Starkenburgh et al. 2008).

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2\ e$$

1.3.2 Heterotrophic nitrification

Heterotrophic nitrification can take place via pathways related to the inorganic transformation of nitrogen as described, or through organic nitrogen pathways through the oxidation of ammonium by heterotrophic bacteria or fungi (Ward. 2013). The process of heterotrophic nitrification is brought about through the presence of organic or inorganic compounds (Obaton et al. 1968). Recent studies demonstrate that heterotrophic nitrification by denitrifying bacteria such as *Psuedomonas stutzeri*

oxidize ammonium and produce N_2 gas in waste water treatment plants (Zhang et al. 2011). Fungi have been known to have a capacity to oxidize ammonium to nitrate (Hora and Iyengar. 1960). Studies have demonstrated, with the use of an antibiotic inhibition method using cycloheximide (Eukarotic inhibitor) and streptomycin sulphate (prokaryotic inhibitor) that fungi are able to produce both nitrate in both forest and grassland soils (Stroo et al. 1989; Laughlin et al. 2008; Castaldi and Smith. 1998) demonstrated that cycloheximide inhibited NO_3^- production in a forest soil. Laughlin et al. (2008) clearly demonstrated that the oxidation of NH_4^+ and the production of NO_3^- were completely inhibited by cycloheximide in a ^{15}N tracing study. Therefore, the importance of heterotrophic nitrification should not be overlooked.

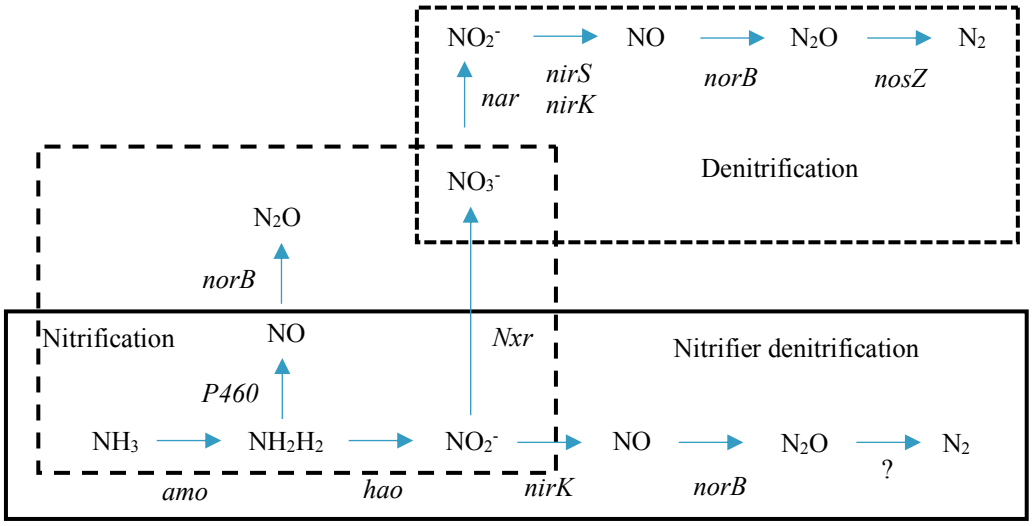


Figure 1.1. Sources of N_2O. Pathways of NH_4^+, NO_2^- , NO_3^-, NO, N_2O and N_2 with their repective gene regulators. Adapted from Wrage et al . 2001.

1.3.3 Denitrification

The subsequent dissimilation of NO_3^- to N_2O and finally to N_2 ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) is known as nitrification coupled denitrification (NCD) and conforms to the more classic view of N_2O production in environmental systems (Davidson and Seitzinger. 2006). Organisms capable of denitrification are facultative-anaerobes that utilise NO_3^- when O_2 becomes limiting in the environment. Denitrifiers utilise a number of enzymes (Fig. 1.1). Denitrification is primarily a bacterial respiratory process and is mediated by four enzymatic reduction steps; nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS) (Heylen et al. 2006) (Fig. 1.1). NO_3^- is first reduced to NO_2^- by the nitrate reductase gene (*narG*) and is considered the first step in the pathway of N_2O production (Averill and Tiedje. 1982). Nitrate reductase contains a respiratory membrane-bound nitrate reductase and a secondary periplasmic nitrate reductase (*napA*) that is responsible for anaerobic and aerobic NO_3^- reduction (Potter et al. 2001). The second step in the pathway is the reduction of NO_2^- to NO (nitric oxide), catalysed by the copper coded *nirK* gene. NO is then converted to N_2O by the *norB* gene and then finally to N_2 by the *nosZ* gene.

Denitrifiers utilise N oxides as the terminal electron acceptor in the generation of ATP and are phylogenetically diverse, spanning three kingdoms: bacteria, archaea, and eukarya. While ubiquitous in soil (Firestone et al. 1980; Tiedje. 1988), Most prokaryotic and all eukaryotic denitrifiers are heterotrophs, however, chemolithotrophic denitrifying bacteria and archaea, which couple the reduction of N-oxides (NO and N_2O) to the oxidation of inorganic compounds, have also been identified (Zumft. 1997; Fernandez et al. 2008). All these microorganisms can use NO_2^- instead of NO_3^- for denitrification. In soil, complete denitrification of NO_3^- to N_2

predominantly occurs through the synergistic activities of microbial consortia (Zumft. 1997; Wallenstein et al. 2006).

Approximately one third of sequenced bacterial denitrifiers lack N_2O reductase (Philippot et al. 2010) and therefore evolve N_2O as the final denitrification product. The denitrification systems of most fungi also lack N_2O reductase (Kobayashi et al. 1996). An increasing number of publications have identified the importance of fungal nitrous oxide emissions (Laughlin et al. 2009; Pendergast-Miller et al. 2010; Herold et al. 2012; Jirout et al. 2013; Chen et al. 2014; Rex et al. 2018). Fungal nitric oxide reductase (P450nor) is a cytochrome p450-containing enzyme that receives electrons directly from NADH for the reduction of NO to N_2O (Nakaharaet al. 1993; Zhou et al. 2001). In contrast to bacteria, low levels of O_2 (hypoxia) are required to generate NADH from the oxidation of citrate in the tricarboxylic acid cycle (Ma et al. 2008) for fungal production of N_2O. Combinations of formate and hypoxia (versus anaerobiosis) are thought to be essential for the fungal reduction of nitrate to N_2O and as much as 65% of soil-emitted N_2O can be attributable to fungi. However, this was found only in soils from water-accumulating landforms (Ma et al. 2008). pH has been demonstrated to be an important factor in regulating the concentrations of N_2O emitted from fungi. Chen et al. (2014) demonstrated that fungal N_2O production was significantly higher in more acidic soils. Using streptomycin and cycloheximide to inhibit bacteria and fungi respectively, they observed that N_2O fluxes for soils at pH 5.5 were more affected by cycloheximide treatment whereas streptomycin inhibited N_2O production for soils at higher pH. This indicated that fungi were the most dominant producers of N_2O in acidic soils. Laughlin et al. (2009) demonstrated that in a grassland soil treated with cyloheximide reduced N_2O production by 89% in comparison to streptomycin which only reduced N_2O emissions by 23%. This was again confirmed by Laughlin et

al. (2009) in another grassland soil with cycloheximide treatment almost completely inhibiting N_2O production. Chen et al. (2015) demonstrated, with the use of streptomycin and cycloheximide, that fungi were either comparable to bacteria in N_2O production or, contributed more to N_2O production across diverse set of ecosystems.

1.3.4. Other potential pathways of nitrous oxide production in soil

1.3.4.1. Hydroxylamine Oxidoreductase (HAO): Contribution to the formation of nitrogen compounds used in the production of N_2O

HAO is a large and very complex homotrimer, with a molecular weight of 68 kDa per monomer, that catalyses the oxidation of NH_2OH to NO_2^-. Each monomer contains seven *c*-type hemes and a novel eighth heme known as P460 (Arciero and Hooper. 1997; Igarashi et al. 1997). This has a Soret absorbance at 463 nm in the reduced state, hence it is known as P460 heme (Arciero et al. 1998). The P460 is known to be the active site to which NH_2OH binds and is then oxidized and is comprised of a Fe centre (Arciero and Hooper. 1997). The role of the *c*-hemes that P460 is attached appears to be the transfer of an e^- from the P460 active site to one or more e^- acceptors such as Cytochrome *c*-554, an abundant protein found in the periplasm of *N. europea* (Bergmann and Hooper. 1994). HAO ultimately converts NH_2OH to NO_2^-. However, Hooper and Terry (1979) later observed the production of NO through dehydrogenation of NH_2OH under anaerobic conditions describing it as an intermediary step in the production of NO_2^-, as shown below;

$$(1).\ NH_2OH \rightarrow HNO + 2H^+ + 2e^-$$

$$(2).\ HNO \rightarrow NO^+ + H^+ + 2e^-$$

$$(3).\ NO^+ + H_2O \rightarrow NO_2^- + 2H^+$$

∴ in a combined two step reduction of hydroxylamine the following reaction is;

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4\ e^-$$

Maalcke et al. (2014) described the reaction as follows;

$$NH_2OH \rightarrow NO + 3H^+ + 3e^- \ (E_0' = -0.030\ V)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \ (E_0' = +0.065V)$$

Hooper and Terry (1979) demonstrated, *in vitro*, the ability of the HAO in *Nitrosomonas europea* to catalyse the oxidation of NH_2OH to NO and N_2O specifically and not through the reduction of NO_2^- or NO_3^- to form NO in O_2 limited conditions. However, the ability of this process to proceed was also found in aerobic conditions by Firestone and Davidson (1989) in soils. A study by Kozlowski et al. (2014) showed that within *N. europea*, N_2O production was only formed through the reduction of nitric oxide by NorB nitric oxide reductase. HAO, therefore contributes significantly to the ability of AOB in the production of N_2O through two possible pathways (Fig. 2). Recently a study by Caranto et al. (2016) observed that P460 activity in *Nitrosomonas europaea* did not form NO_2^- under aerobic conditions, as the NO_2^- formed in their study was not stoichiometrically associated with NH_2OH concentrations, and that rather the P460 enzyme was important in the formation of

N_2O under anaerobic conditions. Below is presented an adapted version of N_2O production pathway of *N. europaea*;

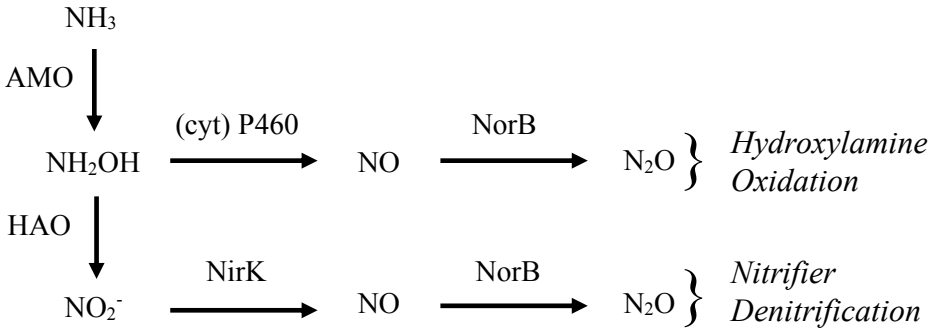


Figure 1.2. Schematic of N reduction pathways in chemolithotrophic nitrification of *N.europaea.* Adapted from Kozowski et al. (2014)

1.3.4.2. Nitrite reduction to nitric oxide (NO_2^- → NO) by Nir genes in chemolithotrophic nitrification

Nitrifying bacteria have the capability of producing significant amount of N_2O from NO_2^- *in-vitro* as demonstrated by Goreau et al. (1980) using *Nitrosomonas* spp. which had been demonstrated to occur naturally in soil (Lipshultz et al. 1981). Most ammonia-oxidizing bacteria studied to date have gene homologues to copper-containing nitrite reductase (*nirK*) but are not thought to possess a *nirS* homologue (Jason et al. 2007). Beaumont et al. (2004) identified that the gene in *N. europaea* was active in both aerobic and anaerobic conditions and was regulated by NO_2^- concentrations. *N. europea,* have been found to have a higher expression of *nirK* at higher concentrations of NO_2^- (Beaumont et al. 2004). *Nitrospira*. Spp, now considered to be the most dominant AOB in soils (Daims et al. 2015), was also demonstrated to produce N_2O by Shaw et al. (2006). In further work, Shaw et al. (2006)

demonstrated that *Nitrospira* spp. produced N_2O in aerated cultures when supplied with NO_2^-. There have been indications as to why ammonia oxidizers reduce NO_2^-. Poth and Focht. (1985) suggested that aerobic NO_2^- reduction may have been to reduce NO_2^- from nitrite oxidizing bacteria, thus decreasing competition for oxygen by removing their substrate. However, with the increased knowledge on ammonia oxidizers such as *Nitrospira* spp., that are capable of performing full nitrification (Daims et al. 2015), this may be less likely and that N_2O emissions from nitrifier may be simply as a response to increased NO_2^- and the subsequent reduction to N_2O is a way to reduce concentrations of toxic NO_2^-. Loss of ammonia monooxygenase activity has been observed on exposure to nitrite (Stein and Arp. 1998) and work by Beaumont et al. (2004) demonstrated that expression of *nirK* in *N.europaea* was greater at increasing concentrations of NO_2^-, therefore suggesting that the expression of *nirK* in *N.europaea* is used as a defence against toxic NO_2^- that is produced during nitrification. However, in 2011 Cua and Stein (2011) demonstrated that the expression of *amoA* in relation to nitrite toxicity was different amongst species of AOB.

Oxygen is indicated as another important regulating factors for the productions of N_2O in ammonia oxidizing microorganisms. Zhu et al. (2013) demonstrated that with different substrates, urea and $(NH_4)_2SO_4$, the contribution of nitrifiers to the production of N_2O increased with decreasing oxygen concentrations, with the highest yields occurring at 0.5% O_2, this agreed with earlier observations by Goreau et al. (1980).

1.4. Impact of management on microbial N_2O production in soil and grassland soils

N_2O production by both nitrification and denitrification are reliant on the amount of available oxygen in the soil. The impact that soil structure plays in the emission rate of N_2O is well noted through alterations and reductions in soil total porosity and

available air-filled pore spaces due to increased water content as a result of compaction from agricultural machinery and livestock (Beauchamp 1997; Ball et al. 2008; Ball. 2013; Butterbach-Ball et al. 2013). This decrease in air-filled pore spaces due to the increased bulk density of the soil reduces the ability of oxygen to pass through the system, which can give rise to both nitrifier and denitrifier N_2O emissions depending on the other environmental factors present in the soil at the time.

Agricultural soils are vulnerable to compaction due to the use of increasingly heavy agricultural machinery and presence of livestock when the soil is more susceptible to compaction i.e. soil moisture close to field capacity. Bhandral et al. (2007) demonstrated a seven-fold increase in soil N_2O flux from grassland soils with compaction gradients ranging from 1.4 g cm^{-3} in the 0.2 cm portions of the soils and 1.23 g cm^{-3} in the slightly deeper portions of the soil (4-6 cm). The deformation of soil internal structure, through compaction from farm traffic and the increase in water content during periods of rainfall enhance the conditions to which N_2O is emitted through either reduction in total porosity or increased in soil water content, or through a combination of both, which in turn reduce the number of air-filled pores available therefore reducing the aerobic conditions of the soil (Sexstone et al. 1985; Mosier. 1994; Pilegaard. 2013; Signor. 2013). Soil compaction occurs through an increase in force applied to the soil surface that deforms the internal soil matrix, which increases soil bulk density and results in a reduction of a soils total porosity (Richard et al. 2001; Ball et al. 2008). The percentage of available air in the soil is regulated by both the total percentage pore space and the soil moisture content (%MC) as the reduction in available pores reduces the ability of soil water to move through the soil matrix and drain away. This balance between moisture content and available air-filled pore space can change dramatically with soil compaction. A reduction in total porosity means an

increase in the percentage of water-filled pore space (%WFPS). Therefore, due to this changing dynamic as a result of soil compaction means that conditions which favour the emissions of N_2O are enhanced through the reduction in the amount of available oxygen that can flow through the soil system which either takes places through nitrifier denitrification or heterotrophic denitrification (Bateman and Baggs. 2005).

The study by Bhandral et al. (2007) also evaluated the impact of an N source on N_2O fluxes. They demonstrated that NO_3^- application to a compacted grassland soil increased total N_2O emissions by roughly 7 times that of urine, which gave the second highest flux of N_2O. Given that NO_3^- provided the highest fluxes of N_2O it was assumed that the denitrifying soil community was the dominant group of microbial N_2O production (Bhandral et al. 2007). It is common that grasslands are applied with either urea or slurry, however, as discussed previously urine can play a substantial role in soil N_2O fluxes in grassland grazed by cattle.

1.5 *Conclusion*

An improved understanding is needed of the impact of compaction on soil N_2O emissions from grasslands. It has been well established that the main impact of soil compaction on the increase in soil N_2O flux is due to the reduction of available air-filled pores and therefore the reduction of oxygen that is able to diffuse efficiently through the soil matrix. However, in this respect there are still processes to be studied, especially the importance of the soil microbial communities.

The literature indicates that there are at least 3 main regulating factors with regard to N_2O emissions. The most obvious and notable is that reductions in O_2 leads to the expression or certain genes that are responsible for the production of N_2O. This reduction in O_2 not only leads to typical denitrification, either nitrifier coupled

denitrification (NCD) or fertilizer denitrification (FD), however, it has been demonstrated that reductions of O_2 can also lead to increases in N_2O emissions from nitrifying soil microbial communities (Wrage et al. 2004). In combination with conditions that may induce stress responses, i.e. toxic amounts of NO_2^- leading to the increased expression of *nirK* in nitrifying microorganisms (Beaumont et al. 2004), the conditions that lead to activation of nitrifier nitrous oxide emissions is a key area to be explored. This type of denitrification needs further study especially alongside the use of N inhibitors. The deposition of high amounts of nitrogen either in the form of fertilisers, urea and slurry, or through the deposition of cattle urine can allow large quantities of NH_4^+/NH_3 to be added to the soil environment. This in combination with the N inhibitor dicyandiamide (DCD) which prolong concentrations of NH_4^+ in the soil and reductions in a soils capacity to diffuse oxygen through the soil matrix, either through compaction or increased water content (WFPS) have the ability to induce the conditions described that cause nitrifier denitrification (ND).

Although denitrification is an important aspect to consider when studying N_2O emissions, it is important to evaluate the source of NO_3^- that regulates the denitrification. Nitrification is arguably the most important factor to study with regard to most fertilizer applications, as this process is responsible for converting applied ammonium (NH_4^+) to nitrite (NO_2^-) and then eventually to nitrate (NO_3^-). As archaea only possess a homologous *amoA* gene to that of AOB and fungi are not known to possess an *amoA* gene DCD has no effect on either ammonia oxidizing archaea at the level currently used in agriculture (Zhang et al. 2012), or, fungi's ability to oxidize ammonium.

Within this thesis the following areas will be explored with regard to 'Managing nitrous oxide emissions through an improved understanding of soil microbial

communities'. As oxygen is arguably the most important controlling factor with regard to N_2O and N_2 emissions, the study of how compaction alters the internal soil matrix and therefore soil pore space size distribution and connectivity, soil water retention, air permeability rate, pore continuity, effect pore blocking and soil gas diffusion rates will be studied. This will provide essential background information to help understand the conditions in the soil that contribute to N_2O emissions. First, a laboratory replication of the field compaction trials will be used to identify the main regulatory soil physical factors, such as bulk density and oxygen diffusion rates in relation to fluxes of N_2O. As bacteria and fungi have been demonstrated to actively oxidize ammonium in grasslands soils (Laughlin et al. 2008), a second experiment aims to identify the dominant ammonia oxidizers in the grassland systems that was part of this study by using an antibiotic inhibition method. This is then followed by a third experiment that aims to understand efficacy of DCD in grassland soil when receiving different fertilisers, whilst also evaluating the use of the use antibiotics used in the second study to distinguish between bacteria and archaea. Finally, the thesis finishes by addressing the efficacy of DCD in urine and slurry treated compacted mesocosms by study the impact on soil nitrifying and denitrifying microbial communities.

The main aims to be investigated throughout this study are;

(1). Evaluate the effect of increasing bulk density of a grassland soil on N_2O emissions

(2). Study the efficacy of the nitrification inhibitor dicyandiamide (DCD) on soil ammonium oxidation in a grassland soil.

(3). Understand the effect of different nitrogen sources, commonly found in grassland soil on different microbial communities.

(4). Evaluate the efficacy of DCD on N_2O emissions on compacted grassland soil receiving cattle urine and slurry treatments.

CHAPTER 2. UNDERSTANDING THE EFFECT OF SOIL COMPACTION AND WATER-FILLED PORE SPACE ON SOIL PHYSICAL PARAMETERS REGULATING SOIL N_2O AND CO_2 FLUX

2.1 INTRODUCTION

Soil gaseous emissions are a direct result of microbial activity. Soil microorganisms, like all living organisms, need food sources in order to grow, survive and reproduce these come from inputs of nutrient sources to a soil system. These sources are governed by a number of factors such as natural C and N inputs from plant-microbe interactions and the decomposition of organic material. Grasslands are generally limited by nitrogen (LeBauer & Treseder. 2008), where fertiliser input is common to increase yield. The input of nitrogen can creates an excess of nitrogen, which is subsequently released as N_2O, or, leached out through the soil matrix into groundwater as diffuse pollution.

Although both carbon and nitrogen are important for micro-organism cell reproduction with micro-organisms, soil conditions have a significant role in both the regulation of both the C and N compounds utilised by soil micro-organisms. Oxygen (O_2) availability is a crucial factor in the nitrogen cycle as both autrophic and hetertrophic bacteria, as well as soil fungi produce N_2O in response to changes in oxygen concentrations (Zhou et al. 2000; Zuba et al. 2013). Soil compaction increases soil bulk density, which in turn reduces total air-filled pore space, therefore redcuing the amount of oxygen available in the soil. As soil compaction not only reduces air-filled pore space, but significantly alters soil pore connectiveity this may ultimately lead to the accumulation of nutrients leading to 'hotspots' for N_2O production in the soil (Laudone et al . 2011; Loick et al. 2017). Only a handful of researchers have studied the impact of diffusion rates in combination with other physical characteristics

such as gas diffusion, air permeability and pore tortuosity on N_2O and CO_2 fluxes (Panday and Nkongolo. 2016), therfore this chapter will aim to improve the understanding of the effects of compaction on soil physical structure and it's relationship to soil gaseous flux.

OBJECTIVE: *Evaluating the Impact of Bulk Density and Water Filled Pore Space (WFPS) in a Grassland Soil on N_2O Emissions.*

The following experiment aimed to investigate;

(1) The effect of soil bulk density and soil percentage water-filled pore space as singular and combined factors on the flux rate of both N_2O and CO_2 fluxes in soil.

HYPOTHESIS:

'Nitrous oxide fluxes are regulated due to changes in oxygen availability through increased bulk density'

2.2 MATERIALS AND METHODS

2.2.1 Soil Sample Preparation

Soil was sampled to a depth of 10 cm from a short-term grassland compaction experiment from SRUC Crichton Farm, Dumfries, Scotland (N55:02:19, W3:36:06). The field was an imperfectly (poorly) drained silty loam, of the Stirling soil series (30% clay, 14% sand and 55% silt) that overlies red sandstone as the parent material (pH 5.7, K and P medium to high), which had been in grass (perennial ryegrass) for 5 years.

Field moist soil was air dried to a moisture content (MC) between 10-15%. Once dried, the soil was sieved to < 2mm. Samples were ordered into treatment factors, in total there were 2 treatment factors; three (3) bulk densities 1.1, 1.2 and 1.4 (g cm3) and two (2) WFPS%, 60% and 80%. All samples were replicated in sets of 4 therefore total sample numbers for each day equated to 24, providing a total number of 240 samples (n=4).Soil samples were prepared by compacting to a height of 50 mm inside 180 mL plastic containers, by compacting two 25 mm layers and using a spatula to disturb the top of the first layer to attempt to minimise layer effects, using a constructed compacting device with weight weighing 6.5 kg (Appendix; Fig. 7.1). The containers dimensions had a height of 100 mm, with an internal diameter of 50 mm. The force applied by the weight was calculated as follows;

$$\text{Force (N)} = m \cdot g \cdot h/d$$

Where Force in Newtons (N) is equal to m = mass multiplied by g = acceleration due to gravity (9.81 m s^{-1}), multiplied by h = distance travelled and d = slow down distance.

Compaction force for bulk densities of 1.1 and 1.2 g cm^3 was calculated to be 1592.5 N when dropped from a height of 25 mm; bulk densities of 1.2 g cm^3 had two impacts from the weight onto the surface of the soil column in order to achieve the desired bulk density. The bulk density of 1.4 g cm^3 was achieved with a calculated force of 3185 N applied 3 times to the soil surface.

2.2.2 Soil microcosm preparation

Soil was air-dried soil to 15% MC before being compacted into containers in order to achieve desired bulk densities and to allow the adjustment of %WFPS treatments. After compaction into 180 mL cylindrical containers (Scientific Laboratory Supplies), soil samples were adjusted to 40% WFPS. This was calculated from the desired gravimetric moisture content from a re-arrangement of the WFPS equation (below); (particle density was assumed to be 2.65 g cm^3 and soil total porosity was calculated as indicated below;

WFPS = (%MC (θg) · Dry Bulk Density (ρ))/ (Dry Bulk Density (ρ)/(Particle Density (P_D), Effectively;

WFPS (ϕ_{WF}) = Volumetric Water Content (θ_w) /Total Porosity (ϕ);

$$\phi_{WF} = \theta_w/\phi$$

Therefore, the calculation for gravimetric water (g), and mL of H_2O, needed for each bulk density at both 60% and 80% WFPS was;

$$\theta_g = (\phi_{WF} \cdot \phi)/\rho$$

Air-filled pore space (ϕ_{AF}) was calculated from the difference in water-filled pores and total porosity (ϕ_{AF}).

$$\phi_{AF} = \phi - (\phi \cdot \phi_{WF})$$

Water volume was calculated by multiplying the gravimetric water content by the weight of soil, but also taking into account water that was already present in the soil. This equaled the final volume of 40% water-filled pore space for each individual compaction treatment. Samples were covered with parafilm; to allow air circulation but prevent moisture loss from the soil. Samples were then incubated for 4 days at 20^{o}C, to allow re-establishment of microbial communities to avoid any peak respiration rates at the start of the experiment as observed in other studies (Bateman and Baggs. 2005)

2.2.3 Application of urea

After the initial 4-day incubation, soils had 128 kg ha^{-1} Urea (60 kg ha^{-1} N), field equivalent (DEFRA RB209 Handbook) applied to the soil surface. Three (3) mL of urea solution, with a concentration of 8.3 mg mL^{-1} urea, was added to the soil surface equaling a total concentration of 25 mg urea per sample. Soil samples were then brought to their respective %WFPS by addition of H_2O from the initial calculated solution volume (mL).

2.2.4 Soil sampling and storage

Soils were sampled for gas emissions and then destructively sampled every 7 days over a 28 day period. Samples were homogenised within sample bags and stored at 4^{o}C. Soil samples were analysed for soil ammonium (NH_4^+-N) and nitrate (NO_3^- - N) within 5 days of sampling.

2.2.5 Experimental design

Three (3) bulk density treatments (1.1 g cm^3, 1.2 g cm^3 and 1.4 g cm^3), two (2) water-filled pore space treatments (60% and 80% WFPS), five (5) sampling points (0, 7, 14, 21 and 28 Days) and four (4) replications of each sample treatment were used for the purpose of this experiment, equalling 120 samples in total.

2.2.6 Soil gas sampling: Nitrous Oxide (N_2O) and Carbon dioxide CO_2

Soil gas emissions were determined in-situ inside the sample 180 mL containers. At each sampling point, before lids were secured, soil core surfaces were purged 3 times with air, using a 50 mL gas tight syringe; this helped to evacuate the container of any excess concentrations of soil gases. Samples were taken from the calculated headspace of 98.4 mL container with an air-tight screw top lid. Vaccum grease to used to ensure an air-tight fit and a modified three-way valve gas as a sampling port. N_2O linearity was assured previously by measuring at 0, 30 and 60 mins. Once sealed an initial time, 0, gas sample was taken. 30 mL of gas was sampled using the 50 mL gas tight syringe, modified with two plastic three way valves and syringe needle. Before the sample was taken, 30 mL of air was introduced into the sealed container to ensure a sufficient amount of gas could be taken so as not to introduce a vaccum into the sample, the air was then circulated using the syringe to mix the gas by taking a 30 mL

sample and returning it back into the sample container. This action was repeated three times. The final 30 mL gas sample was then injected into a pre-evacuated 22 mL gas chromatograph (GC) vial through a rubber septum using a needle attached to the syringe.

Samples were analyzed for N_2O and CO_2 using an Agilent Gas Chromatogram (GC) against pre-determined standards (Air Products). All gas values obtained from the GC were corrected for the initial dilution (1.3x). The following calculation was used to determine the rate of gas flux μg g^{-1} h^{-1} and amount of N_2O-N;

$$N_2O\ (\mu g\ mL^{-1}) \cdot 98.4\ mL\ (\text{headspace of container}) = \text{Total Mass of } N_2O\ (\mu g)$$

Where N_2O (μg mL^{-1}) is the measured concentration of N_2O taken from the GC and 98.4 mL is equal to the headspace in the sampling chamber. Gas flux was then calculated using the following formula;

$$\text{Gas Flux } (\mu g\ m^2\ h^{-1}) = \frac{\delta c}{\delta t} \cdot \frac{\rho \cdot V \cdot (Mw(N_2O-N))}{R \cdot T \cdot A}$$

Where ρ = atmospheric air pressure at sea level (101.3000k Pa), V = volume of N_2O in the sample (μg), Mw = molecular weight of N_2O-N = 44.013 g mol^{-1} x 0.6365 (0.6365 is the percentage of N in N_2O which is equal to 28.0143 g mol^{-1}), R = universal gas coefficient (8.31 J mol^{-1} K^{-1}) and T = Temperature at which gas samples were taken in Kelvin (273.15°K + 20°C) and A = soil microcosm surface area (m^2).

2.2.7 Soil Ammonium and Nitrate Concentrations

Ten (g) of soil was weighed and placed into 250 mL plastic containers, to which 20 mL of 2 M potassium chloride (KCl) solution was added. Soil ammonium and nitrate were then extracted, by shaking on an orbital shaker at 200 rpm for 2 hours. After the two-hour extraction period, soil slurries were decanted into 15 mL polyethylene conical centrifuge tubes. The tubes were then centrifuged at 13,500 rpm to separate soil suspension from extracted solution. Once centrifuged, the supernatent was decanted into a 10 mL plastic test tube, stoppered and stored at 4^{o}C until analyzed. Ammonium and nitrate concentrations were determined using a SKALAR San ++ Automated Wet Chemistry Analyzer (Continuous Flow Analyzer), with concentrations determined against calibration standards for both ammonium and nitrate. If sample concentrations fell outside of the upper end of the calibration standards, samples were diluted as necessary.

2.2.8 Gas Diffusion, Pore Connectivity and Tortuosity, Air permeability

2.2.8.1 Soil Gas Diffusion Rates

Using a similar design to that used by Schjønning (1985) based on the work by Taylor (1949), following the principle as suggested by Currie (1960a), soil gas diffusion coefficients were measured. A soil sample was placed into air-tight contact with a closed chamber (Fig 2.1). Oxygen concentrations in the chamber were monitored by an O_2 sensor (calibrated to measure in a range between 0% - 100% O_2) (Apogee instruments). The oxygen sensor was initially calibrated to atmospheric levels in the lab (20.9%). The diffusion chamber was flushed with O_2 free N_2 until levels in the chamber read 0% O_2, after which the slide plate that disconnected the soil core

from the chamber was opened, and O_2 from the soil core atmosphere was allowed to flow into the chamber. Readings of O_2 were taken every minute, for the first 10 minutes, and then every 5 minutes subsequently. A measurement series typically took 1.5 h to 3 h depending on the development of O_2 concentrations within the chamber.

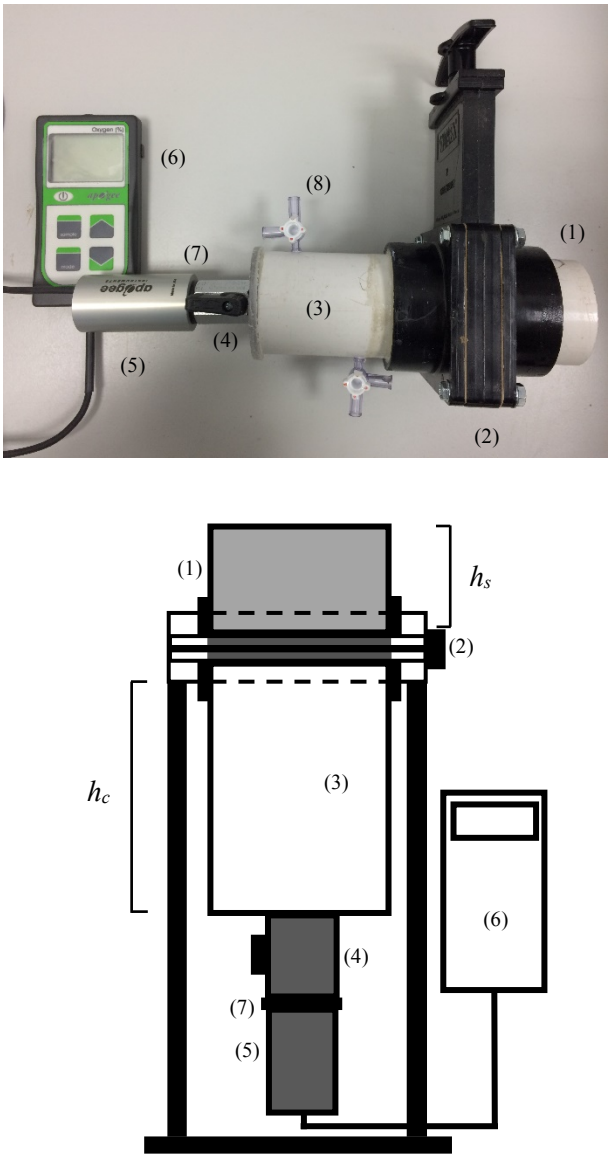


Figure 2.1 Constructed oxygen diffusion apparatus. (1) soil sample, (2) air tight gate valve, (3) Diffusion Chamber, (4) Oxygen sensor valve, (5) Oxygen sensor, (6) Digital readout for oxygen sensor, (7) Rubber O-ring, (8), Flush gas inlet and outlet valves. h_s = soil sample height and h_c = Diffusion chamber height.

The gas diffusion coefficient was derived from the change in time and the logarithm of the O_2 concentration in the chamber. Using work conducted by Schjønning, (1985), soil gas diffusion coefficients were calculated using the following principles.

Assuming that the diffusion of the gases can be estimated by steady state conditions from the experimental measurements taken, Fick's first law can be expressed as;

$$JA = \frac{dq}{dt} = -D_p \frac{\Delta C_t}{h_s} A$$

where J is the diffusive flux [ML^{-2} T^{-1}], *dq* is the amount of gas [M] diffusing in the time interval *dt* [T], *Dp* is the effective diffusion coefficient of the soil [L^2 T^{-1}], ΔCt is the difference in O_2 concentration between the two ends of the sample [ML^{-3}] at time t, A is the cross-sectional area available for diffusion [L^2], and hs is the diffusion pathway (length of soil sample) [L]. The relation between concentration change in the diffusion chamber and the amount of gas diffusing through the sample can be given by;

$$\frac{dq}{dt} = \frac{d\Delta C_t}{dt} h_c A$$

Where h_c was the height of the cylindrical diffusion chamber [L]. The apparatus was constructed so the cross-sectional area of the diffusion chamber and the sample soil surface were identical, A.

Combining Eq. [1] and [2] renders;

$$D_p \Delta C_t = -h_s h_c \frac{d\Delta C_t}{dt}$$

Integration yields (ΔCt = ΔC0 at t = 0, ΔCt = ΔCt at t = t);

$$\ln\left(\frac{\Delta C_t}{\Delta C_0}\right) = -\frac{D_p}{h_s h_c} \leftrightarrow \ln\left(\frac{\Delta C_t}{\Delta C_0}\right) = k_1 t$$

Therefore, the diffusion coefficient, D_p, could be calculated from the dimensions of the diffusion chamber, soil sample and the slope, k_1, from the relationship between the diffusion time (s) and the logarithm of the concentration difference between the diffusion chamber and the atmosphere.

$$D_p = -h_s h_c k_1$$

The difference between the diffusion chamber was calculated by subtracting the value of O_2 in the diffusion chamber at t from the atmospheric O_2, and then finding the natural logarithm of this value, or, through normalizing the data as follows;

$$C_r = \frac{C_g - C_s}{C_0 - C_s}$$

Where C_r = normalized O_2 concentration, C_g = is the concentration of O_2 (%) at t, C_0 is the concentrations of O_2 (%) at t = 0 and C_s = the measured atmospheric concentration in the atmosphere (20.9%).

Both calculations, although providing different values, gave the same soil diffusion coefficient (k_1) when regressed against t. The relative diffusion coefficient was calculated by dividing D_p by the gas diffusion coefficient of O_2 in free air, 0.205 cm s^{-1}.

2.2.8.2 Soil Pore Tortuosity

Considering soil pores as tubes, tortuosity is the ratio, L/Ls, of tube length, L, to sample length, Ls. Ball (1981) showed that L/Ls can be calculated as;

$$\frac{L}{L_s} = \tau = \sqrt{\frac{\varepsilon}{D_s/D_o}}$$

Where τ = tortuosity, ε = soil air-filled pore space and D_s/D_o = relative soil gas diffusion.

2.2.8.3. Specific Diffusivity (SD) and Buckingham X

Gradwell (1964) suggested the use of specific diffusivity (SD) as a value to indicate the tortuosity of a soil pore system and this was calculated as follows;

$$SD = \frac{D_s/D_o}{\varepsilon}$$

With a values close to 1 indicating a non-tortuous system, i.e. a soil system where soil tubes are effective straight and 0 equaling a highly tortuous system for specific diffusivity.

Chamindu Deepagoda et al, (2012) suggested the use of the Buckingham X value, a value proposed by Buckingham (1904) that connected the relationship between the relative diffusivity, Ds /D_o, and the soil pore fraction open to diffusion, as a value that could be used as an estimation of soil pore connectivity and tortuosity of soil pores. Buckingham suggested that a value of X = 2 could adequately predict relative gaseous diffusion through a soil system.

$$\frac{D_s}{D_o} = \varepsilon^x$$

Where D_s/D_o = relative soil gas diffusion coefficient, ε = soil air-filled pore space and $x = 2$

However, the value of X could be calculated as follows using the measured gas diffusion coefficient (Schjønning et al. 2013) which can therefore be used as an indicator of soil pore tortuosity;

$$X = \frac{\log(D_s/D_o)}{\log(\varepsilon)}$$

Buckingham X values will produce values of 1 for a non-tortuous system and values of 3 for a highly tortuous system, these are much the same as Ball's method (1981) for indicating soil tortuosity.

2.2.8.4. Soil Air Permeability

Soil air permeability was calculated using the method of Ball et al. (1981). The same samples that were used for measurements of soil gas diffusion coefficients were used to calculate air permeability. An air tight cylinder was attached to the top of the soil sample and tightened using the screw fastening. Air was then passed through the samples at flow rates of 4 mL min^{-1} and 8 mL min^{-1} as regulated by a mass flow controller (Aalborg) (Fig 2.3?). At each flow rate the pressure was recorded using a differential pressure transmitter (Furness Controls). Air permeability was calculated using the following formula;

$$K_1 = \text{Flow rate (4) (mL min}^{-1}) / (\Delta_0 - \Delta_1) \cdot 0.7934$$

$$K_2 = \text{Flow rate (8) (mL min}^{-1}) / (\Delta_0 - \Delta_1) \cdot 0.7934$$

Where Δ_0 = measured preseeure in the headspace of the empty chamber, Δ_1 = measured pressure in the headspace of the measured sample and 0.7934 conversion factor specific to sample size which is calculated by;

$$\frac{\text{Flow (cm m}^3\text{ min}^{-1})}{\text{Pressure difference (mL min}^{-1})} \text{ X } \frac{\eta \cdot L_s}{A_s} \text{ x } \frac{1 \text{ x } 10^8}{60 \text{ x } 98.1}$$

=

$$\frac{\text{Flow (cm m}^3\text{ min}^{-1})}{\text{Pressure difference (mL min}^{-1})} \text{ x } 4.67 \text{ x } 10^{-5} \text{ x } \frac{1 \text{ x } 10^8}{60 \text{ x } 98.1}$$

Where η = air visocity in Poise (1.83×10^{-4}), L_s = length of sample (5 cm), A_s = area of sample (19.62 cm^2) and $\frac{1 \text{ x } 10^8}{60 \text{ x } 98.1}$ = conversion factor, which then preceeds to;

$$(K_1 \cdot K_2)^{0.5} = \text{Air permeability } (K_a, \mu m^2)$$

These calculations gave different soil physical properties for the diferent sample bulk densities and water filled pore spaces (Table 2.1).

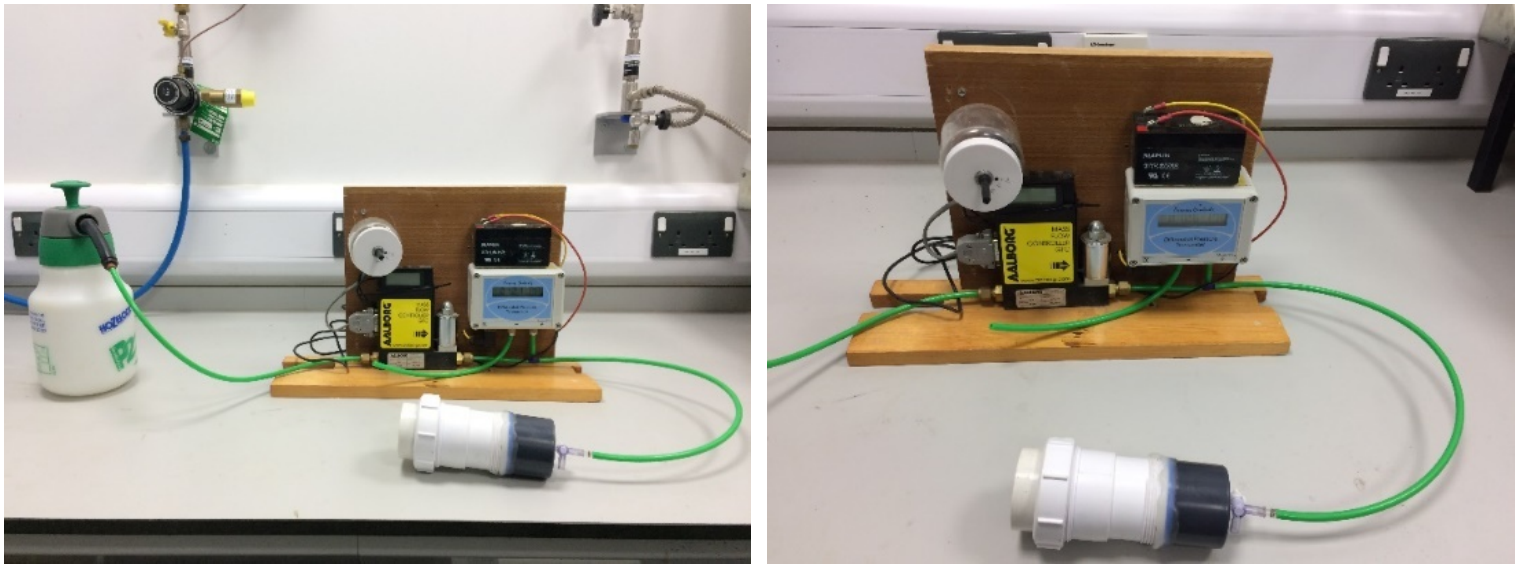


Figure 2.2 Air permeability measuring system, developed by Dr Bruce Ball.

Table 2.1 Soil sample physical properties at -10 kPa.

Soil Sample Physical Properties	**Sample Bulk Density & Water-filled Pore Space**		
Samples at -10 kPa	1.1 g cm^3	1.2 g cm^3	1.4 g cm^3
	40%	50%	60%
Effective pore radius (μm)	38.7	21.2	6.19
Number of air-filled pores cm^{-2}	4.1	2.0	2.6
Air Permeability [K_a (μm^2)]	1.15	0.70	0.12
Specific air permeability [K_a/ε (μm^2)]	4.0	3.2	2.0
Diffusion coefficient (D_s) cm^2 s^{-1}	0.025	0.025	0.01
Relative diffusivity (D_s/D_o)	0.12	0.12	0.05
Specific diffusivity [(D_s/D_o)/ε]	0.42	0.55	0.36
Tortuosity [$\varepsilon/(D_s/D_o)]^{0.5}$	1.7	1.5	1.9
Buckingham X [log(D_s/D_o)/log(ε)]	2.0	1.6	2.0
Total porosity (Φ), cm^3 cm^{-3}	0.59	0.55	0.47

Table 2.2 Physical properties of soil samples at 60% and 80% WFPS. Standard errors of sample means (SEM) for air permeability and oxygen diffusion coefficients. (n = 4)

Soil Sample Physical Properties	**Sample Bulk Density & Water-filled Pore Space**					
Experimental Conditions	1.1 g cm^3		1.2 g cm^3		1.4 g cm^3	
	60%	80%	60%	80%	60%	80%
Air permeability [K_a (μm^2)]	0.84 (±0.01)	0.2 (±0.03)	0.44 (±0.00)	0.1 (±0.03)	0.12 (±0.03)	0.07 (±0.00)
Number of air-filled pores cm^{-2}	0.15	0.15	0.27	0.32	2.6	0.82
Specific air permeability [K_a/ε (μm^2)]	3.5	1.6	2	0.9	0.6	0.7
Diffusion coefficient (D_s) cm^2 s^{-1}	0.005 (±0.00)	0.0025 (±0.00)	0.005 (±0.00)	0.0025 (±0.00)	0.01 (±0.00)	0.004 (±0.00)
Relative diffusivity (D_s/D_o)	0.024 (±0.00)	0.012 (±0.00)	0.024 (±0.00)	0.012 (±0.00)	0.05 (±0.00)	0.02 (±0.00)
Specific diffusivity [(D_s/D_o)/ε]	0.1	0.1	0.11	0.11	0.26	0.2
Tortuosity [$\varepsilon/(D_s/D_o)]^{0.5}$	3.2	3.2	3.0	3.0	1.9	2.2
Buckingham X [log(D_s/D_o)/log(ε)]	2.3	2.0	2.1	1.8	2.0	1.5
Air filled pores (ε), cm^3 cm^{-3}	0.24	0.12	0.22	0.11	0.19	0.09

2.2.9 Statistical Analysis

Data were analysed using Genstat (17th edition), General ANOVA were used to analyze the impact of soil physical characteristics alone and in combination with N concentrations in the form of NH_4^+ and NO_3^- on N_2O flux. In order to normalize data, N_2O, NH_4^+ and NO_3^- data were log10 transformed, and CO_2 data was log10 (x+10) transformed. Where approproate two times the standard error of the mean (SEM) of samples was used to discern differences, with difference estimated to be $P \leq 0.05$ when there were observed gaps between the two times the mean sample standard error of the mean (SEM).

2.3. RESULTS

2.3.1 Soil N_2O flux

Analysis of log(*e*) tranformed N_2O fluxes revealed an interaction between WFPS x Day (Table 2.3), as N_2O flux's peaked on day 7 of the experiment for all sample treatments, including the control (1.1 cm^3 + 60% WFPS) (Fig. 2.3) except 1.2 g cm^3 + 60% WFPS (Fig. 2.3 (a)). 1.1 g cm^3 + 60% WFPS emitted less ($P \leq 0.05$) N_2O than the 1.1 g cm^3 + 80% WFPS treatment. with N_2O fluxes 10 times higher in the 80% WFPS treatment; 0.18 ± 0.003 (SE) N_2O-N g m^2 h^{-1} and 1.8 ± 0.5 (SE) N_2O-N g m^2 h^{-1}, respectively. A similar result was observed for the 1.2 g cm^3 bulk density soil with the 80% WFPS treatment, where the N_2O fluxes were 300 times higher than the 60% WFPS treatment with 6.6 ± (1.4 SE) and 0.025 ± (0.0025 SE) g m^2 h^{-1} N_2O-N, respectively. The largest peaks were observed in the 1.4 g cm^3 bulk density soils for both WFPS treatments, however these did not significnatly differ from one another. Bulk density did demonstrate an effect on soil N_2O emissions with a graduated increase for the 80% WFPS treatment. Cumulative total N_2O fluxes also significantly increased with WFPS as observed by the difference between 1.1 and 1.2 g cm^3 at 60% WFPS when comapred to their 80% WFPS treatment counterparts (Table. 2.3; Fig. 2.4, $P \leq 0.05$).

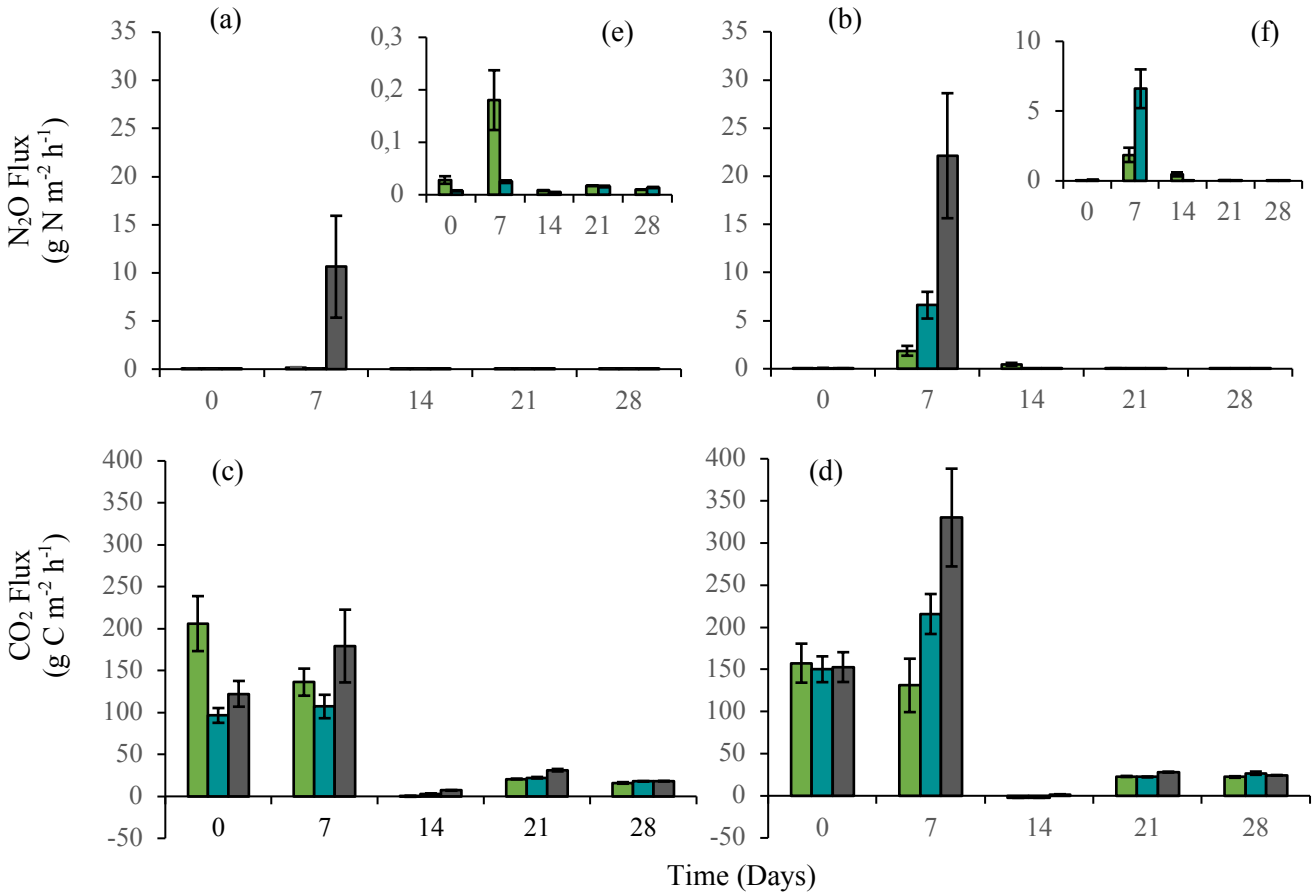


Figure 2.3. N_2O at 60% WFPS (a) and 80% WFPS (b) and CO_2 concentrations 60% WFPS (c) and 80%WFPS (d) for bulk density treatments (1.1 g cm^3 (green bars), 1.2 g cm^3 (teal bars) and 1.4 g cm^3 (grey bars). Graphs (e) and (f) show the N_2O concentrations that are too low to be seen on graphs (a) and (b). Error bars respresent standard error of sample means (SEM). n=(4).

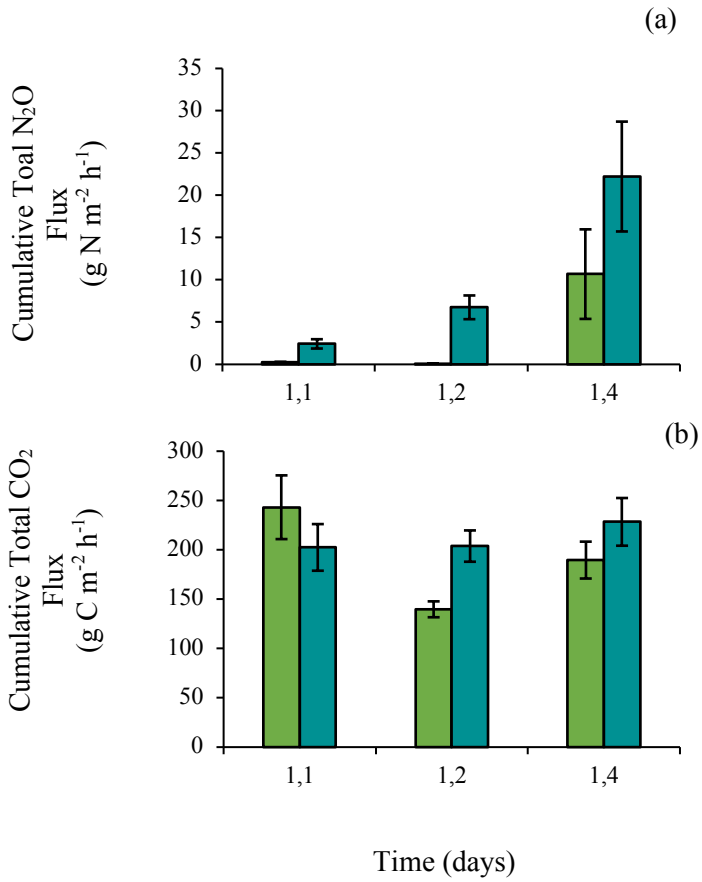


Figure 2.4 Cumulative N_2O and CO_2 fluxes from start to finish of the experiments for bulk density treatments. Graph (a) are cumulative fluxes for N_2O fluxes and (b) are cumulative fluxes for CO_2 fluxes. 60% WFPS is shown with the green bars and 80% WFPS is shown with the teal bars. Error bars represent standard error of sample mean (SEM). n = (4)

2.3.2 Soil CO_2 flux

Soil CO_2 fluxes were observed to follow a similar pattern to those of N_2O, with a decrease after Day 7 of the experiment (Table 2.3; Time, $P < 0.001$; Fig 2.3 (a) and (b))

2.3.3 Ammonium (NH_4^+) and Nitrate (NO_3^-) concentrations

Soil ammonium concentrations were altered through the interaction between WFPS x Time (Table 2.3, $P < 0.001$). Soil NH_4^+ concentrations significantly ($P \leq 0.05$) increased upon addition of urea by Day 7, with concentrations ranging between 14 and 20 μg g^{-1} soil NH_4^+-N, with the higher concentrations, 16 (± 3.6 SE), 17 (± 4.1 SE), 19 (± 5.2 SE) and 20 (± 5.0 SE) μg g^{-1} soil NH_4^+-N measured in bulk density samples 1.1 + 80, 1.2 + 80, 1.4 + 80 and 1.4 + 60 g/cm^3, respectively (Fig. 2.5). Ammonium concentrations also demonstrated a significant intereaction between Bulk Density x WFPS for the 1.2 g/cm^3 samples between 60% and 80% water-filled pore space on Day 7 and for the 1.4 g cm^3 60% and 80% WFPS treatment for Days 14 to 28.

Soil NO_3^--N concentrations were measured between 58 μg g^{-1} soil NO_3^--N (1.4 g cm^3 + 80% WFPS) to 85 μg g^{-1} soil NO_3^- (1.1 g/cm^3 + 60% WFPS) at the start of the experiment and demonstrated little change from Day 0 to Day 7 relative to both WFPS treatments. However, it was observed that the 1.2 g cm^3 bulk density increased ($P \leq 0.05$) soil NO_3^- concentration which was opposite to the lower concetrations of NH_4^+ on Day 7.

Table 2.3 ANOVA general model output for soil treatment effects on soil gas (N_2O and CO_2), mineral N (NH_4^+ and NO_3^-). Treatments include soil bulk density (ρ_b), water-filled pore space (WFPS) and time (day). Values $P < 0.05$ are highlighted in bold. (n = 4)..

Effect	**log10 N_2O**	**Cumulative Total log10 N_2O**	**log10 (x+10) CO_2**	**Cumulative Total log10 (x+10) CO_2**	**Log10 NH_4^+**	**Log10 NO_3^-**
ρb	0.601	0.738	0.161	0.602	**<0.001**	**0.032**
WFPS	**0.002**	**0.003**	0.522	0.479	**<0.001**	**0.015**
Time	**<0.001**	-	**<0.001**	-	**<0.001**	**<0.001**
ρb·WFPS	0.531	0.470	0.417	0.521	**0.031**	0.801
ρb·Time	0.144	-	0.585	-	0.077	0.218
WFPS·Time	**0.003**	-	0.101	-	**<0.001**	0.924
ρb·WFPS.Time	0.150	-	0.612	-	0.999	0.977

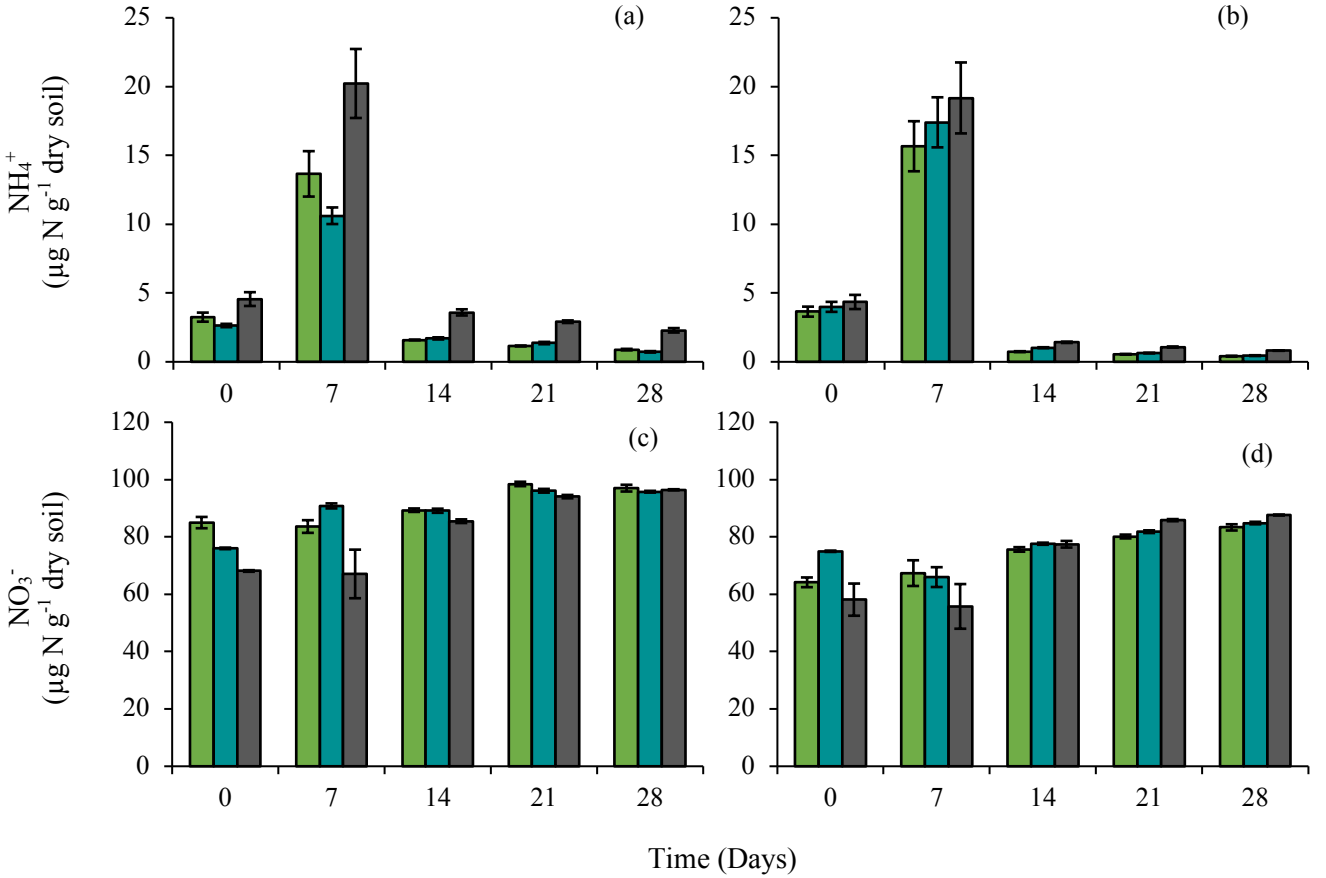


Figure 2.5. NH_4^+ at 60% WFPS (a) and 80% WFPS (b) and NO_3^- concentrations 60% WFPS (c) and 80%WFPS (d) for bulk density treatments (1.1 g cm^3 (green bars), 1.2 g cm^3 (teal bars) and 1.4 g cm^3 (grey bars). Error bars respresent standard error of sample means (SEM). n=(4).

2.4. DISSCUSION

2.4.1Understanding the effect of compaction and WFPS on the source of soil N_2O flux

Soil N_2O emissions significantly (Table 2.3; $P = 0.003$) increased as a result of soil WFPS peaking on Day 7 of the experiment. Soil bulk density demonstrated a trend, although not significantly to increase soil N_2O fluxes (Table. 2.1; Fig. 2.3), with higher emissions rates for all soil bulk density treatments at 80% WFPS when compared to the 60% WFPS treatment. This was also observed for cumulative total N_2O fluxes (Fig. 2.4 (a)). WFPS has previously been noted as a regulating factor in terms of N_2O release from soil (Castellano et al. 2009). The study by Castellano et al. (2009) found strong consistent observations that N_2O flux rate was a Gaussian function of WFPS i.e. that peak fluxes occurred at peak WFPS consistant with the results from this study. Recent work by Panday and Nkongolo. (2016) demonstrated that gas diffusion and soil pore totuosity were also useful predictors of N_2O fluxes, with decreases in gas diffusion coefficients and increased soil pore tortuosity related to increased soil N_2O fluxes. However, the measured gas diffusion coefficients in this study in relation to fluxes of N_2O are contrary to the observations by Panday and Nkongolo. (2016) as the soil tested here, under the conditions presented, the estimates of the soil gas diffusion coefficient for each sample treatment increased with increasing bulk density, with both the 1.4 60% and 80% WFPS treatments measuring the highest relative gas diffusion rates (Table 2.2). Rather interestingly, however, is that CO_2 fluxes relative to gas diffusion coefficients and soil pore tortuosity measurements are in agreement with the findings by Panday and Nkongolo. (2016), who observed that CO_2 fluxes increased

with increase gas diffusion coefficients and soil pore tortuosity. As N_2O fluxes emulated CO_2 fluxes (Fig 2.3), this can made the source of the N_2O hard to distinguish. Soil anaerobisis has been reported to begin at a relative diffusivity (D_s/D_o) of < 0.02 (Stepniewski. 1981), therefore both the 1.1 and 1.2 g cm^3 treatments at the 80% WFPS treatment would seem to be under soil anerobisis (Table 2.2), conditions that are known to be optimal for denitrification (Bremner. 1997). However, both the 60% and 80% WFPS in the 1.4 g cm^3 soils were not in a state of anearobsis as soil D_s/D_o values were measured at 0.05 and 0.02 respectively (Table 2.2), which suggested that soil N_2O fluxes may have occurred as a result of nitrifier denitrificaiton if soils were not under anaerobisis. Rapid anoxia through increased CO_2 concentration has been suggested to stimulate denitrificaiton by induceing anerobisis in the microsites of the soil (Sherlock and Goh. 1983). In this instance, CO_2 concentrations could have induced anerobisis in the soil, especially due to the lower volume of air-filled pores in comparison to both 1.1. g cm^3 and 1.2 g cm^3 bulk density treatments (Table 2.2). This could suggest that N_2O production was as a result of denitrification. However, as no mesurements were taken to indicate otherwise, i.e. the source of N_2O prodcution, this is just speculation. As neither soil potential nitrification activity nor nitrate reductase activity were measured in this experiment, as they were in Chapter 5, it is more difficult to identify the source of N_2O fluxes. Bateman and Baggs. (2005) studied the relative contributions to nitrication and denitrification from soils at different water-filled pores spaces. In their study, using a silt loam soil (17% sand, 68% silt and 15% clay) similar to the soil used in the current experiment, they observed that the relative contribution between nitrifers and denitrifiers after fertilisation with ^{15}N-labelled NH_4NO_3 for autotrophic nitrification on Days 3, 7 and 24 of the experiment were 89.91% 87.1% and 81.0% respectively and that denitrificaiton and/or heterotrophic nitrificaiton were

10.08%, 12.9% and 19% for Days 3, 7 and 24 respectively for soils maintianed at 60% WFPS. Soils that were maintained at 70% WFPS were observed to produce N_2O in response to denitrification and/or hetertrophic nitrification (100% of accounted ^{15}N-labelled N_2O-N fluxes) (Bateman and Baggs. 2005). Therfore, in the current study it is reasonable to assume that soil maintainted at 60% WFPS would likely have emitted N_2O as a result of autotrophic nitrificaiton and that N_2O fluxes in the 80% WFPS would have been as a result of denitrification activty. This is further supported by the lower concentrations of NO_3^- observed in the 80% WFPS treatments in comparison to the 60% WFPS treatments (Fig. 2.5), which would suggest that more NO_3^- was lost as N_2O between measurements, agreeing with the higher N_2O fluxes that were observed in the 80% WFPS treatment. Nitrifier nitrous oxide emissions emitted from soils have been shown to occur at concentrations of 0.5% to 20% of O_2 in a range of soil types (Zhu et al. 2013). The study by Zhu et al. (2013) used labelled ^{15}N-^{18}O to distinguish the pathway for N_2O emissions. They observed that most of the N_2O that was emitted was mainly attributable to NH_3 oxidation between the oxygen concentations range of 20% to 0.5% O_2 and that only 0% O_2 were solely as a result of hetertrophic denitrificaiton (Zhu et al. 2013). This is in comparison to the work by Bateman and Baggs (2005), who observed that once soil WFPS had reached 70% the dominant source of N_2O was from hetertrophic nitrifiers of denitrifiers and this would suggest that above 70% WFPS O_2 concentrations are close to or at 0% O_2 and denitrification would be the dominanting system. However, as discussed earlier in this chapter the soil gas diffusion coefficients in these samples would suggest otherwise in relation to the suggested gas diffusion rates. As samples were incubated without plant cover, CO_2 fluxes were most likely regulated by the amount of organic carbon input into the soil. Li et al. (2004) reported that litter or root exclusion significantly reduced soil

respiration in secondary forests and pine plantations, therefore the same may be said for the current experiment, given the soil CO_2 fluxes peaked soon after N addition and then reduced to concentrations lower than measured at the start of the experiment. Given the high concentrations of nitrate in the samples it would be expected that soil N_2O emissions should have persisted for longer periods. Both oxygen and nitrate are factors that regulate the reduction of NO_3^- to N_2O and finally to N_2 (Del Grosso et al. 2000). The oxygen concentrations in the 80% WFPS treatment in the current study would have been suffcicent for respiration of NO_3^- to proceed. This would indicate for the 80% WFPS treatments that carbon was a limiting factor, as it is assumed that N_2O fluxes from the 60% WFPS treatment was as a result of nitrifier denitrification and would therefore would be less reliant on the presence of carbon. As there was no variation from in the gas diffusion data it was assumed that the values were fixed factors, such as compaction and %WFPS treatments, and treated as such. It has been noted that soil physical characteristics can change with microbial activity, for example, increased pore connectivity through respiration (Helliwell et al. 2014). Helliwell et al. (2014) observed that pore volume and connectivity increased alonside soil microbial respiration with glucose amendments to the soil. This was not deemed relevant to the current study as the incubation period for the experiment only ran for 28 days, and significant changes were only observed in the study by Helliwell et al. (2014) after 4 weeks of sample incubation. In addition, in the study by Helliwell et al. (2014) a glucose substrate solution was added every two days, this simulated plant root carbon secretions which in turn stimulated microbial activity rates was included in the study by Helliwell et al. (2014). As this was not a factor in the current study it was assumed that any structural changes, due to microbial respiration, would have not been significant enough to alter measured soil gas diffusion and air permeability rates.

2.4.2 Notes on soil gas diffusion rates and soil physical structure in relation to gas fluxes

Soil gas diffusion coefficients are normally found to decrease in compacted soils (Xu et al. 1992; Situala et al. 2000), however, the increased gas diffusion in the higher bulk density soils were attributed to the increased straightening of soil pore tubes, as seen from the calculation of tortuosity factors using the Buckingham X equation as both specific diffusivity and tortuosity had values close to 1 which came closer to a value of 1 with increasing bulk density. Measurements of soil pore tortuosity factors in this study revealed that increased compaction produced increased tortuosity factors, i.e. closer to 1. This increased straightening of the pores would have shortened the path along which the gases would have to diffuse. Soil gas diffusion coefficients have been observed to increase with increasing bulk densities in a study by Fujikawa and Miyaki. (2005), they found that gas diffusion coefficients increased with increased bulk densities in both undisturbed and repacked soil columns. The current study agrees with that of Fujikawa and Miyaki. (2005) as gas diffusion increased with increased soil bulk density, in addition samples in the current study had similar air-filled pore space volumes to those observed by Fujikawa and Miyaki (2005). A study by Schjonning et al. (2013), noted an increased soil gas diffusion coefficients in field compacted samples compared to those seen in the control, and they attributed the increased gas diffusion in the compacted soils due to decreased soil pore tortuosity, therefore as pore totuosity decreases the pathway for gases to diffuse becomes shorter and therfore releasing higher concentrations of gas from the soil. The highest peaks of gas fluxes occurred in soils both at highest oxygen diffusion and lowest tortuosities. As it has been generally observed that gas diffusion coefficients decrease with increased compaction, (Xu et al. 1992; Situala et al. 2000), and as compaction treatments in

samples that were maintianed under the same matric potential were observed to decrease with increasing compaction, it is assumed that the reductions in soil gas diffusion and pore tortuosity is related to the reduction in soil moisture contents that were variable in each sample treatment in order to achieve a uniform %WFPS treatment in all sample bulk density treatments. Both Compaction and water saturation of the soils are suggested to be the main barriers of soil gas diffusion, with water being a more effective barrier (Moldrup et al. 2000), therfore this would provide solid reasoning for the increased gas diffusion rates in the higher bulk density treatments used in the current study.

2.4.3 Limitations to the current experiment

There are several limiations to the current experiment, mainly through the lack of the measurement of biological indicators of nitrifcation and nitrate reducing activities. The measurement of potential nitrifcaiton rates would have allowed an estimation of the contribution of the nitrfying soil microbial community in the production of N_2O emissions. Additionally, the use of inhibition methods, such as acetylene alongside ^{15}N labelling of the urea N would have allowed the realtive contribtion of different microbial groups the production of the soil N_2O gas fluxes measured.

The inclusion of a nitrate reductase enzyme assay would have allowed the estimation of the contribution of the nitrate reducing communities and therefore an indirect undertstanding of the contribution of denitrifers to N_2O fluxes. It was planned to study the relative abundance of AOB, AOA, *nirK* and *nosZ* genes in order to relate these to the fluxes of N_2O, as this would have enabled a more complete understanding of the microbial processes that occured in the soil during the experiment.

Although N_2O emissions recorded in this experiment were extremely high and were considerably greater to any fluxes measured in other studies that were conducted either in the field or within the laboratory. It has been noted that the emission rate of N_2O from the particular soil used for the experimental soil columns are some of the highest found across Europe (Rees et al. 2013). However, although the concentrations measured were much greater than any measured in other published studies, the patterns demonstrated relationships to soil the WFPS were similar to those found in similar studies of soil compaction effects on soil N_2O emissions (Oenema et al. 1997, Ruser et al. 2006; Hammonts et al. 2013). As gas standards were found to be accurate and no error found with the GC instrumentation, the only possible explanation for the large concentrations would be as a result of the gas sampling methodology. It is suggested that as air was injected into the sample, in order to ensure that no vaccum occurred in the headspace of the samples when taking gas samples, that this could have created a potential artefact in gas concentrations itself through increased gas diffusion from the soil through reduced pressure. In addition, is thought that the extra volume of air injected into the system, which was 1/3 higher ,as the headspace which was 98.4 mL and the amount that was injected was 30 mL, would have increased pressure in the headspace and the subsequent removal of 30 mL would then have reduced that pressure; s this was repeated 3 times it would only have exacerbated any artefact induced from the methodology. This methodology could in addition have led to the temporary displacement of water in the soil and produced an ebullition effect. As noted in a study by Vachaud et al. (1974) ebullition events can occur during water infiltration which can cause soil gases to erupt past the wetting front during the infiltration process. Due to the increased pressure in the system, water may have been forced down to the lower portions of the soil columns which would have forced gases out from the

remaining air-filled pores through the displacement of the water in the soil pore system (Fig. 2.6).

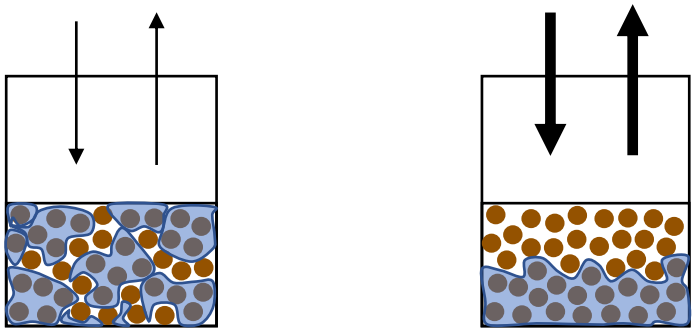


Figure 2.6. Diagram of potential mechanism for increased gas fluxes from soil. Narrow arrows represent a normal atmospheric pressure and resultant gas fluxes. Wider arrows represent an increased atmospheric pressure and resultant increase in gas fluxes from through through the displacement of gas by the movent of water through the soil system

2.5. CONCLUSION

- Soil WFPS content was found to significantly regulate soil N_2O emissions,
- Interestingly it was observed the samples that emitted the highest N_2O fluxes at the higher WFPS also demonstrated the highest rate of O_2 diffusion.
- It is suggested that soil N_2O fluxes were regulated by both soil nitrifying (60% WFPS) and denitrifying microbial communities (80% WFPS), however, due to no other corborating evidence no firm conclusion could be draw from this study.

- Soil N_2O fluxes were noted to be extremely high comapred to other studies and is most likely as a result of an artefact introduced due to the sampling procedure.
- It is recommended that a further study of the interactions between soil gas diffusion coefficients and activities of the nitrifying and denitrifying soil microbial communtiy should take place as this would improve the understanding of the understanding of soil gas diffusion on these activities of these soil microbial communities as these are still limited.

CHAPTER 3. ELUCIDATING MICROBIAL GROUP CONTRIBUTIONS TO AMMOINIUM OXIDATION IN A GRASSLAND SOIL RECIVING DIFFERENT COMPACTION TREATMENTS

3.1. INTRODUCTION

Soil nitrification is a key regulating step in the production release of sources of potential nitrous oxide (N_2O) into the atmosphere. Although ammonia oxidizing bacteria are commonly associated with this process, however, research has demonstrated that fungi are capable of oxidizing ammonium to nitrate. Schmidt (1954) first demonstrated the importance of fungal nitrate (NO_3^-) production. Later Hora and Iyengar. (1960) corroborated Schmidt's findings by demonstrating the ability of Aspergillus and Penicillium, of which both are commonly found in grasslands soil (Jirout et al. 2012) to produce both nitrite and nitrate and nitrite respectively. Their results, however, showed that no nitrification was observed in media containing ammonium sulphate. Hirsch et al. (1961) on the other hand, did show that *Aspergillus* was able to form NO_3^-, not but not nitrite (NO_2^-) from incubations with ammonium phosphate; therefore, indicating that fungi were able to oxidize ammonium to nitrate. Studies have confirmed fungal ammonium oxidation in different soils. Stroo et al. (1989) demonstrated the ability of fungi to oxidize ammonium to nitrate, using an antibiotic protein synthesis inhibitor streptomycin and a eukaryotic protein synthesis inhibitor, cycloheximide, in an acid forest soil in different pH ranges (3.2 – 6.1), with maximum NO_3^- formation occurring at a pH range of 4-5. Laughlin et al. (2008) clearly demonstrated an effect of cycloheximide on reducing NH_4^+ oxidation and NO_3^- production in a grassland soil (pH = 6.1). Fungi have been found to increase in numbers, alongside denitrifying bacteria in compacted soils in corn fields within the top layer of the soil (0 – 10 cm) (Pupin et al. 2009). Shestak and Busse. (2005)

observed increased fungal PLFA biomass in soils following compaction. Therefore, soil compaction in grasslands may increase the biomass of soil fungi that could potentially contribute to soil ammonium oxidation.

Using the method described by Laughlin et al. (2008) the impact of DCD of grassland nitrifying communities was studied using both the antibiotic streptomycin, a well-known bacterial protein synthesis inhibitor, and cycloheximide, a well-known eukaryotic protein synthesis inhibitor in a preliminary style experiment. The use of this inhibition method will help to distinguish the dominant nitrifying soil microbial community in the soil used in this study and the effectiveness of DCD on reducing soil nitrification potential.

AIM

The aim of this experiment was to discern whether streptomycin and cycloheximide could be used to regulate microbial activity and therefore provide information to what extent autotrophic and heterotrophic microorganisms contribute to nitrification in a grassland soil that has received different compaction treatments and to assess the effect of DCD on non-target ammonia oxidizing microbial groups.

HYPOTHESIS

(1). Heterotrophic nitrification dominates ammonium oxidation in grassland soil

(2). DCD does not reduce nitrate production due to heterotrophic ammonia oxidation

3.2. MATERIALS & METHODS

3.2.1 Soil sample collection

Soils were collected from the runover area for each compaction treatment plot; this is an area that received the same compaction treatment as the rest of the compaction plot in the field as shown below in Fig. 3.1. Soils were classified as described in chapter 2.

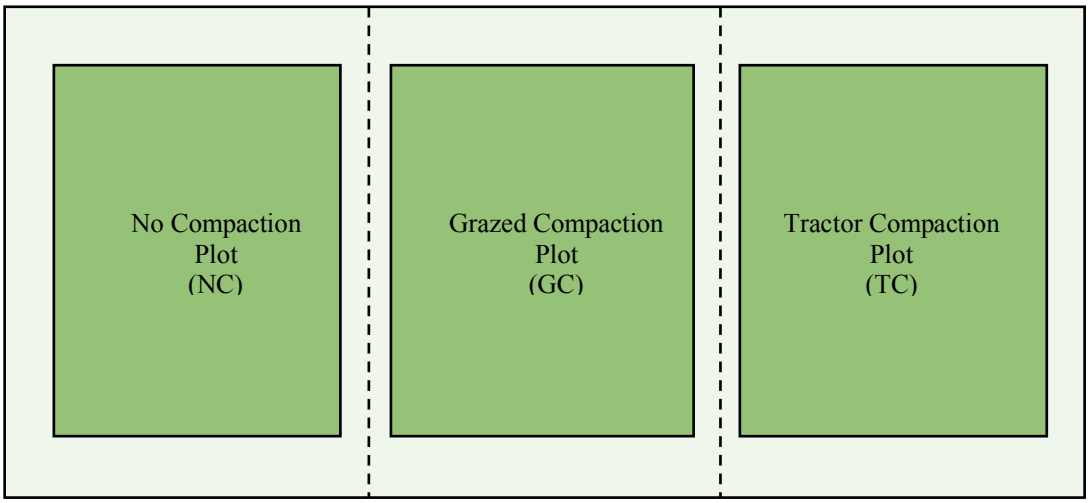


Figure. 3.1. Outline of field compaction plot at SRUC Crichton grassland compaction experiment. Light green area highlights 'runoff' section where samples were collected. Cattle grazing 'runoff' and Tractor 'runoff' areas are highlighted by the dashed lines.

3.2.2 Soil sample treatments

Soils from Non-Compacted (NC), Grazed Compacted (GC), and Tractor Compacted (TC) field plots were sieved to < 5 mm and air-dried to 15% MC. 15 (g) of each plot soil sample was placed into 162 (3 Compaction treatments x 3 replications x 3 inhibitor treatments x 2 DCD treatments x 3 sampling points (0, 3 and 5 Days)

sterile 250 ml plastic containers, covered with parafilm and allowed to incubate at 20°C for 24 hours. In order to apply solutions to equal 60% WFPS soil height was measured in the 250 mL containers and a bulk density value was calculated. Bulk densities of 0.84 g cm^3 (NC), 0.79 g cm^3 (GC) and 0.75 g cm^3 (TC) were calculated in the pots by measuring the height of the soil in the container and calculating the bulk density by using the diameter of the containers. This was performed in order to assess the amount of solution that needed to be applied to bring samples to 60% WFPS.

Adapting a method by Laughlin et al. (2008), after the 24-hour period soils were inhibited with antibacterial, streptomycin sulphate (Sigma Aldrich, UK) and antifungal, cycloheximide (Sigma Aldrich, UK) inhibitors were applied in 2 mL solutions equalling concentrations of 4 mg g^{-1} dry soil; control soils were applied with the equivalent volume of de-ionised water only. Samples were covered with parafilm and left to incubate again at 20°C for 24 hours in order for active populations to become inhibited. After 24 hours 100 µM of N of ammonium sulphate ($(NH_4)_2SO_4$) and of 100 µM of C in the form of sodium acetate ($C_2H_3NaO_2$) was applied to the soil to provide a source of carbon that would be found in grassland soils either from deposition from cattle slurry or manure (Laughlin et al. 2009) and was used in the similar study by Laughlin et al. (2008). Sodium acetate was used to represent volatile fatty acid (VFA) concentrations of C found present in slurry (Paul et al. 1989). A 250x dilution of an N inhibitor which has the active nitrification inhibitor component dicyandiamide (DCD) was added to half of the samples at a rate of 8.8 µg g^{-1} dry soil.

Both nutrient solutions and DCD were applied to the soil at combined volume of 2 ml; de-ionised water was applied instead of DCD in the case of non-DCD treated samples. De-ionised water was used to bring samples up to 60% WFPS where necessary. Containers were then re-covered with parafilm and left to incubate. Samples

were extracted for mineral N at 0, 72, and 96 hours. 5 (g) subsamples from control treatments for each compaction treatment were taken and stored at -80ºC for further analysis.

3.2.3 Soil mineral N extraction and analysis

As described in Chapter 2.

3.2.4 Statistical analysis

Genstat 17th Edition was used to perform a general linear model using factorial design. Antibitoic, DCD, Compaction and Day were used as factors. This design was used to distinguish the effects of antibiotics, DCD and compaction treatments on ammonium and nitrate concentrations.

3.3. RESULTS

3.3.1 Ammonium concentrations

NH_4^+ concentrations were significantly altered by interactions between Inhibitor x DCD x Compaction x Day (Table 3.1, $P < 0.001$). It was easily observed that both streptomycin and cycloheximide significantly reduced the rate of ammonium reduction in the each of the compacted soils in comparison to respective control soils (Fig. 3.2 (a), (b) and (c), $P \leq 0.05$). The effect of antibiotic on ammonium concentrations was dependent on the different compaction treatments (Table 3.1; Antibiotic x Compaction, $P = 0.003$; Fig. 3.2). Non-compacted (NC) soils demonstrated an increase in NH_4^+ concentrations when compared to Day 0 for cycloheximide treated soils (Fig. 3.2 (a), $P \leq 0.05$). The opposite was observed for STM treated soils, with significant reductions in NH_4^+ concentrations on Days 3 and 5 (Fig. 3.2 (a), $P \leq 0.05$). Grazed compacted (GC) soils displayed a similar effect with NH_4^+ concentrations increasing on Days 3 and 5 (Fig. 3.2 (a), $P \leq 0.05$) when compared to Day 0 for cycloheximide treated soils. A similar effect was observed for streptomycin treated samples, however, NH_4^+ reduced at a slower rate than observed for streptomycin treated NC samples; STM treated samples for GC samples only became significantly different on Day 5 when compared to Day 0 for STM treated samples. The main variation between samples occurred in Tractor Compacted (TC) soils (Fig. 3.2 (c)). Although the effects of both streptomycin and cycloheximide demonstrated the same apparent inhibitory effect in reducing the rate of reduction in NH_4^+ concentrations, there were observed decreases in cycloheximide treated samples which were not apparent for both NC and GC soils when compared to cycloheximide treated soils NH_4^+ concentrations. STM treated soils displayed declines in NH_4^+

concentrations, however, the rates of NH_4^+ decline were higher in the TC than observed in both NC and GC soils, with NC and GC soils demonstrating 20 and 21 % declines in NH_4^+ respectively and the TC soil demonstrating a 63% reduction in NH_4^+ concentrations by Day 5 of the experiment.

DCD treatment significantly increased NH_4^+ concentrations in all soil compaction types for all antibiotic treatments (Fig. 3.2 (a), (b) and (c)). The effect of both streptomycin and cycloheximide significantly altered when samples were treated with DCD (Table. 3.1; Antibiotic x DCD, $P < 0.001$) when compared to non-DCD treated with both streptomycin and cycloheximide treatments either significantly ($P \leq 0.05$) increasing NH_4^+ concentrations or demonstrating no significant changes when compared to Day 0 concentrations. However, this effect was subject to compaction treatment (Table 3.1; Antibiotic x DCD x Compaction, $P < 0.005$). Although no significant change was observed in both NC and GC soils (Fig. 3.2 (a) and (b)), TC soils demonstrated decreased NH_4^+ concentrations when compared to Day 0 in both control + DCD and streptomycin + DCD treated samples, soils treated with cycloheximide offered no significant decreases in NH_4^+ when compared with Day 0 concentrations (Fig. 3.2 (c)). The effect of compaction treatment was also observed to change the impact of DCD on NH_4^+ concentrations as it was generally observed that NH_4^+ concentrations did not decrease significantly when DCD was applied to the soil (Fig. 3.2 (a) and (b)). However, in the TC soils, even with the application of DCD, NH_4^+ concentrations decreased significantly (Table 3.1 DCD x Compaction x Day, $P < 0.001$; Fig. 3.2 (c), $P \leq 0.05$). This effect was however dependant on the antibiotic treatment, as cycloheximide treated soils did not demonstrate any significant decreases in soil NH_4^+.

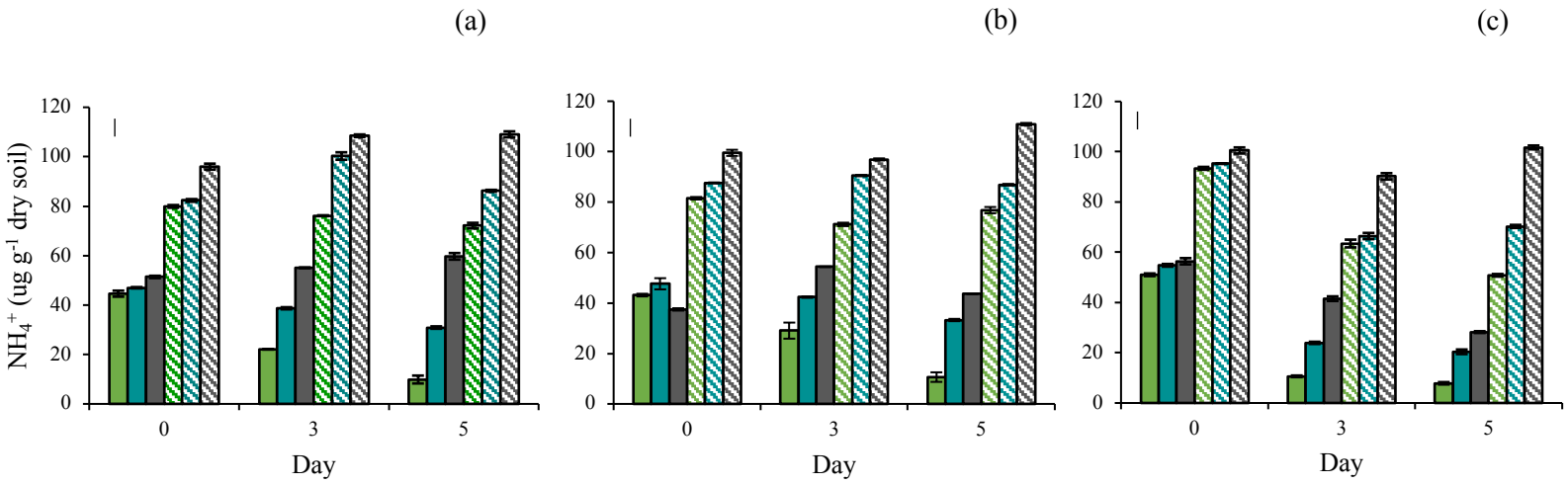


Figure 3.2. NH_4^+ conecntrations for non compacted (NC) (a), Grazed compacted (GC) (b) and Tractor compacted (TC) (c) soils. Green bars = control soils, Teal bars = Streptomycin treated soils, Grey bars = Cycloheximide treated soils, Green dashed bars = Control soils + DCD, Teal dashed bars = Streptomycin treated soils + DCD and Grey dashed bars = Cycloheximide treated soils. Error bars represent standard error of sample means (± SEM). LSD ($P \leq 0.05$) bars are locate at the top left of each graph and represent the LSD between all sample treatments for all graphs.

Table 3.1 ANOVA table for effect of Antibiotic, DCD and Compaction treatments on NH_4^+ concentrations during the experiment.

Source		NH_4^+	
	df	*F*	*P*
Antibiotic	2	424.01	**< 0.001**
DCD	1	5499.28	**< 0.001**
Compaction	2	62.61	**< 0.001**
Day	2	137.63	**< 0.001**
Antibiotic x DCD	2	12.70	**< 0.001**
Antibiotic x Compaction	4	4.31	**0.003**
DCD x Compaction	2	0.84	0.433
Inhibitor x Day	4	55.83	**< 0.001**
DCD x Day	2	43.21	**< 0.001**
Compaction x Day	4	68.98	**< 0.001**
Antibiotic x DCD x Compaction	4	4.00	**0.005**
Antibiotic x DCD x Day	4	3.07	**0.019**
Antibiotic x Compaction x Day	4	1.07	0.393
DCD x Compaction x Day	8	6.44	**< 0.001**
Antibiotic x DCD x Compaction x Day	4	7.22	**< 0.001**
Total	156		

3.3.2 Nitrate concentrations

Nitrate concentrations changed significantly with the interaction between Antibiotic x DCD x Compaction x Day (Table 3.2, $P < 0.001$). Soil nitrate concentrations significantly decreased as a result of both streptomycin and cycloheximide additions for the non- DCD treated soils (Fig. 3.3 (a), (b) and (c), $P \leq 0.05$). The effect of antibiotic varied significantly during the experiment between the different compaction treatment soils. NO_3^- concentrations in the Streptomycin treated soils were significantly lower than the control NO_3^- concentrations for both the NC and GC soils (Fig. 3.3 (a) and (b), $P \leq 0.05$), with 20% and 15 % (NC) and 8% and 15% (GC) reductions on Days 3 and 5 respectively. TC soils showed no significant decrease in soil NO_3^- concentrations when treated with streptomycin, with NO_3^- concentrations actually demonstrating increases in soil NO_3^- on Day 5 ($P \leq 0.05$).

Cycloheximide significantly reduced soil NO_3^- concentrations for all the compaction treatments in all days compared to the control treatment (Fig. 3.3 (a), (b) and (c); $P \leq 0.05$). NC soils treated with cycloheximide lowered increases NO_3^- concentrations by 39% and 41 % on Days 3 and 5 respectively when compared to the control soils (Fig. 3.3 (a), $P \leq 0.05$). GC soils treated with cycloheximide were also observed to significantly decrease NO_3^- concentrations by 22% on Day 3 and by 28% on Day 5 compared to the control. Cycloheximide treatment in TC soils significantly reduced NO_3^- concentrations by 28% (Fig. 3.3 (c), $P \leq 0.05$) on Day 3, however, by Day 5 no differences in NO_3^- concentrations were overserved between cycloheximide and the control treatment (Fig. 3.3 (c)).

DCD treatment significantly reduced NO_3^- concentrations for both NC and GC soils (Fig. 3.3. (a) and (b), $P \leq 0.05$) in comparison to the non-DCD treated soil (Table

3.2; DCD, DCD = $P < 0.001$). The effect of antibiotic treatment was significantly altered by DCD during the course of the experiment (Table 3.2; Antibiotic x DCD x Day = $P < 0.001$). Not only did DCD treatment reduce the amount of NO_3^- produced in all soils, but the DCD treated soils reduced any significant difference between the control and streptomycin treated soils for both NC and GC that was observed in the non-DCD treated soils (Fig. 3.3 (a) and (b)), with similar concentrations of NO_3^- in the control and streptomycin treatments. Cycloheximide reduced NO_3^- in all soils from the various field compaction treatments on Days 3 and 5 in comparison to both control and streptomycin treatments (NC: Day 3 = $P \leq 0.05$, Day 5 = $P \leq 0.05$ (Fig. 3. 3 (a)); GC: Day 3 = $P \leq 0.05$, Day 5 = $P \leq 0.05$ (Fig. 3.3 (b)); TC: Day 3 = $P \leq 0.05$ (Fig. 3.3 (c)). TC soils demonstrated the largest increases in NO_3^- concentrations for the DCD treatment in both control and streptomycin treated soils compared to Day 0. Cycloheximide significantly reduced increases in NO_3^- with no significant changes observed for Day 3 with NO_3^- concentrations 43% less than control soils. By Day 5 the cycloheximide treated soils had increased in comparison to both Day 0 and Day 3, however, in relation to the control treatment on Day 5, cycloheximide treated soils still had 43% NO_3^- in the soil (Fig. 3.3, $P \leq 0.05$).

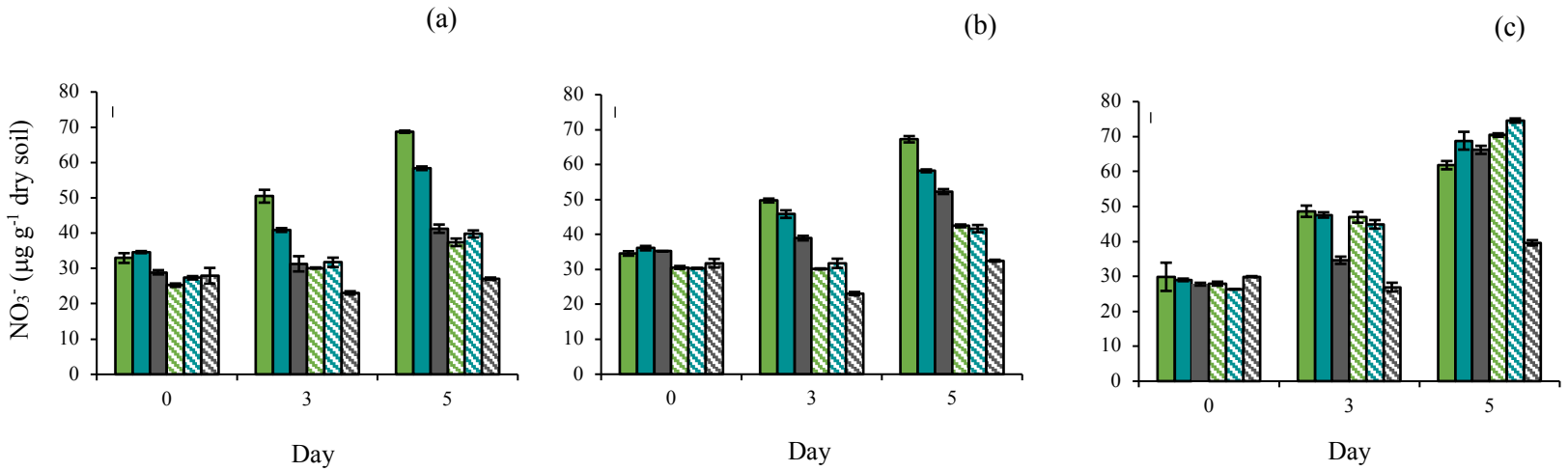


Figure 3.3. NO_3^- conecntrations for non compacted (NC) (a), Grazed compacted (GC) (b) and Tractor compacted (TC) (c) soils. Green bars = control soils, Teal bars = Streptomycin treated soils, Grey bars = Cycloheximide treated soils, Green dashed bars = Control soils + DCD, Teal dashed bars = Streptomycin treated soils + DCD and Grey dashed bars = Cycloheximide treated soils. Error bars represent standard error of sample means (± SEM). LSD ($P \leq 0.05$) bars are locate at the top left of each graph and represent the LSD between all sample treatments for all graphs

Table 3.2 ANOVA table for effect of Antibiotic, DCD and Compaction treatments on NO_3^- concentrations during the experiment.

Source		NO_3^-	
	df	*F*	*P*
Antibiotic	2	201.05	**< 0.001**
DCD	1	391.34	**< 0.001**
Compaction	2	127.85	**< 0.001**
Day	2	1784.18	**< 0.001**
Antibiotic x DCD	2	46.60	**< 0.001**
Antibiotic x Compaction	4	29.69	**< 0.001**
DCD x Compaction	2	30.79	**< 0.001**
Antibiotic x Day	4	85.98	**< 0.001**
DCD x Day	2	100.59	**< 0.001**
Compaction x Day	4	126.92	**< 0.001**
Antibiotic x DCD x Compaction	4	10.55	**<0.001**
Antibiotic x DCD x Day	4	61.46	**< 0.001**
Antibiotic x Compaction x Day	4	11.32	**< 0.001**
DCD x Compaction x Day	8	13.40	**< 0.001**
Antibiotic x DCD x Compaction x Day	4	34.97	**< 0.001**
Total	156		

3.4. DISCUSSION

3.4.1 The effect of antibiotics on soil NH_4^+oxidation and NO_3^- production

The application of both streptomycin and cycloheximide significantly reduced soil NH_4^+ oxidation. Cycloheximide completely inhibited NH_4^+ oxidation for NC and GC soils (Fig.3.2 (a) and (b)) when compared to Day 0 samples, with ammonium concentrations increased during the incubation, and significantly reducing the rate at which NH_4^+ was oxidized in the TC soil (Fig. 3.2 (c)). A similar pattern evolved for NO_3^- concentrations. The results in this study, with regard to the inhibition of NH_4^+ oxidation and NO_3^- production by cycloheximide, have great similarity to those by obtained Laughlin et al. (2008) of which the study was based around. Laughlin et al. (2008) observed as significant reductions of both NH_4^+ oxidation and NO_3^- production in unlabelled and labelled fractions of NH_4^+ and NO_3^- in a grassland soil treated with cycloheximide. Cycloheximide has been shown to have no negative effects on soil bacterial communities and more specifically types of ammonia oxidizing bacteria that are found in soils, i.e. *Nitrosomonas europaea* (Ingham and Coleman. 1984; Vajrala et al. 2014). The application of cycloheximide should therefore not have negatively impacted on NH_4^+ oxidation rates in the soils studied if autotrophic nitrification was the sole process that was occurring. This would indicate that the dominant group that was utilising NH_4^+ were heterotrophic in nature. Streptomycin treatment also reduced the rate at which NH_4^+ was oxidized in the soil in the current study. As cycloheximide completely inhibited ammonium oxidation in the soils for both NC and GC soils, the partial inhibition by streptomycin would appear contradictory. As a preliminary study was not conducted to determine a maximum inhibitory concentration, it is acknowledged that streptomycin may not have fully inhibited soil AOB, however, as

cycloheximide completely reduced NH_4^+ oxidation in two of the three soils it could is strongly suggested that the dominant nitrifying communities in the soil were heterotopic in nature. Significant increases ($P \leq 0.05$) in soil NH_4^+ were observed in the cycloheximide treated soils (Fig. 3.2 (a) and (b)), this was due to lysis of microbial cells that would have released amino acids, sugars and peptides (Badalucco et al. 2014). A study by Ingham and Coleman (1984) demonstrated the effects of both streptomycin and cycloheximide on target and non-target microorganisms in the soil. Their results showed that streptomycin at a concentration of 3 mg g^{-1} soil had a non-target effect on fungi, reducing the activity of the fungal population in the soil by 25%. The reason that the streptomycin treated soils also displayed an apparent reduction in NH_4^+ oxidation, which were calculated to be between 20 and 21% for the NC and GC soils, a similar percentage to the observed reductions in fungal activity in the study by Ingham and Coleman (1984) could suggest that streptomycin reduced the activity of the fungal population in the soil; this may explain how cycloheximide completely inhibited NH_4^+ oxidation and streptomycin still reduced NH_4^+ oxidation in the soil. Variations in NH_4^+ concentrations were observed between soils sampled from different compaction plots treated with cycloheximide (Fig. 3.2). Both GC and TC + No DCD treated soils demonstrated significantly lower NH_4^+ concentrations by Day 5, but more pronounced in the TC soils (Fig. 3.2 (b) and (c); $P \leq 0.05$) in comparison to the NC + No DCD treated soils, which only demonstrated significantly increased NH_4^+ concentrations (Fig. 3.2 (a); $P \leq 0.05$). A similar pattern was observed for NO_3^- concentrations (Fig. 3.3 (a), (b) and (c)), with significantly higher NO_3^- concentrations in both the GC and TC soils in comparison to the NC soil. However, these differences between the GC and TC soils compared with the NC soil could have been as a result of a higher abundance of fungi in the section of the field where samples were taken

from the TC plot, as it has been demonstrated that microbial biomass N increased with increased compaction (Breland and Hansen. 1996). The increased microbial biomass N in the study by Breland and Hansen (1996) was suggested be due to the changes in pore size distribution, which has been observed to significantly decreases the abundance of pore sizes > 30 μm and between 30 and 0.2 μm, but significantly increase soil pore sizes of < 0.2 μm (Shestak and Busse. 2005), making regions of the soil inaccessible to nematodes that would predate on soil bacteria and fungi (> 30 μm) and that the pores would only be available to bacteria and fungi (≤ 3 μm) were increased by 12.7 – 15.6%. This could potentially explain the observed increased NH_4^+ oxidation in antibiotic treated soils in comparison to the NC soil, as both GC (Day 5; $P \leq 0.05$, cycloheximide treatment) and TC (Days 3 and 5; $P \leq 0.05$, cycloheximide and streptomycin treatment) demonstrated decreases in soil NH_4^+ oxidation, which would have seen a decrease in soil pore size distribution as a result of compaction by either cattle traffic or tractor compaction. In addition, Breland and Hansen. (1996) suggested that soil organic matter was increased due to increased physical protection in the soil as a result of compaction due to decreased accessibility to soil microbes (Van Veen and Kuikman. 1990). The subsequent liberation of organic material from the soil can therefore be subjected to increased bursts of microbial activity (Van Veen and Kuikman. 1990). As the TC was observed to be less inhibited by either streptomycin or cycloheximide than both NC and GC soils, the fact that soils were sampled and subsequently sieved from a tractor compacted plot could suggest that both an increased abundance of soil fungi, and or, heterotrophic bacteria in combination with the subsequent bursts of activity that followed soil sample collect and soil sieving which would have liberated organic matter from the soil. This is, however, only a theoretical

pathway as no measurements of bulk density or pore size distribution were taken from the soils at the time of sampling that would shed any further light on this mechanism.

DCD significantly increased NH_4^+ concentrations in all sample treatments. DCD is known to be used a slow release fertiliser (Amberger. 1989), therefore the addition of DCD was clearly the source of increased NH_4^+ concentrations in this study (Fig. 3.2). The use of the nitrification inhibitor DCD, which is known to deactivate the activities of the ammonia monooxygenase enzyme in ammonia oxidizing bacteria (AOB) (Amberger. 1989). The fact that NH_4^+ oxidation (Fig. 3.2 (a), (b) and (c); Day 5, $P \leq 0.05$), and NO_3^- (Fig. 3.3 (a) and (b); Day 5, $P \leq 0.05$) was still observed to proceed in the DCD the treated soils for soils in the control treatment, and between Days 3 and 5 for the streptomycin treatment, whereas soils receiving cycloheximide demonstrated either no change or increased NH_4^+ would further suggest that heterotrophic ammonium oxidation was taking place, especially as both NH_4^+ oxidation and NO_3^- production was significantly inhibited by cycloheximide. The study by Laughlin et al. (2008) also observed the inhibition of the oxidation of ammonium and of inorganic N to NO_3^- by cycloheximide, suggesting that fungi can oxidize NH_4^+ and organic N simultaneously, indicating that fungi may be able to simultaneously oxidize both inorganic ammonium and organic N to produce NO_3^- in grassland soils. In this regard, it could perhaps have been possible that DCD, which is an organic N compound (Amberger. 1989) was being metabolised by the active fungal population in the soil as NH_4^+ concentrations, which were observed to increase with DCD application in the control soils, subsequent started to significantly ($P \leq 0.05$) decline by Day 5 followed by increased NO_3^- concentrations, which were both inhibited by cycloheximide (Fig. 3.2 and 3.3).

3.4.2 Alternate routes of NH_4^+ Oxidation

Soil archaea have recently been shown to be inhibited by cycloheximide (Vajrala et al. 2014). The study by Vajrala et al. (2014) demonstrated that CHX was capable of preventing the incorporation of $^{14}CO_2$ into cellular proteins of *Nitrosopumilus maritimus*; an ammonia oxidizing archaeon, which prevented protein synthesis in archaea. This would have prevented the incorporation of NH_3 into the cells of *N. maritimus* indicating that cycloheximide significantly altered the ability of *N. maritimus* to oxidize NH_3 once cellular turnover was inhibited. It is therefore possible that the inhibitory affect by cycloheximide may have been due to an unintentional inhibition of soil AOA.

3.5 Notes on Current Experiment

One of the aims for this experiment was to perform gene abundance analysis on AOB, AOA and Fungal ITS genes. Samples from control streptomycin and cycloheximide treated soils would have been used to correlate gene abundance with NH_4^+ reduction and NO_3^- production; unfortunately, this could not be carried out. As understanding that the pathway of ammonium oxidation in fungi is not linked to growth, as it is in AOB (Marshall and Alexander. 1961). As β-*N*-acetylglucosaminidase activity has been demonstrated to be a useful indicator of fungal biomass in soil, although it is also present in a limited number of bacteria that utilise chitin (Miller et al. 1998), which would rule out AOB. However, a recent study by Xue et al. (2016) found significant correlations between β-*N*-acetylglucosaminidase and AOA communities containing *Nitrosophaera* sp. And *Nitrososta*lea sp. in the soil, however there was no correlation between AOB

communities containing *Nitrosomonas* spp and *Nitrosospira* spp. β-*N*-acetylglucosaminidase. Therefore, the use of this enzyme assay method may perhaps provide a simple, quick and useful indicator of the activity of soil fungal/AOA activity in comparison to AOB ammonium oxidation in grassland soils, which be a useful counterpart to the cycloheximide inhibition method. A study of the expression of both AOB and AOA AMO genes in the soil would provide a better estimate of the activity rate in antibiotic treated soils. This would provide not only an improved understanding of bacterial, archaeal and fungal ammonium oxidation, but also the efficacy of the antibiotic inhibition method. A further suggested improvement to the antibiotic inhibition method to help ensure sufficient application of cycloheximide and streptomycin would be the use of sample cores larger in diameter, but only 25 – 50 mm in height placed on water tension tables, such as the soil cores described in Chapter 4. This would allow a greater application rate, in terms of antibiotic solution, that would pass through all available pores in the soil sample, whilst the samples would be maintained at the desired %WFPS's. This would also allow the study of the effects of compaction that are found in grassland soils on both autotrophic and heterotrophic nitrification in soil.

Earlier studies have demonstrated that the pathway of ammonium oxidation in the fungus *Aspergillus* flavus is not linked to growth as it is in AOB (Marshall and Alexander. 1961), and that heterotrophic nitrification in *A.flavus* obtained energy for growth from an organic substrate (Van Gool and Schmidt. 1972); moreover, it was proposed that the role of nitrification in fungi is related to energy maintenance of the resting mycelium (Van Gool and Schmidt. (1972). As Laughlin et al. (2008) observed the simultaneous oxidation of both ammonium and organic N in a grassland soil, a study elucidating the relationship between ammonium oxidation and organic N

utilisation in fungi in soil would seem to be a logical step that could provide information for the reason for fungi to oxidize NH_4^+. This could either be achieved through ^{15}N labelling of both NH_4^+ and organic N, perhaps in combination with acetylene inhibition which would inhibit both soil AOB and AOA communities. As DCD treated soils initially increased soil NH_4^+concentrations (Fig. 3.2 (a), (b) and (c), $P \leq 0.05$; Table 3.1, $P < 0.001$) which was then subsequently reduced, this could potentially indicate that DCD was being metabolised in the soil by microorganisms as has been suggested by other studies (Kelliher et al. 2008)

3.6. CONCLUSION

This study demonstrated that cycloheximide application to soil significantly reduced NH_4^+ oxidation and that soil NO_3^- concentrations demonstrated little or no increase during the incubation as a result of cycloheximide treatment. Although there were reductions in NH_4^+ oxidation with the streptomycin treatment, the fact that cycloheximide completely inhibited NH_4^+ would indicate that the effect of streptomycin may have been as a result of an artefact of the experimental design in relation to the concentration of streptomycin that was applied. This would suggest further that cycloheximide inhibited the dominant nitrifying microbial communities in the soil and that understanding of the nature of cycloheximide these microbes were of a fungal origin. The slight but significant variation in NH_4^+oxidation between soils sampled from different compaction treatments indicated that soil compaction may have a significant impact on the activity of these heterotrophic soil NH_4^+ oxidizers, as compaction has been observed to increase fungal biomass in other studies and due to the increased protection of soil organic matter, may stimulate fungal ammonium oxidation due to lack of available organic N that would be the prominent source of N

for these heterotrophic microorganisms. As nitrification inhibitor such as DCD used in this study are commonly used to inhibit active bacterial nitrifying populations in the soil, the indication that heterotrophic ammonium oxidation and nitrate production in a grassland soil can still proceed after the application of DCD is of great importance in understanding the mechanisms that contribute to nitrification and subsequent N_2O emissions from grassland soils.

CHAPTER 4. UNDERSTANDING THE EFFICACY OF DCD ON NITRIFICATION ACTIVITY IN A GRASSLAND SOIL IN RESPONSE TO VARYING N INPUTS: MICROBIAL DIFFERENTIATION USING A SELECTIVE INHIBITOR TECHNIQUE

4.1. INTRODUCTION

The use of the nitrification inhibitor, dicyandiamide (DCD), has been used in the mitigation of grassland N_2O emissions, however, although DCD has been reported to reduced N_2O between 50-80%, emissions from grassland, especially after nitrogen, inputs therefore leaving a wide margin of uncertainty for its effectiveness. Di and Cameron (2002) demonstrated that the use of DCD reduced N_2O emissions by 82% following urine applications. Di and Cameron (2003), again demonstrated that DCD reduced N_2O emissions by 78%. Further work by Di and Cameron (2008) demonstrated that N_2O was reduced again by 72%, however, the emissions of N_2O attributed to nitrifiers indicated that the application of DCD enhanced production of nitrifier N_2O in soils treated with urea (25 kg urea-N ha^{-1}) and urea (25 kg urea-N ha^{-1}) + urine (1000 kg urea-N ha^{-1}). DCD applications to slurry fertiliser treatments have demonstrated lower reductions in N_2O emissions when Merino et al. (2001) demonstrated that 56% more N_2O was emitted in slurry fertiliser treatments when compared to the slurry treatment without DCD. It was shown that DCD reduced slurry N_2O emissions from denitrifiers (78.7% of total N_2O) more than from nitrifiers (36.5% reduction in N_2O) when compared with total N_2O fluxes (Merino et al. (2001). Vallejo et al. (2005) also demonstrated similar reductions in N_2O emissions from soils receiving pig slurry, with reductions of 46% of total N_2O emitted attributable to DCD

treated soils. These studies indicate that the nitrogen source applied to the soil produces contrasting results in relation to both N_2O emissions and the effects of DCD.

Recent years have shown an increased understanding of soil ammonium oxidizing archaea AOA. Although possessing an AMO gene homologous to that of ammonia oxidizing bacteria (AOB), it has been demonstrated that DCD does not have any effect on reducing the size of this soil nitrifying microbial community (Podolyan et al. 2014). Further research has demonstrated that DCD has reduced the growth of *Thaumarchaeal* AOA belonging to the group 1.1a associated and 1.1b., in strongly acid soils (pH < 4.5) at an application rate of 50 mg kg^{-1} (Zhang et al. 2012), thereby indicating that DCD has the ability to reduce the growth of AOA in soil. As discussed briefly in Chapter 3, a study by Vajrala et al. (2014) demonstrated that the application of cycloheximide to a culture of *Nitrosopumilus maritimus* reduced the incorporation of $^{14}CO_2$-C into cellular proteins. Cyloheximide has the potential to be used as a way to estimate AOA contribution to soil nitrification rates. Cycloheximide is a eukarytotic protein synthesis inhibitor and has been commonly used to inhibit fungi in studies aiming differentiating ammonium reduction and nitrate production in both forest and grassland soils between bacteria and fungi (Stroo et al. 1986; Castaldi and Smith. (1998); Laughlin and Stevens. (2002); Chapter 3). Taken together with the recent research by Vajrala et al. (2014), the impact of cycloheximide in the soil microbial community may therefore be more widespread than currently assumed.

The main aim of the work in this chapter by using a standard enzyme assay (Kandeler. 1996), was to assess the potential nitrification efficacy of DCD on soil nitrifiers commonly found after grassland N inputs of urea, slurry and bovine urine. A secondary aim of the study was to evaluate the effect of cycloheximide on soil potential

nitrification enzyme activity, and thereby indicate if there is any influence on soil ammonia oxidation potential.

Aims and Hypothesis

The aims of this study were to;

(1) Determine the efficacy of dicyandiamide on soil nitrification potential in soil receiving different sources of N typically found in grassland soils.
(2) Assess the influence of cycloheximide on soil potential nitrification for an improved understanding of its effects on grassland soil ammonium oxidizers

Therefore, the following chapter will assess two hypothesis's

'DCD efficacy is relative to the type of nitrogen inputs commonly found in a grassland soil'

'Cycloheximide does not reduce potential nitrification enzyme assay activity'

4.2. MATERIALS & METHODS

4.2.1. Soil Microcosm Preparation

Soil was sampled as described in chapter 2. Twenty (20) g, dry weight equivalent, soil microcosms were prepared by hand compacting into 100 (H) mm x 25 mm (D) PVC cylinders, to a height of 50 mm in two 25 (H) mm layers to a bulk density of 1.1 g cm^3 at 32% moisture content. As described previously in chapter 2, samples were compacted into two layers, but by hand for current experiment due to the smaller size of the samples. The first 25 mm layer was compacted into the bottom of the 100 mm PVC cylinder. The top of the layer was then disturbed using a spatula, in order to minimize any layering effect between the two 25 mm layers. The second 25 mm layer was added and compacted to the determined 50 mm level marked on the tubes. Sand tension tables were employed to create a matric potential in the soil equivalent to 6 kPa. A preliminary experiment identified that samples at a bulk density of 1.1 g cm^3 at 6 kPa (60 cm H_2O) maintained a WFPS of 60%. 250 μm nylon mesh was attached to the bottom of all samples to reduce any loss of soil that could occur. Samples were then placed onto sand tension tables and left to equilibrate to 60% WFPS for 4 days before N treatments were added.

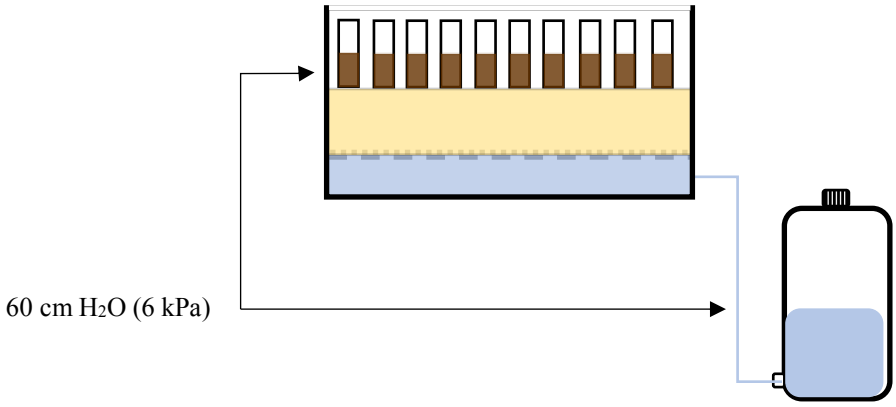


Figure 4.1. Diagram of sand tension table for maintaing moisture content in soils during the experiment. As indicated by the arrows a 60 cm distance from the centre of the container to the centre of the soil samples equals to -6 kPa

4.2.2 Collection and storage of cattle urine and cattle slurry

Urine was collected from Frisian Dairy cows and slurry from slurry storage tanks at Crichton dairy farm in Dumfries, Scotland. Samples were stored in 1 L containers and frozen immediately. Frozen samples of both urine and slurry were broken down into smaller quantities, homogenised and sub samples analysed for total soluble carbon (TC) dissolved inorganic (IC) and (DOC) organic carbon, total soluble nitrogen, as well as NH_4^+. The remaining frozen urine and slurry was kept at -20^{o}C for further use. As slurry was applied in liquid form, once the slurry had been defrosted it was hand compressed in order to remove any heavy solid material, i.e. hay, so that the liquid fraction could be applied to respective samples. Nitrogen and Carbon concentrations for both slurry and urine are shown in Appendix; Table. 7.1.

It is noted that by removing the heavy organic fraction, i.e. hay, does not fully represent field application, however, the main focus in the case of slurry was the more soluble fraction of the slurry, and given that the experiment is run only over a short

period (8 Days), it is assumed that sufficient degradation of the more solid material would not have occurred over this period to apply further nutrients to the soil.

4.2.3 Fertiliser treatments

All nitrogen sources were applied at a rate equivalent to field application or field deposition rates. Urea was applied at a rate of 60 kg ha^{-1} urea-N (DEFRA, RB209 Handbook) urine deposition has been measured to equate to 5 L m^{-2} equivalent (Misslebrook et al. 2014). The guideline for slurry is 50 m^3 ha^{-1} equivalent (DEFRA, RB209, Handbook). Using these application volumes as a guideline the application of N treatments is as follows;

$$\text{Radius of universal tube } \pi \cdot 1 \text{ cm}^2 = 3.14 \text{ cm}^2$$

$$\equiv 0.000314 \text{ m}^2$$

$$\equiv 0.0000000314 \text{ hectares}$$

As an application rate for urine and Slurry were the same, 50 m^3 ha^{-1} is roughly equal to a volume of 5 L m^{-2} which is equivalent to 0.5 mL cm^{-2}, the following calculation was used to determine the concentrations, in liquid form, should be applied to each soil sample surface inside the universal tubes;

$$5 \text{ L m}^{-2} \equiv 0.5 \text{ mL cm}^{-2}$$

Therefore;

$$0.5 \text{ mL} \cdot 3.14 = 1.57 \text{ mL}$$

As the concentrations being applied were of such small quantity, to ensure sufficient application of N, application volumes were rounded up to 2 mL. This increased the concentration of urine and slurry fertiliser by 21.5%, thereby increasing the field equivalent application rate to 60.75 m^3 ha^{-1} for slurry application and 6.075 L m^{-2} equivalent for urine application. As slurry was applied by volume, rather than weight, 1 mL of slurry was weighed on a weighing balance to determine the weight equivalent of liquid slurry being applied. It was found that 1 mL of slurry was equivalent to 1 (g).

As both urine and slurry were applied at volumes of 2 mL per microcosm, for comparison, both the urea and no nitrogen treatment (de-ionised water control) treatments were also applied at volumes of 2 mL. Urea was applied at the field equivalent rate of 60-N kg ha^{-1} (DEFRA, RB209 Handbook), therefore the as the volume of solution being applied was 2 mL, the following calculation details the actual weighted concentrations of urea used for the experiment;

$$128 \text{ kg of Urea (60 kg urea- N ha}^{-1} \text{ equivalent)} = 128 \cdot 0.00000003141593$$

$$= 4 \text{ mg urea-N per soil column}$$

As urea solution had to be applied at a volume of 2 mL, the concentration of granular urea was halved; therefore 2 (g) of urea were added to 1 litre of de-ionised H_2O in order to achieve a concentration of 2 mg mL^{-1}.

4.2.4 DCD application

In order to replicate field conditions as much as possible, the application rate of 10 kg ha^{-1} DCD was applied to each sample receiving DCD treatments. The following

calculation details the concentration of DCD applied to each sample receiving DCD treatment;

$$10\ kg \cdot 0.00000003141593 = 0.0000003141593\ (kg) = 0.0003141593\ (g) = 0.314\ (mg) = 314\ \mu g\ DCD\ per\ soil\ column$$

A solution containing 157 mg of DCD L^{-1} was created to apply at a volume of 2 mL which would then equal 314 μg DCD mL^{-1}).

4.2.5 Ammonium analysis

As the previous methodology for ammonium analysis that was described in chapter 2 was unavailable, an adapted method of Kandler. (1996) for available ammonium concentrations was used and described as follows.

Three (3) g of fresh soil were weighed into a 50 mL centrifuge tube (Fisher Scientific, UK) and 15 mL of 2M potassium chloride (KCl) (Fisher Scietific, UK) added. Samples were placed horizontally on an orbital shaker extracted for 2 hours. Samples were then centrifuged for 10 mins at 4500 r.p.m and filtered through a 0.22μm Merck Millipore syringe filter into a 25 mL universal tube. Once filtered, 2.5 mL of sample was pipetted into a 4 mL cuvette, which contained 1.2 mL of solution containing 0.3M sodium hydroxide (NaOH) (Fisher Scientific, UK), 1.06 M sodium salicylate ($C_7H_5NaO_3$) (Acros Organics, UK) solution and de-ionised H_2O. Finally, 0.8 mL of sodium dichloroisocyanurate ($C_3Cl_2N_3NaO_3$) (Fisher Scientific, UK) was added to bring about colour development. Samples were left to stand for 30 mins at room

temperature to allow colour development; the colour formed is a teal to green colour. Sample absorbance was measured on a Hitachi U-2010 spectrophotometer at 660 nm.

4.2.6 Total carbon (TC), Dissolved organic carbon (DOC), inorganic carbon (IC) and total nitrogen (TN)

Three (3) g of fresh soil were weighed into a 50 mL centrifuge tube and 15 mL of potassium sulphate solution (K_2SO_4) added. The resultant slurry was shaken at 300 r.p.m for 30 mins at room temperature to extract total carbon and total nitrogen from the soil. The slurries were then centrifuged at 4500 r.p.m for 10 minutes to separate the extractant. After centrifugation, the samples were filtered through a Millipore syringe filter (0.22µm) (Millex-GP), this filtration step is required in order to measure only the dissolved fraction of organic carbon by removing particulate organic carbon. Samples were diluted 1:10 with milliQ de-ionised water into 30 mL glass test tubes and measured for total carbon (TC), inorganic carbon (IC) and total nitrogen (TN) on a Shimadzu TOC:TN analyser.

4.2.7 Soil potential nitrification activity

Using an adapted method developed by Kandeler (1996), 3 g soil was suspended in 20 ml of a 2 mM ammonium sulphate ($(NH4)_2SO_4$) (Fisher Scientific) and 10 mM sodium chlorate ($NaClO_3$) (Acros Organics) solution, an inhibitor of nitrite oxidation to nitrate. Samples were shaken for 5 hrs at 200 r.p.m at 25°C, and were extracted with 2 M Potassium Chloride (KCl) after being shaken and then centrifuged at 4500 r.p.m. for 5 min. Sample supernatants were analysed for nitrite (NO_2^-) by a using the Griess colorimetric procedure (Shinn. 1941); briefly, 2.35 mL of sample was mixed with 1.2 mL colour reagent, (2 g of sulphanilamide ($C_6H_8N_2O_2S$) (Sigma-Aldrich) and 0.1 g N-

(1-naphthyl)-ethylenediamine dihydrochloride ($C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl$) (Sigma-Aldrich) mixed in 130 ml de-ionised water brought to 150 mL with 20 ml concentrated phosphoric acid (H_3PO_4) and then 480 µl of 0.1% Sodium dichloroisocyanurate dihydrate ($C_3Cl_2N_3NaO_3 \cdot 2H_2O$) (Sigma-Aldrich) was added. Samples were left at room temperature for 30 mins, to allow full colour development, then analysed spectrophotometrically at 520 nm (U-2010 spectrophotometer (Hitachi)). The colour for the assay, once developed, produced a purple/red colour complex and was stable for 4 hrs at room temperature (20-23ºC). The method stated is an active measure of the potential production of NO_2^- in the soil. Production of NO_2^- requires the activity of two enzymes present in both autotrophic nitrifying bacteria and archaea, ammonia monoxygenase (AMO) and hydroxylamine oxidoreductase (HAO) present in bacteria and its homolog in archaea.

4.2.8. Potential nitrification using the inhibitors Cycloheximide, Streptomycin and C_2H_2

Three (3) g soil were added to a 100 mL serum bottle to which 1 mL of either, de-ionised H_2O, or, 1 mL of cycloheximide or streptomycin solution equalling 4 mg g^{-1} dry soil (12 mg mL^{-1}) were added. Cycloheximide is a known eukaryotic protein elongation inhibitor, however, as discussed in chapter 4, Vajrala et al. (2014) demonstrated its ability to inhibit the marine ammonia oxidizing archaeon (*N. martimus*); therefore, the effects on autotrophic nitrification enzyme assay used in this chapter. Streptomycin (STM) (4 mg g^{-1} dry soil (12 mg mL^{-1})) was used to inhibit prokaryotic protein synthesis and serve as a comparative mode of inhibition for potential nitrification activity from ammonia oxidizing bacteria (AOB). Inhibitor

concentrations (4 mg g^{-1} dry soil) for both cycloheximide and streptomycin were determined as were used in the previous study in chapter 3.

As cycloheximide is traditionally to inhibit eukaryotes, a set of sub-samples were treated with acetone-free acetylene (C_2H_2), by passing acetylene gas through concentrated sulphuric acid and then cleaning by passing through de-ionised water (Appendix; Fig. 7.2). Both bacterial and archaeal AMO enzymes have been shown to be completely inhibited by the addition of C_2H_2 (Offre et al. 2009) acting as a suicide inhibitor. Concentrations of C_2H_2 ranging from 100 Pa (0.1 kPa) to 1000 Pa (1 kPa) have demonstrated no significant differences in inhibition rate (Offre et al. 2009). Therefore, in order to ensure sufficient inhibition of AOB and AOA AMO enzyme activity, 1 kPa C_2H_2 (i.e. 1 mL C_2H_2 gas in 100 mL headspace) was used to inhibit both bacterial and archaeal AMO genes and served as a control to determine as to the nature of NO_2^- production. C_2H_2 therefore served as an indicator of any fungal NO_2^- production as concentrations up to 5 kPa have been demonstrated to have no effect of fungal nitrification with concentrations between (Schimel et al. 1984; Stams et al. 1990; De Boer et al. 2001), After treatments were applied, samples were left to incubate for 16 hours at 20ºC in the dark. After treatment with cycloheximide, streptomycin and C_2H_2, samples were assessed for potential nitrification activity, as described previously (4.2.6).

4.2.9. Cumulative potential nitrification rates

Cumulative nitrification potentials were calculated by adding potential nitrification rate values together for each day of the experiment, i.e. Day_0 + Day_3 + Day_5 + Day $_8$. Average cumulative potential nitrification rates were calculated from the four single replicates that had been summed together.

4.2.10. Statistical analysis

Data were analysed using Genstat 17th edition (VSN International, Hemel Hempstead, UK) using General ANOVA models to assess the impact of treatment factors, i.e. Fertiliser, DCD, Day on nitrification activity NH_4^+, total soluble carbon, total soluble nitrogen, dissolved organic carbon and inorganic carbon using a split-plot design. As inhibitors treatments were used only as pre-treatments for the potential nitrification enzyme assay treatment, and therefore not as treatment factors for the experiment, they were excluded from the factorial design and analysed as Y-Variates within the model. 95% confidence LSD bars were used to distinguish significant differences between sample means, indicating a significant difference of $P \leq 0.05$. Correlations between potential nitrification rate and carbon, or nitrogen components of the soil were analysed by linear regression. To indicate significant differences of $P \leq 0.05$ between mean values on graphs for NH_4^+, total nitrogen, total organic carbon, dissolved organic carbon and dissolved inorganic carbon LSD was determined from the ANOVA output.

4.3 RESULTS

4.3.1. Impact of fertiliser on soil chemical parameters: NH_4^+, TN, TC, DOC, and IC

Fertiliser type had a significantly increased concentrations of NH_4^+, TN, C, DOC and IC concentrations in soils treated with urine and significant increases in TC and DOC when treated with slurry when compared to control soils (Fig. 4.2; Table 4.1). The relationship between soil chemical parameters and fertiliser type varied with respect to DCD treatment (for NH_4^+, TC and DOC), Day (for NH_4^+, TN, TC, DOC and IC) and DCD x Day (for NH_4^+, TN, TC, DOC and IC) as evident by the two-way and three-way interactions (Table 4.1).

Urine treatment increased soil NH_4^+ concentrations for each day of the experiment when compared to control + No DCD, urea + No DCD and slurry + No DCD treatments ($P \leq 0.05$: Fig. 4.2 (a)), which remained similar to the control soil NH_4^+ concentrations (Fig. 4.2 (b)). Peak concentrations of NH_4^+ were observed on Day 3 in the urine + No DCD treatment, with concentrations reaching 165 (± SE 24.1) μg g^{-1} dry soil. DCD further increased soil NH_4^+ concentrations in urine treated soils (Fig. 4.2 (c)), with concentrations reaching 446.5 (± 110) μg g^{-1} dry soil, 2.7 times higher than the urine + No DCD treatment ($P \leq 0.05$).

Soil TC, DOC concentrations in the non-DCD treated samples increased as a result of urine and slurry treatment when compared to the control + No DCD ($P \leq 0.05$) with concentrations reaching 184 (± SE 2.6) μg g^{-1} dry, 158 (± SE 3.6) μg g^{-1} dry soil Day 3 for urine + No DCD and 82.7 (± SE 1.8) μg g^{-1} dry soil and 88.8 (± SE 2.2) μg g^{-1} dry soil for slurry + No DCD respectively (Fig. 4.3). Soil TC and DOC concentrations for slurry + No DCD increased to 141.2 (± SE 17.7) μg g^{-1} dry soil and 132.4 (± SE 17.4) μg g^{-1} dry soil by Day 5 respectively when compared to Day 3

concentrations ($P \leq 0.05$) and were comparable with TC and DOC concentrations observed in the urine + No DCD treated soils for Days 5 and 8. DCD reduced TC and DOC concentrations for slurry treated samples, with significant differences ($P \leq 0.05$) observed on Day 8 for slurry + no DCD (TC = 161.9 (± SE 18.5) μg g^{-1} dry soil; (DOC = 140.8 (± SE 12.7) μg g^{-1} dry soil) and slurry + DCD treatments (TC = 105.6 (± SE 3.1) μg g^{-1} dry soil; (DOC = 95.6 (± SE 3.1) μg g^{-1} dry soil) (Fig. 4.3 (a-d))

Inorganic carbon concentrations were observed to increase mostly in urine + No DCD and urine + DCD treated soils, with inorganic carbon concentrations peaking on Day 3, 25 (± SE 1.4) μg g^{-1} dry soil (Fig. 4.3 (e) and Day 5 for urine + DCD 25 (± SE 1.4) μg g^{-1} dry soil). Total nitrogen concentrations significantly ($P \leq 0.05$) increased for all sample treatments when compared to the control soil (Fig. 5.3 (g)), with both urea and slurry measuring at similar concentrations of total nitrogen on Day 3 (321.0 (± SE 12.7) μg g^{-1} dry soil and (370.5 (± SE 13.1) μg g^{-1} dry soil respectively), remaining relatively unchanged throughout the rest of the experiment (Fig. 4.3). Urine treated samples increased total nitrogen concentrations in comparison to the control and both urea and slurry treatments in both DCD treatments ($P \leq 0.05$).

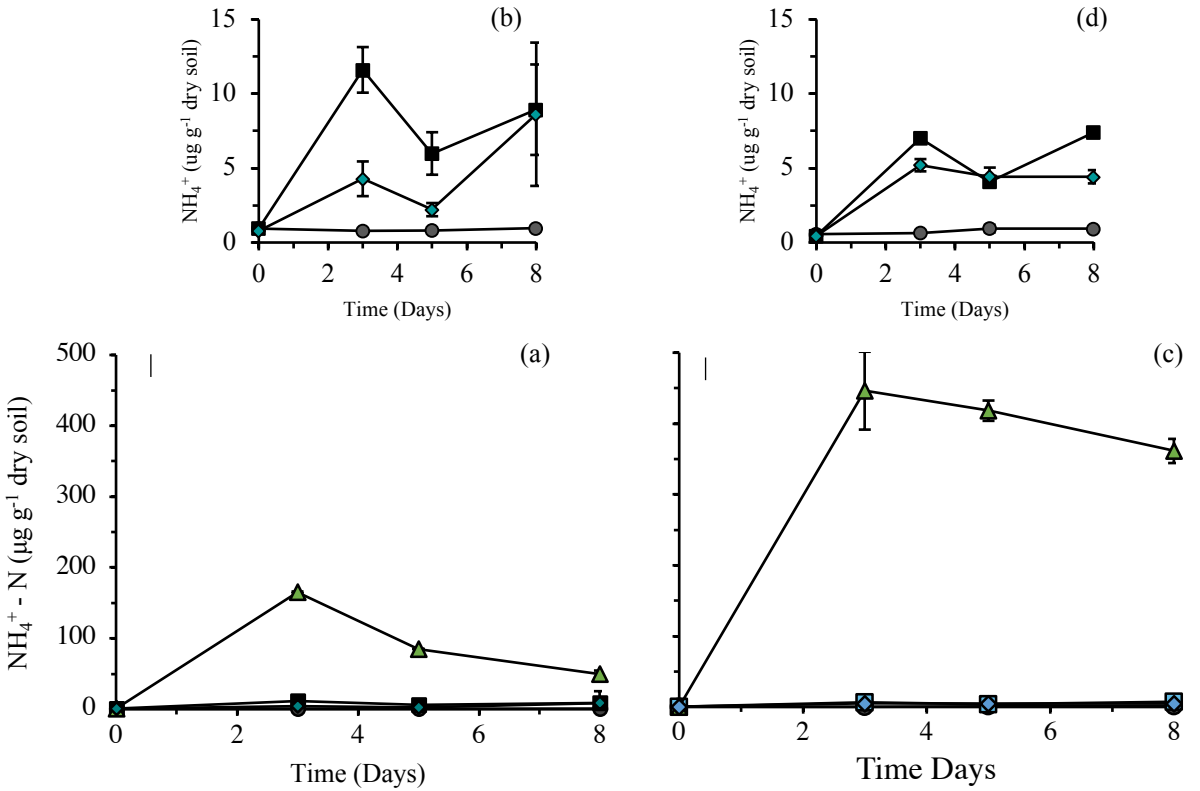


Figure 4.2. Ammonium concentrations in soils treated with water (control), urea, urine and slurry. Graphs (a) display samples treated without DCD and graph (b) shows samples treated with DCD. Graphs (b) and (d) show NH_4^+ - N concentrations for water (control), urea and slurry fertiliser treatments that aren't clearly visable on graphs (a) and (d). Error bars represent standard error of the mean (SEM). LSD ($P \leq 0.05$) are displayed in the top left corner of graphs. n = 4.

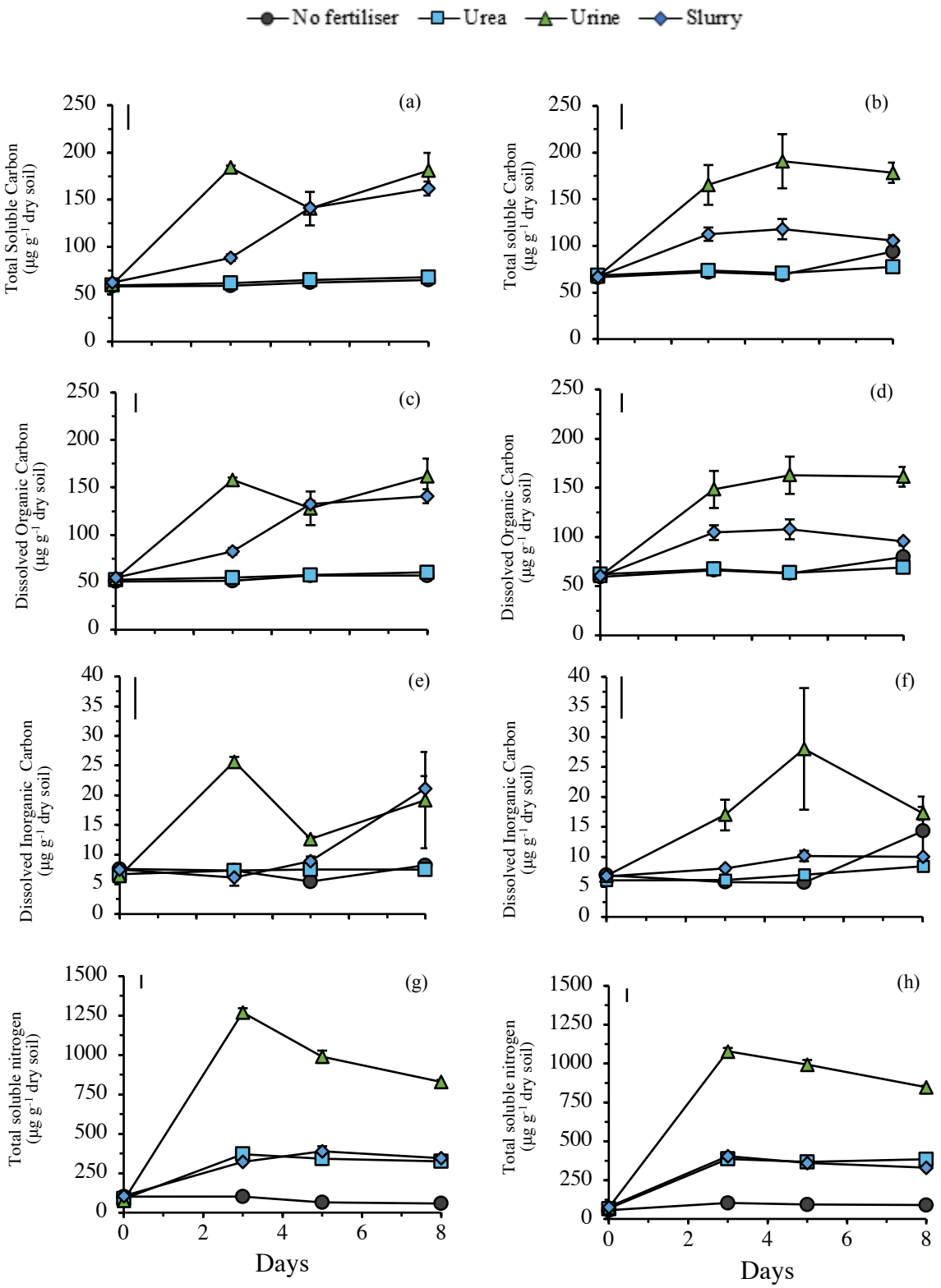


Figure 4.3. Soil total soluble carbon (TC), dissolved organic carbon (DOC), dissolved inorganic nitrogen (DIC) and total soluble nitrogen (TN) for fetiliser treatemnts. Samples without DCD treatment Grapsh (a) (c) (e) and (g) and samples with DCD are displayed in graphs (b), (d), (f), and (h). Error bars represent standard error of the mean. LSD ($P \leq 0.05$) bars are displayed in the top left of each graph. n = (4).

Tabel 4.1. Three-Way ANOVA for effects of fertiliser DCD and Day on ammonium NH_4^+, total nitrogen (TN), total carbon (TC), dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC)

		NH_4^+		TN		TC		DOC		DIC	
Treatment	DF	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
DCD	1	187.79	**< 0.001**	0.11	0.743	1.88	0.183	3.00	0.096	0.00	0.955
Day	3	69.38	**< 0.001**	278.72	**< 0.001**	46.02	**< 0.001**	56.92	**< 0.001**	7.93	**< 0.001**
DCD x Day	3	21.52	**< 0.001**	1.06	0.385	0.96	0.428	1.15	0.350	2.40	0.093
Fertiliser	3	547.36	**< 0.001**	810.70	**< 0.001**	138.72	**< 0.001**	176.98	**< 0.001**	22.37	**< 0.001**
Fertiliser x DCD	3	209.93	**< 0.001**	1.79	0.156	3.56	**0.018**	4.48	**0.006**	0.72	0.541
Fertiliser x Day	9	67.26	**< 0.001**	101.86	**< 0.001**	16.38	**< 0.001**	20.72	**< 0.001**	3.98	**< 0.001**
Fertiliser x DCD x Day	9	23.79	**< 0.001**	2.55	**0.013**	4.18	**< 0.001**	4.03	**< 0.001**	2.91	**0.005**

4.3.2. Response of potential nitrifying enzyme activities (AMO+HAO) to N source, Dicyandiamide (DCD)

Soil potential nitrification activity rates are shown in Fig. 4.4 (a-d). Three-way ANOVA (Table 4.2) demonstrated significant differences between fertiliser treatments ($P < 0.001$), DCD treatment ($P < 0.001$), time (Day) ($P < 0.001$), for NP and NP + CHX, NP + STM. Significant interactions between, fertiliser x DCD ($P < 0.001$), Fertiliser x Day ($P < 0.001$) and Fertiliser x DCD x Day ($P < 0.001$) were observed for both NP (Table 4.2). NP + CHX activity rates demonstrated significant interactions between fertiliser x DCD ($P < 0.001$), Fertiliser x Day ($P = 0.001$) (Table 4.2). Interactions between Fertiliser x Day were observed for NP + STM and interactions between Fertiliser x DCD and Fertiliser x Day were observed for NP +C_2H_2 (Table 5.2)

Increases in NP activity were observed for both Urine + No DCD and Slurry + No DCD treatments in comparison to both control and urea treated soils on Days 3, 5 and 8 of the experiment ($P \leq 0.05$; Fig. 4.4 (a)). Urine + No DCD produced the highest rates of potential nitrification activity in comparison to all fertiliser treatments (Fig. 4.1; (a) Table 4.1; $P \leq 0.05$), with the exception of Day 5 which produced a mean nitrification activity rate similar to Slurry + No DCD treated soils (urine + No DCD = 2.96 (± SE 0.19) µg NO_2^- $5h^{-1}$ g^{-1} dry soil; Slurry + No DCD = 2.90 (± SE 0.18) µg NO_2^- $5h^{-1}$ g^{-1} dry soil). Peak rates of nitrification potential were reached on Day 8 in the urine + No DCD treated soil 7.03 (± SE1.0) µg NO_2^- $5h^{-1}$ g^{-1} dry soil. NP was significantly reduced ($P \leq 0.05$) in all fertiliser treatments with inclusion of DCD to fertiliser treatments (Fig. 4.4 (a)), with the most notable effect observed for urine

treated samples, which had nitrification potential rates reduced by 6.5, 2.5 and 12.6 times on days 3, 5 and 8 respectively ($P \leq 0.05$).

NP + CHX activity followed the same pattern as observed for NP activity, with significant increases from the control soils related to both urine + No DCD and slurry + No DCD treatments. However, NP + CHX activity was lower than that observed for NP activity in urine treatment on each Day of the experiment ($P \leq 0.05$). Activity rates reduced from 4.94 (± SE 0.17) to 1.85 (± SE 0.05) µg NO_2^- $5h^{-1}$ g^{-1} dry soil on Day 3, from 2.96 (± SE 0.19) 1.5 (± SE 0.08) µg NO_2^- $5h^{-1}$ g^{-1} dry soil on day 5, and from 7.03 (± SE 0.10) to 3.04 (± SE 0.10) µg NO_2^- $5h^{-1}$ g^{-1} dry soil, equalling to differences in activity of 63% for Day 3, 50% for Day 5 and 67% for Day 8 respectively (Fig. 4.4).

For slurry treated soils NP + CHX activity increased by 9% when compared to NP on Day 3 however, by Days 5 and 8, NP + CHX activity had decreased by 21% and 10% respectively when compared to NP activity. In contrast to what was observed for NP activity, NP + CHX activity for both urine and slurry treatments remained similar throughout the experiment. DCD reduced activity for NP + CHX on Days 5 and 8 ($P \leq 005$) for urine treated samples and on Days 3, 5 and 8 for slurry treated samples.

NP+STM activity rates, much like NP + CHX were significantly lower than NP for each day of the experiment ($P \leq 0.05$) (Fig. 4.4 (c)). Again, urine and slurry treatments showed significant increases from control and urea samples ($P < 0.05$) but demonstrated no significant variation from one another (Fig. 4.4 (c)). Peak NP + STM concentrations were observed for urine + No DCD treated samples 2.8 (± SE 0.17) µg NO_2^- $5h^{-1}$ g^{-1}. DCD decreased NP + STM activity significantly ($P \leq 0.05$) on Days 3, 5 and 8 ($P \leq 0.05$). No activity was observed for NP + C_2H_2.

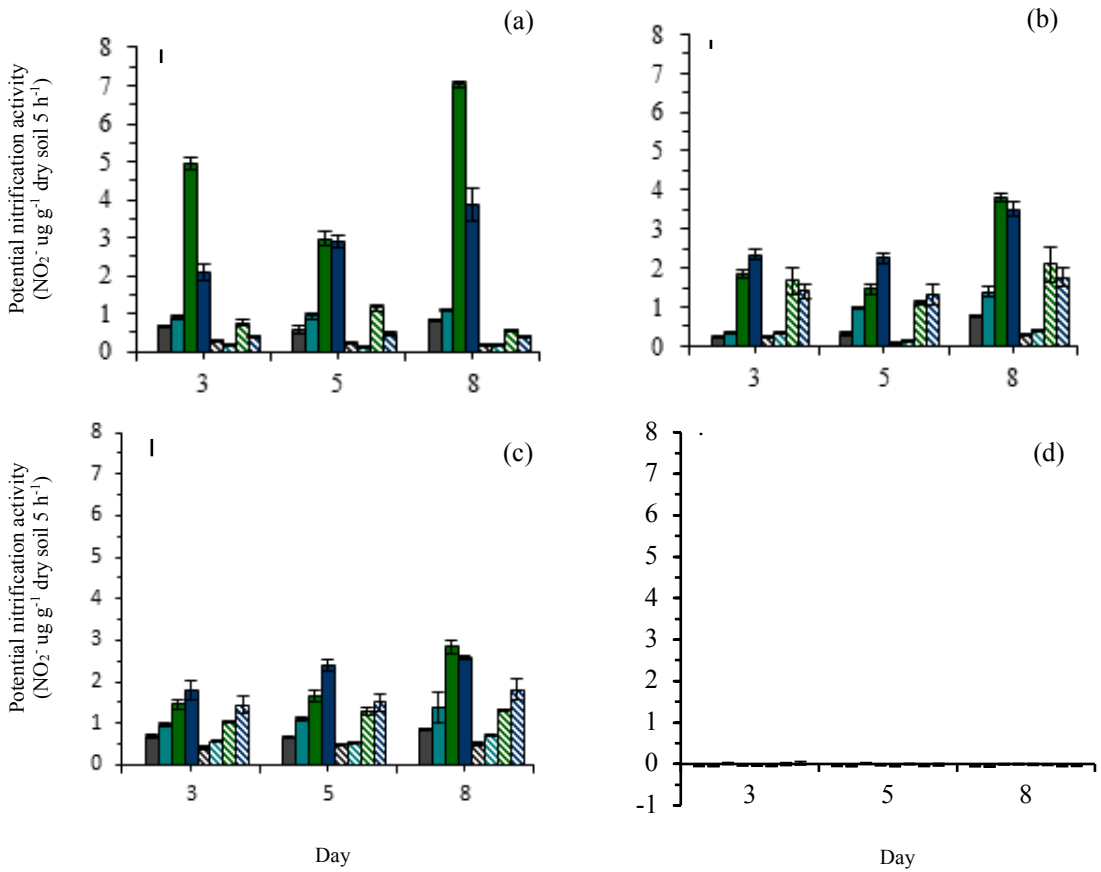


Figure. 4.4. Graphs for (a) Nitrification potentials (NP), (b) cycloheximide affected nitrification potential (NP + CHX), (c) streptomycin affected nitrification potential (NP + STM) and (d) acetylene affected nitrification potential (NP + C_2H_2). Standard errors of mean are displayed on bars. LSD bars are displayed in the top left of each graphs ($P \leq 0.05$) n = (4) (LSD value for C_2H_2 graph is too low to bee seen).

Tabel 4.2. Three-Way ANOVA for effects of fertiliser DCD and Day on nitrificaiton potential (NP), cycloheximide affected nitrification potential (NP + CHX), streptomycin affected nitrification potential (NP + STM) and actylene affected nitrification potential (NP + C_2H_2)

		NP		NP + CHX		NP + STM		NP + C_2H_2	
Treatment	DF	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
DCD	1	1451.62	**< 0.001**	85.66	**< 0.001**	89.69	**< 0.001**	0.01	0.614
Day	2	50.47	**< 0.001**	12.28	**< 0.001**	25.48	**< 0.001**	3.17	0.711
DCD x Day	2	76.31	**< 0.001**	4.43	**0.027**	9.48	**0.002**	0.96	0.632
Fertiliser	3	478.74	**< 0.001**	189.38	**< 0.001**	94.48	**< 0.001**	4.78	**0.017**
Fertiliser x DCD	3	269.45	**< 0.001**	8.04	**< 0.001**	1.46	0.236	9.23	**0.003**
Fertiliser x Day	6	22.38	**< 0.001**	4.31	**0.001**	2.91	**0.016**	2.22	0.336
Fertiliser x DCD x Day	6	38.86	**< 0.001**	1.87	**0.103**	1.18	0.330	1.20	0.100

4.3.3. Cumulative Potential Nitrification Activity

Fertiliser treatments demonstrated significant interactions with both DCD and inhibitors (Fig 4.5). Urine treatment demonstrated the highest rate of cumulative potential nitrification, with values reaching 15.6 (± SE 0.39) μg NO_2^- $5h^{-1}$ g^{-1} dry soil., followed by slurry 15.6 (± SE 0.39) μg NO_2^- $5h^{-1}$ g^{-1} dry soil. NP + CHX and NP + STM treated soils demonstrated lower cumulative potential nitrification in the no fertiliser and urine treated soils when compared to the controls ($P \leq 0.05$). DCD treatment significantly lowered nitrification potential in all samples.

4.3.4. Response of Nitrification Potential to Carbon and Nitrogen

Nitrification potential demonstrated significant strong correlations between dissolved organic carbon ($R^2 = 0.85$; $P < 0.001$), inorganic carbon ($R^2 = 0.66$: $P < 0.001$) NH_4^+ ($R^2 = 0.46$; $P < 0.001$) and total soluble nitrogen ($R^2 = 0.66$; $P < 0.001$) (Fig. 4.6 (a) and (b)). Significant, but weaker correlations were also observed for the abovementioned relationships but with DCD (Fig. 4.6 (a) and (b)).

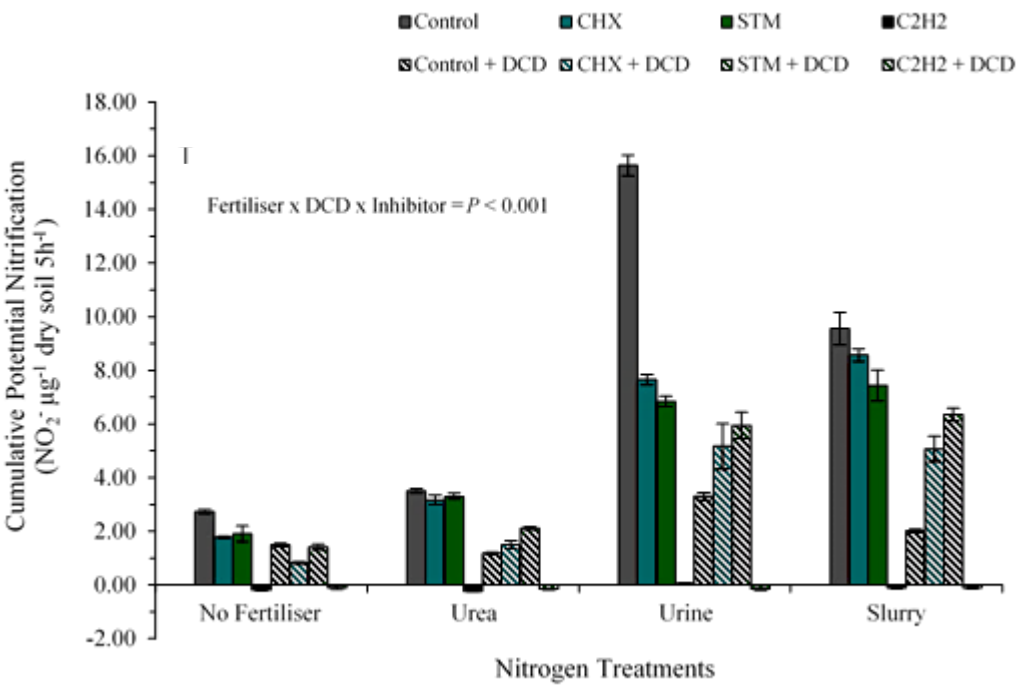


Figure 4.5. Effect of cycloheximide (CHX), streptomycin (STM) and acetylene (C_2H_2) on cumulative nitrificaiton potential for soil fetiliser treatments water (control), urea, urine and slurry with and without the application of the nitrification inhibitor DCD. Error bars represent the standard error of the mean (SEM). LSD is displayed in the top left of the graph ($P \leq 0.05$). n = (4)

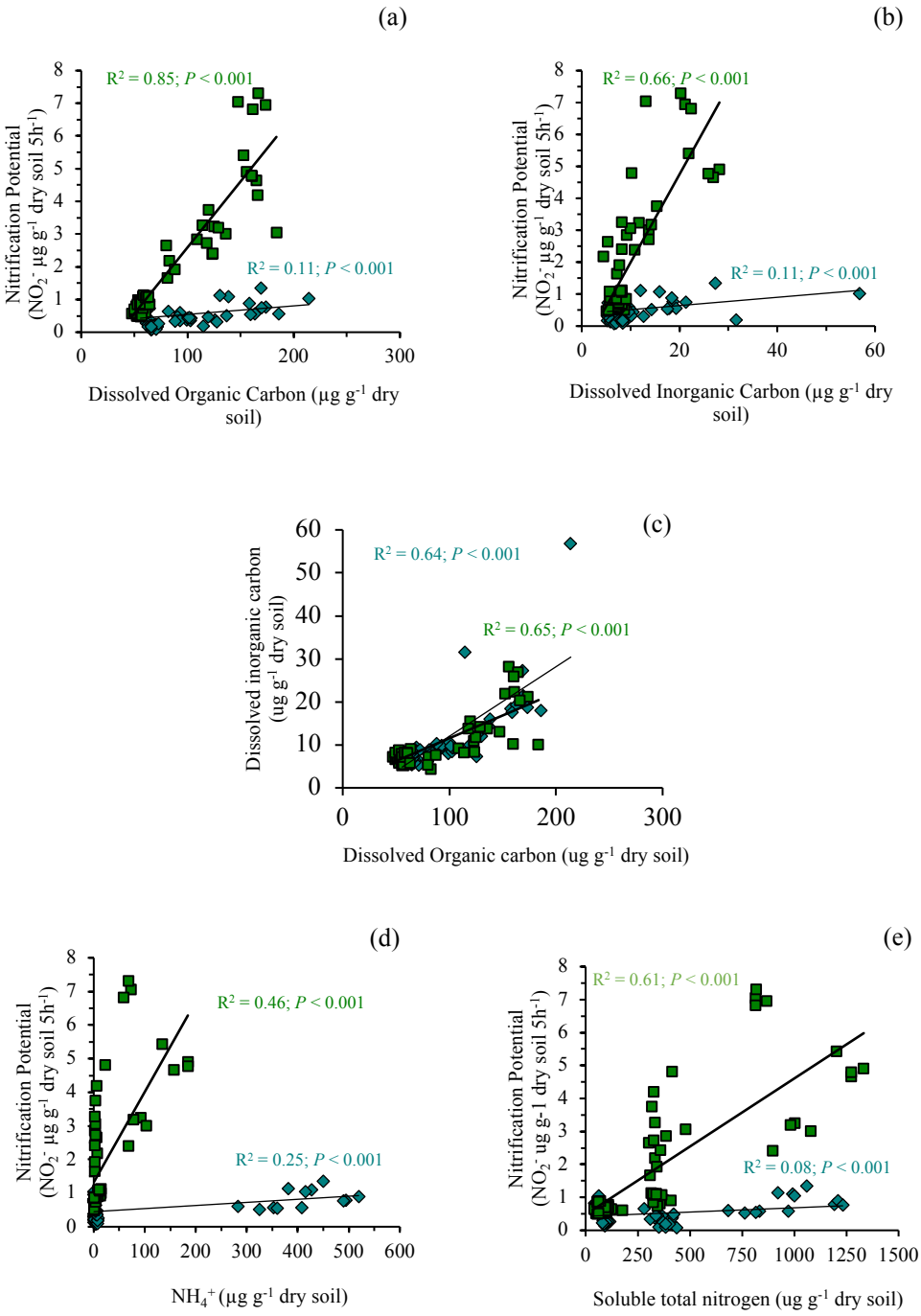


Figure 4.6. Graphs (a), (b) , (d) and (e) show correlations between dissolved organic carbon, inorganic carbon, ammonium (NH_4^+) and total nitrogen. Graph (c) shows correlations between dissolved organic carbon and inorganic carbon. Green squares indicate non-DCD treated samples and blue diamonds indicates DCD treated samples. (n) = 64 for No DCD treatment, (n) = 64 for DCD treatment

4.4. DISCUSSION

4.4.1. Effects of DCD in soils treated with different fertilisers

The results indicated by this study demonstrate that DCD effectively reduces nitrification potential within the range of nitrogen inputs commonly found in grasslands, thus rejecting the null hypothesis that DCD efficacy is related to typical grassland nitrogen inputs. No significant differences were observed for potential nitrification rates in soils treated with DCD, with the exception of Day 5 for the urine treated samples (Fig. 4.4 (a)), which demonstrated slight increases in NO_2^-; although potential nitrification activity was still observed in the samples (Fig. 4.5).

DCD significantly inhibited potential nitrification in all fertiliser treatments (Fig. 4.4 (a)). However, cumulative potential nitrification was observed over the whole experiment, indicating that nitrification was still active throughout the experiment (Fig. 4.5). Zacherl and Amberger (1990) demonstrated that DCD at a concentration of 100 ppm in cultures of *Nitrosomonas europaea*, which was roughly equal to 12.5 kg ha^{-1} (10 kg ha^{-1} equivalent was used in this study), only inhibited ammonia oxidation in *N.europaea* by 56%. As previously discussed other studies have shown that N_2O emissions were not completely inhibited by a DCD application, especially when urine was deposited on soils. Podolyan et al. 2014 showed that in soils treated with urine, with DCD was applied at 10 kg ha^{-1}, gene copy numbers of AOB were only reduced by half during the study, and AOA numbers were not affected by either the urine or DCD applications. As 56 % inhibition of ammonia oxidation was observed in the study by Zacherl and Amberger (1990), the work by Podolyan et al. (2014) agreed that DCD at this concentration did not fully inhibit AOB in soil. However, it reduced the rate at which they increased, which would explain the continued potential nitrification rates and that they were not completely inhibited, most likely due to the reduced growth of

AOB. Although, this may have only been true only for the urine treated soils, as the increased in NH_4^+ concentrations were significantly higher the other fertiliser treatments. It has been shown that acidic grassland soils, similar to the one used in this study, are largely dominated by soil ammonia oxidizing archaea (AOA) rather than their bacterial counterpart, soil ammonia oxidizing bacteria (AOB) (Nicol et al. 2008; Prosser and Nicol. 2012; Gan et al. 2016) with pH a dominant factor for AOB:AOA ratios (Zahlnina et al. 2012). This suggests that in the current study the control, urea and slurry treated soils that the pontential nitrifcation activities were as a result of soil AOA communities, given that the soil used had a more natural pH of 5.7, which has been observed to be optimal for AOA (Gubry-Rangin et al. 2011). Concentration tolerances have been observed between the ranges 1 mM up to 20 mM of NH_4^+ in certain species of soil and marine AOA (Hatzenpichler. 2012), as the concentrations used in the potential nitrificaiton method used 2 mM $(NH_4^+)_2SO_4$, this was not sufficient enough to have had any negative impact on the activity of AOA. Yet, DCD was observed to demonstrate similar rates of inihibition in all fertiliser treatments. Although commonly confirmed not to affect AOA groups in soil (Di et al. 2009, 2010: O'Callaghan et al. 2010: Gong et al. 2013: Chen et al. 2015), DCD has been shown to reduce increases in gene abundance of AOA. Work by Shen et al. (2013) showed that the population of the ammonia oxidizing archaeon *N. Viennensis* in pure culture reduced by as much as 50% when DCD was present at a concentration of 800 μM. Zhang et al. (2012) also established DCD was able to supress the activities of AOA belonging to the Crenarchaeota group 1.1a associated and 1.1b. in strongly acid soils (pH < 4.5) at a field application rate of 50 mg kg^{-1}. On a per gram of soil basis, the former study would be equal to 67 mg g^{-1} dry soil and in the study by Zhang et al. (2012) it would be equal to 50 μg g^{-1} soil. As the DCD concentrations used in the

current experiment were equal to 12.56 ug g^{-1} soil, this suggests that the concentrations were not great enough to fully inhibit the AOA populations in soil, and AOA could still significantly contribute to ammonia oxidation in the soils with lower concentrations of NH_4^+. The use of cycloheximide was employed in order to potentially distinguish between the AOB and AOA as it has been demonstrated to inhibit cellular turnover in AOA (Vajrala et al. 2014).

4.4.3 Potential sources of potential nitrification activity

As has been shown, all potential nitrification rates were inhibited by acetylene gas (Fig. 4.4 and 4.5), a method that has been commonly used to distinguish between autotrophic and heterotrophic nitrification rates in soils in other studies (Schimel 1984; Pennington and Ellis (1993); Pedersen 1999). It has been demonstrated that any nitrite produced was as a result of autotrophic ammonium oxidation, as acetylene completely inhibits AMO in both AOB and AOA (Hyman. 1990; Offre et al 2009; Gubry-Rangin et al. (201); Vajrala et al. 2014). This confirmed that all potential nitrification activity measured in the current experiment was related to either AOB, or, AOA communities, eliminating possible oxidation of NH_4^+, and therefore nitrite production by fungi.

Although cycloheximide inhibited potential nitrification in the control, urine and slurry treatments, only significant inhibition was observed for the urine treatment. Cycloheximide significantly ($P \leq 0.05$; Fig. 4.4 and 4.5) reduced potential nitrification activity in urine treated samples on all days in samples receiving the non-DCD samples. A non-significant trend was observed for the decreased potential nitrification activity in both the control and slurry treatments (Fig. 4.4 and 4.5). In addition, in previous studies cycloheximide has been demonstrated *in-vivo* to inhibit recovery of acetylene inactivated cells in *Nitrosophaera maritimus* (Crenarchaeota group 1.1b)

(Vajrala et al. 2014). A study by Vajrala also demonstrated that a 12 hour cycloheximide (without acetylene) treatment alone reduced the NH_4^+ dependent O_2 uptake rate of *N. maritimus* cells incubated with cycloheximide (200 μg mL^{-1}) to ~ 50% of the uptake seen in samples without the cycloheximide treatment, but still had no effect on *Nitrosomonas europaea*. Consistent with the observations by Vajrala et al (2014), potential nitrification activity, and therefore AMO+HAO activity, were observed to significantly ($P \leq 0.05$) have reduced (by as much as 50%) after a 16 hour incubation with cycloheximide for the control and urine treated soils. Streptomycin also produced similar reductions in potential nitrification in both control and urine soils, and slightly increased the reduction of nitrification potential in the slurry treated soils (Fig. 4.4 (c) and 4.5). This suggests that both AOB and AOA were contributing to similar amounts of potential nitrification activity in the control and urine treatments and although streptomycin demonstrated more inhibition in the slurry treated soils, cycloheximide still had an impact on soil potential nitrification activity. It has been noted that *amoA* gene sequences in AOA from soil are phylogenetically distinct and have been grouped separately in comparison to *amoA* sampled from marine environments (Taylor et al. 2010), such as the one used by Vajrala et al. (2014). However, as the understanding in this area is still expanding no firm conclusion can be drawn with regard to whether the effects of cycloheximide on marine AOA would differ to those of soil AOA, although the effects could be similar. The inhibition by cycloheximide for both the urine and control soils would suggest that AOB and AOA were providing similar contributions to the ammonia oxidation. However, it has been consistently demonstrated that in ammonia oxidizing activity in soils that have received urine deposition has generally been attributed to AOB (Di et al. 2009; 2010; Jia and Conrad. 2009; Podolyan et al. 2014). Sterngren et al (2015) demonstrated that

although AOA dominated in grassland soils, with 6 to 43 times more *amoA* genes of archaeal origin than bacterial, AOB have been shown to drive gross nitrification rates in soils receiving high concentrations of nitrogen in the form of both amino-acids and ammonium chloride (NH_4Cl), with increases in AOB near 10-fold higher than AOA (Sterngren et al. 2015). Verhamme et al. (2011) showed that AOA increased in abundance at concentrations of 0, 20 and 200 μg NH_4^+ g^{-1} soil (0, 1.1 and 11 mM of NH_4^+ respectively) over 28 days at a pH of 7.5, whereas AOB only grew at the higher concentration of NH_4^+, indicating that some species of AOA could actively grow at high concentrations of NH_4^+. This provides evidence that some soil AOA may have contributed to the oxidation of ammonium in the urine treated samples in this study. This needs to be considered along with the fact that in Verhamme's study, increased growth of AOA at 200 μg NH_4^+ g^{-1} soil was only observed 28 days after application, suggesting that the contributions to potential nitrification from AOA in the urine treatment in the current study may not have occurred over the smaller time scale used (8 Days). Overall, it would suggest that in the urine treated samples the reduced nitrification potential due to cycloheximide addition was not as a result of the partial inhibition of AOA.

Cycloheximide demonstrated a trend, albeit not significantly, that reduced potential nitrification activity in both control and slurry treated soils (Fig. 4.4 (b)) and 4.5). Taylor et al. (2010) investigated differential contributions of archaea and bacteria to ammonia oxidation in varying soil and land uses using cycloheximide and kanamycin (a bacterial protein synthesis inhibitor) treatments to inhibit recovered nitrification potential from acetylene inhibition (inhibition of protein synthesis and therefore cell growth). Their work suggested that in a pasture soil, archaea were responsible for nitrification potential due to the partial inhibition by cycloheximide in

the kanamycin-resistant pasture soil that received ≤ 10 μm NH_4^+. This suggested if AOA were inactivated by cycloheximide, some soil AOA must be cycloheximide resistant as only partial inhibition was achieved. The work in this experiment corroborated the findings of Taylor et al. (2010) where the control and slurry treated soils which also had low concentrations of NH_4^+ (1 μg NH_4^+ g^{-1} soil and > 10 μg, but less than 20 μg NH_4^+ g^{-1} dry soil, respectively; Fig. 4.2 (a) and (b)) for the duration of the experiment. The current work and Taylor et al. (2010) demonstrated partial inhibition of soil nitrification potential with cycloheximide treated soils at low concentrations of NH_4^+. However, as it was observed that streptomycin also induced inhibition in these treatments and this suggested that AOB were also responsible for potential nitrification activity in the soils with these fertiliser treatments. A later study by Taylor et al. (2013) demonstrated that AOA were active and even equal contributors to net nitrification depending on NH_4^+ concentrations when compared to AOB. The Taylor et al. (2013) study used an improved method for differentiating between AOB and AOA in soil by using octyne gas to irreversibly inhibit AOB activity, but which had no effect on AOA. At 0 μmol NH_4^+ concentrations, octyne resistant net nitrification was roughly 80% of total net nitrification potential, indicating that AOA contributed more to net nitrification in soils with extremely low amounts of NH_4^+. At 2 μmol NH_4^+ g^{-1} of soil, (equal to 36 μg NH_4^+ g^{-1} soil), octyne resistant net nitrification contributed to roughly half of net nitrification potential. As the control soils in the current work demonstrated an almost 50/50 contribution to potential nitrification with cycloheximide and streptomycin treatment, and the effect of cycloheximide was less in the slurry soil that had higher concentrations of NH_4^+, it could suggest that growth rates for AOA and AOB were inhibited by cycloheximide and streptomycin respectively, and that both groups were actively contributing to ammonia oxidation in

the soil under the conditions presented. As it has been demonstrated that streptomycin has the ability to reduce the growth of soil AOB but not affect soil AOA (Tourna et al. 2011) this would discount any non-target effects of streptomycin on AOA. As cycloheximide was demonstrated by Vajrala et al. (2014) to have no effect on ammonia oxidation in *N.europaea*, suggesting that both AOA and AOB were inhibited independently by their respective target antibiotics.

As inhibition of soil AOA has been shown to be uncertain, the understanding of the responses of AOB and AOA in soil receiving similar fertiliser treatments will help to further understand the results from both the control and slurry treatments in this study. Fortuna et al. (2012) observed that the AOB and not the AOA gene copy numbers increased in soils which received cattle slurry, which would agree with observations from other studies (Di et al. 2009; Podolyan et al. 2014; Stergren et al. 2015) that AOB respond to a greater extent than AOA to N addition in soil. Interestingly, potential nitrification from soils in this study were found to have a stronger correlation with dissolved organic carbon ($R^2 = 0.85$, $P < 0.001$) than NH_4^+ ($R^2 = 0.46$, $P < 0.001$). This finding is opposed to what is normally associated with dissolved organic carbon, which has been associated with decreased nitrification activities. Strauss and Lamberti. (2000) showed that dissolved organic carbon (DOC) had a negative effect on nitrification in aquatic sediments and increased concentrations of dissolved organic carbon reduced the population size or activity of ammonia oxidizers in sediment soils (Strauss and Lamberti. 2002). In contrast, a recent study by Xue et al. (2016) demonstrated that the AOA communities were more strongly associated with sugar hydrolysis associated soil enzymes. Tourna et al. (2011) previously demonstrated that *Nitrososphaera viennensis*, a soil AOA, was able to achieve considerable growth through the additions of low amounts of pyruvate, which

could indicate that AOA may have contributed to some potential nitrification activity in the slurry treatment. Interestingly, however, DOC concentrations were observed to significantly reduce in the slurry treatment when DCD was applied in comparison to the non-DCD treated slurry treatment (Fig. 4.3 (c) and (d)), and NH_4^+ concentrations followed a similar trend (Fig. 4.3 (b) and (d)) for the same treatment, it was also observed that correlations between potential nitrification and DOC, although still significant, was much lower in DCD treated samples (Fig. 4.5). This suggested that DOC was being liberated by ammonia oxidizing microorganisms that were sensitive to the DCD treatment, which as discussed previously in this chapter were at concentrations that would be relative to the AOB activities. Fortuna et al. (2012) discovered that total soil carbon concentrations correlated well with potential nitrification activity which were associated with the increased NH_4^+ concentrations once slurry had been applied. The similarity in DOC and NH_4^+ concentrations patterns in the DCD treated soils would seem to agree with these observations by Fortuna et al. (2012). Sterngren et al. (2015) used gross nitrification rates based on AOB gene count numbers to demonstrate that short term potential nitrification rates (Day 5) were related to AOB activity in nitrogen, sugar and control soil treatments, assuming that all AOB were oxidizing ammonium at maximum capacity. Based on this observation, the short incubation time in this study (0, 3, 5 and 8 Days), would further suggest that all increased potential nitrification activity was as a result of AOB activity, and not AOA. In contrast, AOA activity has been suggested to be tightly linked with organic N mineralization (Levicnik-Hofferle et al. 2012). In the study by Levicnik-Hofferle et al. (2012), ammonia-oxidizing activity of *thaumarchaea* was stimulated after the additions of mineralizable N in the form of either urea, glutamate or yeast extract, this suggested that this group preferentially oxidized ammonia generated through the

mineralization of organic N. As organic N mineralization in slurry has been demonstrated to contribute to significant amounts of crop available N (Bhogal et al. 2016), it could possibly indicate that the increased potential nitrification activity at low NH_4^+ concentrations was due to the cycling of organic N and growth by use of the dissolved organic carbon by AOA, as other literature has indicated increased dissolved organic carbon concentrations would decreases NH_4^+ oxidation activity (Strauss and Lamberti. 2000(a); 2002(b)). AOA are known to be capable of a mixotrophic lifestyle. Jia and Conrad (2009) demonstrated that soil AOA grew without incorporating any CO_2, therefore suggesting that AOA were mixotrophic and sequencing has identified oxidative and tricarboxcylic acid cycle (TCA) genes in the genome of *Crenarhaeum symbiosum* (Hallen et al. 2006). Amino acid uptake in planktonic archaea has been shown by Ouverney and Fuhrman. (2000) and isotopic studies by Teira et al. (2004) and Herndl et al. (2005) also indicated that arcahea Atlantic Ocean were capable of uptaking amino acids, thus indicating the ability of certain archaea to ultilize dissolved organic matter as an energy source.

Interestingly, a contrasting effect of DCD was observed when soils were treated with cycloheximde and streptomycin, with increased potential nitrification observed in the samples (Fig 4.4 (b) and (c)). As, to the authors knowledge, there are no other studies that have incorporated both cycloheximide and streptomycin these results are somewhat puzzling. However, Zhang et al. (2015) proposed that heterotrophic nitrification of organic nitrogen compounds was an important pathway in acidic soils. Therefore it is perhaps possible that heterotrophic microorganisms may have had the opportunity to dominate in the soil once both DCD and either cycloheximide and streptomycin treatments were combined. *Psuedomonas aeruginosa* have been seen to demonstrate both low and high level resistance to streptomycin (Tseng et al. 1972) and

these are known heterotrophic nitrifiers (Obaton et al. 1968; Castignetti and Hollocher 1984). The inhibition by both DCD and cycloheximide and treatments could suggest the increased activity of heterotrophic nitrifiers due to increased substrate availability through reduced competition. In addition, these observations were made while using an enzyme assay that could have provided the ideal conditions for this to take place (De Boer and Kowalchuk. 2001).

The method used in this study was an estimation of potential nitrification enzyme activity, more an indication of the increased activity of AOB and AOA under 'ideal' conditions, and therefore may not be directly comparable to the other studies that observe growth of AOB and AOA through gene abundance in-situ. However, given the large concentrations of ammonium in the urine treatment, and the existing knowledge regarding AOB and AOA responses to urine and slurry N inputs into grassland soils (Di et al. 2009; Podolyan et al. 2014; Fortuna et al. 2012), the effect of cycloheximide would not agree with current understanding. Therefore, a secondary mechanism will be discussed.

4.4.3. Does cycloheximide inhibit soil archaea?

Cycloheximide reduced potential nitrificaiton activity to roughly 50% in the urine and control treatments. This suggested that both bacterial and non-bacterial groups, (potentially archaea) had a 50/50 contribution to ammonium oxidation in the soil used under the experimental conditions. At present to the authors knowledge there are no known side effects of cycloheximide on the activity of *Nitrospira* spp, and cycloheximide has been demonstrated to have no negative effects on the ammonia oxidation activity of *Nitrosomonas europaea*. It can be suggested that cycloheximide may have in fact inhibited arcahaeal nitrificaiotn potential in both the control and slurry treated soils, as the soil NH_4^+ concentrations and chemistry would seemingly

favour AOA. However, one aspect does appear more difficult to explain; the inhibitory affect of cycloheximide on soil urine treated samples. The concentrations of ammonium under the urine application were consistently associated with increased AOB. Tyalor et al. (2013) observed that although AOA could still remain active at high concentrations of NH_4^+ (20 μmol; 360 μg g^{-1} soil) as net nitrification was still present in octyne treated samples, the 50/50 inhibition rate between both cycloheximide and streptomycin treated samples simply doesn't agree with the current understanding of either the growth rate, or, activity of AOA in urine treated soils, or any soils with greater concentrations of NH_4^+. Therefore, there is a suggestion of a second theory of the inhibitory mode of cycloheiximide.

It has been demonstrated that cycloheximide reduced the precence of nitrifiers Griffiths. (1989) observed that both nitrificaiton rates and numbers of ammonium oxidizers were enhanced in the presence of protozoa, which were then subsequently reduced in the presence of eukaryotic inhibitors triton X-100 and more importantly cycloheximide. More recent studies have also observed similar interactions (Petropoulos et al. 2005; Pogue et al. 2007). Petropoulos et al. (2005) identified that although cycloheximide didn't alter the concentrations of bacteria, the rates of production of ammonia, nitrite and nitrate were significantly reduced in activated sludge batch reactors. Petropolous et al. (2005) proposed that the ability of protozans to feed on heterotrophic bacterial population in similar systems may play a role in reducing competition for nitrification substrates and thereby enhance the nitrificaiton rates. An even more recent study by Pogue et al. (2007) identified that even although AOB and NOB remained present in cyloheximide treated activated sludge reactors, they became less active when the protozoa were inhibited. An earlier study by Ingham and Coleman. (1984) also demonstrated that cycloheximdie had a negative impact on

a number of the protozoan microbial communities, mainly amoaeba and ciliates. Badawi et al. (2012) demonstrated the impact of cyclohexiximide on soil portozoa. By observing reduced protazoan preditation on soil bacteria, that were important to the mineralization of the herbicide 2-methyl-4-chlorophenoxyacetic acid in soil slurries. This identified an artefact of protozoan predation on the soil bactiera in the soil slurry vital to the degradation of the pesticide being studied. The study by Taylor et al. (2010) observed an inhibitory effect of cycloheximide on the recovery of net nitrificaiton from acetylene induced inhibtion in pasture soils that were resistant to kanamycin. This would appear to support the findings in this study, indicating that some AOA were inhibited by cycloheximdie, however, in the same study, Taylor et al. (2010) also observed that recovered nitrification potential was inhibited by kanamcyin treatment in a crop soil, thus indicating that AOB were solely responsible for ammonia oxidiation, was also partially inhibited by cycloheximide. These results would seem to agree more with the observations in this study in urine, control and slurry fertiliser treatments. Taylor et al. (2010) issued a cautious warning for the use of cyclohxeimide in relation to archaea due to the results they obtained for the crop soil and as another eukaryotic growth inhibitor, actinomycin D, which had been demonstrated to effectively impact transcriptional activity in other types of archaea (Jager et al. 2002; Andersson et al. 2006), had no effect on pasture soil, which had been partially inhibited by cycloheximide in the study. It is obvious more studies are needed to assess how cycloheximide is influencing recovery of nitrificiation potential. It further suggests that the seconday mode of inihbition; the inhibition of potential nitrificaiton through the biocidal effect of cycloheximide on soil protozoa is increasingly more likely, at least in soils under higher NH_4^+ concentrations.

In relation to the increased NO_2^- production in the antibiotic treated soils that had received DCD treatment, the interaction as described by Petropoulos et al. (2005) may have been due to the temporary inhibition by DCD combined with the reduced growth as a result of either the inhibition of cellular turnover by streptomycin, or, through the reduced predation of heterotrophic bacteria by protoza within the cylcoheximide treatment. Both of these could have potentially lead to the increased availability of NH_4^+ attributable to the inhibition of the ammonia monoxygenase gene by DCD, and also lysis of amino-acids that occurs in the presence of antibitoics (Badalucco et al. 1994).

In the context of the previous discussion it is therefore posssible that cycloheximide does have an inhibitory action on AOB activities in soils, albeit indirectly. This is potentially a crucial observation that may have been overlooked in other studies relating to NH_4^+ oxidation in soils, their bacterial and fungal communities and even in studies of bacterial:fungal N_2O ratios (Chen et al. 2015; Rex et al. 2018), and may help to elucidate the observations in Taylor et al's. (2010) work. In light of the work conducted by Vajrala et al. (2014), the use of cycloheximide was aimed at identifying NH_4^+ oxidation pathways by archaea. However, it seems that a secondary inhibitory method may have been identified as a result of the known effects of cattle urine on both AOB and AOA nitrogen cycling communities in soils. Although the measurement of gene abundances for AOB and AOA were not able to be carried out in this study, it is highly recommended that any future study should consider the effect of cycloheximide on the ratio of AOB:AOA in soil. It is also recommended that counts of active protozoa in the soil should be included as this would help to identify if this is the reason for the reduction in soil potential nitrification activity.

4.5 CONCLUSION

The efficacy of DCD is not significantly reduced in soils receiving different nitrogen sources, but was observed to not completely reduce nitrification potential. Greater concentrations of NH_4^+ could reduce its efficacy in soil due to the different loading rates of NH_4^+ present in different fertilisers, which are known to have differntial impacts on the structure of soil AOB and AOA communities. More importantly was an increased understanding of the effect of the use of cycloheximide in soil nitrogen potential nitrificaiton activity. The use of cycloheximide allowed further insight to the processes of potential nitrication activity, indicating other biotic activities influenced potential nitrificaiton in grassland soils. As nitrification potential, and therefore AMO + HAO activity, was significantly reduced in the presence of the eukaryotic protein elongation inhibitor cycloheximide, we rejected the sub null hypothesis that cycloheximide does not reduce potential nitrification activity in this grassland soil.

This is the first study to the authors knowledge that studied the impact of cycloheximide on soil potential nitrification activity. Therefore, the results from this study could suggest that AOA could have contributed equally of the potential nitrification rates observed in this study for urine, control treatments and partially in the slurry treatment, and that AOA could potentially contribute to NH_4^+ oxidizing activity in soils treated with DCD. The corroboration with other studies develing into the ratios of AOB and AOA using cycloheximide would suggest that AOA may well have been equally important to NH_4^+ oxidation over a range of NH_4^+ concentrations found in the soil due to different fertiliser treatments. However, with the understanding that cylcoheximide has the ability to reduce protozan growth, as demonstrated by previous researchers, which has been observed to influence the activity of soil nitrfying

microorganisms indicates a future study is needed to discern between these two potential pathways. As the effects of cycloheximide are still unclear on soil AOA in soil, further experiments are crucially needed to finally deduce the impact of cycloheximide on soil NH_4^+ oxidiation as neither an inhibition of AOA activity by cycloheximide or the reduction in AOB activies due to inhibiton of protozoa by cycloheximide could be elucidated to in the current study. It is recommended that the use of gene adundance, as was planned for this study, and or mRNA transcriptional gene activity measurements be included in a future study on the impacts of cycloheximide on soil AOA and AOB, and in other studies utilising cycloheximide to distinguish between bacterial and fungal activities, which would seem a logical step in the understanding of the impacts of antibitoics in soil in general. In relation to this study, perhaps a combination of octyne and acetylene inhibiton as used by Taylor et al. (2013) with cycloheximide treatment would elucidate whether or not AOA are inhibited by cycloheximide as octyne resistant nitrificaiton would either be reduced/inhibited, unaffected, or perhaps increased. The measurement of soil protozoa would be a critical addition to AOB and AOA gene abundances, both in soil and in the solution of the potential nitrificaiton enzyme assay, as either inhibitory mode of action suggest that artefacts may be introduced through the use of cycloheximide in N cycling studies in soil.

CHAPTER 5: EFFECT OF BULK DENSITY AND DICYANDIAMIDE (DCD) ON POTENTIAL N_2O FLUXES, NITRIFICATION ACTIVITY AND NITRATE REDUCTASE ACTIVITY IN A GRASSLAND SOIL RECEIVING CATTLE URINE AND CATTLE SLURRY TREATMENTS: A LABORATORY GRASSLAND MESOCOSM EXPERIMENT

5.1. INTRODUCTION

Nitrous Oxide (N_2O) has a global warming potential 298 times that of carbon dioxide (CO_2) (2012). Grasslands are identified as a major sources of N_2O fluxes, with inputs from grazing considered to be some of the highest due to inputs from animal excreta (de Klein et al. 2010). Studies have investigated the impacts of slurry and urine inputs in grasslands on N_2O emissions. Clayton et al. (1997) demosntrated that the loss of N_2O-N as a percentage of N applied was highest under slurry treatment in a grassland. de Klein and Logtestijn. (1994) dmeonstrated that urine treatment significantly increased both denitrificaiton and N_2O emissions. de Klein also investigated the impact of the nitrification inhibitor Dicyandimaide (DCD) on it's ability to reduce N_2O fluxes from urine impacted soils. Their results indicated that DCD was able to significantly reduce N_2O fluxes from soils reciving urine inputs. More recently work by Di and Cameron. 2002, Di et al. 2003 and Di et al (2010) demonstrated that DCD was crucial in reducing the sharp increases in growth of ammonia oxidzing bacteria (AOB) which largely contributed to N_2O fluxes from grassland soils. However, recent studies by Barneze et al. (2015) and Mazzetto et al. (2015) indicated that the efficacy of DCD should be called into question due to its inability in reducing soil N_2O fluxes in soils treated with urine, contrasting the typical results obtained by other sutdies (Di and Cameron. 2002; Di et al. 2009; Di et al. 2010). The studies by Barenze et al. (2015) or Mazzetto et al. (2015) didn't include measurements of nitrfying or denitrying commmunities, therefore the pathway of N_2O

production was not elucidated to. Both Mazzetto et al. (2015) and Barneze et al. (2015) both suggested that the dineffectiveness of DCD was due to degredation by soil microbes at higher soil temperatures. However, degredation of DCD is known to increase in more aerobic soils (Balaine et al . 2015). Balaine et al. (2015) demonstrated that DCD efficacy was greater in soils at higher matric potential under urine amendment. However, the study by Barneze et al. (2015) demonstrated that DCD was still ineffective at WFPS volumes of 80%, therefore disagreeing with the observations by Balaine et al. (2015). Therfore the reasoning for the varying efficacy of DCD in grassland soils receiving urine inputs is still in question. The work in this chapter aims to focus on the efficacy of DCD treatment in soils recieving different compaction treatments in planta with *Lolium perenne* by studying the potential activities of nitrifying and denitrfying microbial communities under summer conditions. As DCD reduces the activity of the ammonia monoxygenase (AMO) gene in soil AOB, the use of potential nitrification activity will be employed to study the impact of both urine, slurry and DCD on the nitryfing communitiy. Nitrate reductase will be used to help understand the denitrificaiton pathway. As nitrate reducatse is considered the first step in denitrification (Averill and Tiedje. 1982) it will help elucidate to the short-term impacts of DCD on the denitrifying soil microbial communties. Under similar conditions to those used in the study by Barneze et al. (2015), 20ºC, which could be considered summer conditions in the UK, the fate of urine and the efficacy of DCD will be elucidated to.

Hypothesis: Increased soil bulk desnity reduces DCD efficacy on N_2O fluxes from cattle dervied nitrogen inputs in a grassland soi

5.2. Materials and Methods

5.2.1 Sample preparation and experimental design

Soil was sampled as discussed in Chapter 2. Soil was sieved to < 2 mm and placed into a plastic container and left at 4^{o}C for one week prior to sample preparation in order to allow even distribution of moisture within the soil.

Using soil at a gravimetric moisture content of 28%, the sieved soil was compacted into a 50 mm diameter PVC tube at a height of 100 mm (cylindrical volume of 196.4 cm^3) using a compactor (Appendix; Fig. 7.1). Each sample was compacted in two 50 mm layers. After the first 50 mm layer was compacted into the 100 mm tube the surface of the soil was lightly un-compacted with a metal spatula in order to create a loose surface for the compaction of the next 50 mm of soil. This loosening enabled the two layers to join together and stop a layering effect occurring. The two 50 mm layers were half the weight of the total calculated 100 mm sample weight. Two bulk densities, 1.1 g cm^3 and 1.3 g cm^3, were used for this experiment with the dry weights for each of the samples as follows;

Bulk density · Sample volume

Therefore;

1.1 · 196.4 g cm^3 = 216.04 grams (g) dry soil

1.3 · 196.4 g cm^3 = 255.32 grams (g) dry soil

Taking the dry weight of each sample into account for a soil moisture content of 28%, the following calculation was made;

$$1.1 \text{ g cm}^3 = 216.04 \cdot 1.28 = 276.5 \text{ grams (g) fresh soil}$$

$$1.3 \text{ g cm}^3 = 255.32 \cdot 1.28 = 326.8 \text{ grams (g) fresh soil}$$

After soil compaction each tube had a 250 µm diameter square mesh attached to the bottom of each sample to prevent any soil loss. The experiment was set up using a factorial design with two types of N inputs (slurry and Fresian cow urine), two bulk densities (1.1 g cm^3 and 1.3 g cm^3), two levels of DCD application (±DCD) and five (5) sampling points (0, 4, 8, 16 and 24 Days). Four (4) replicate soil columns per treatment were set up for each of the sampling points, resulting in a total of 160 soil columns.

5.2.2 Grass germination and sowing

Each replicate tube had 10, unsterilized pre-germinated, grass seeds (Perennial Ryegrass – *Lolium perenne*) planted to a depth of 1 cm. Pre-germination of the grass seeds was achieved by scattering seeds onto a moist paper towel, which was then placed onto aluminium foil and rolled so that all seeds were in darkness with the ends of each roll sealed by being folded over.

Grass seeds were germinated at 20ºC for 7 days so that primary and seminal roots were at ~10 mm in length, by this stage and the mesocotyl was also ~ 10 mm in length.

5.2.3 Grass Growth

Grass were grown under 6400K compact fluorescent lamp (CFL) (lumii) placed at a height of 700 mm from the surface of the soil in a dark room. At this height the soil surface received a photosynthetic active radiation (PAR) value of 150 µmol m^{-2} s^{-1} (Skye Instruments PAR Quantum Sensor). The light source used provided a light

wavelength of ~452 nm which lies within the blue part of the light spectrum. The reason this portion of the light spectrum was used was to (1) regulate the grass leaf elongation rate (Gautier and Varlet-Grancher. 1996) to enable a possible estimation of grass leaf elongation through nitrogen uptake. In theory, using this technique may be used to estimate the efficiency of dicyandiamide (DCD) application on nitrogen uptake and increase in grass yield; and (2), as plants typically respond to blue-light, i.e. photostimulation positively (growth towards light) in shoots, and negatively (growth away from light source) in roots (Kutschera and Briggs 2012; Briggs 2014; Liscum et al. 2014), using a light source with a wavelength in the blue part of the light spectrum is to achieve even grass root growth, as root growth was observed to be seriously effected in soils at higher bulk density in a preliminary experiment using standard mixed spectrum lighting (Appendix; Fig. 7.3). Grass was grown for two weeks, by which time grass length had reached ~ 200 mm. The leaves were then cut down to 40 mm in height in order to enhance further growth. After the grass had been cut, they were again left for a further week (7 days) to achieve a three leaf growth status.

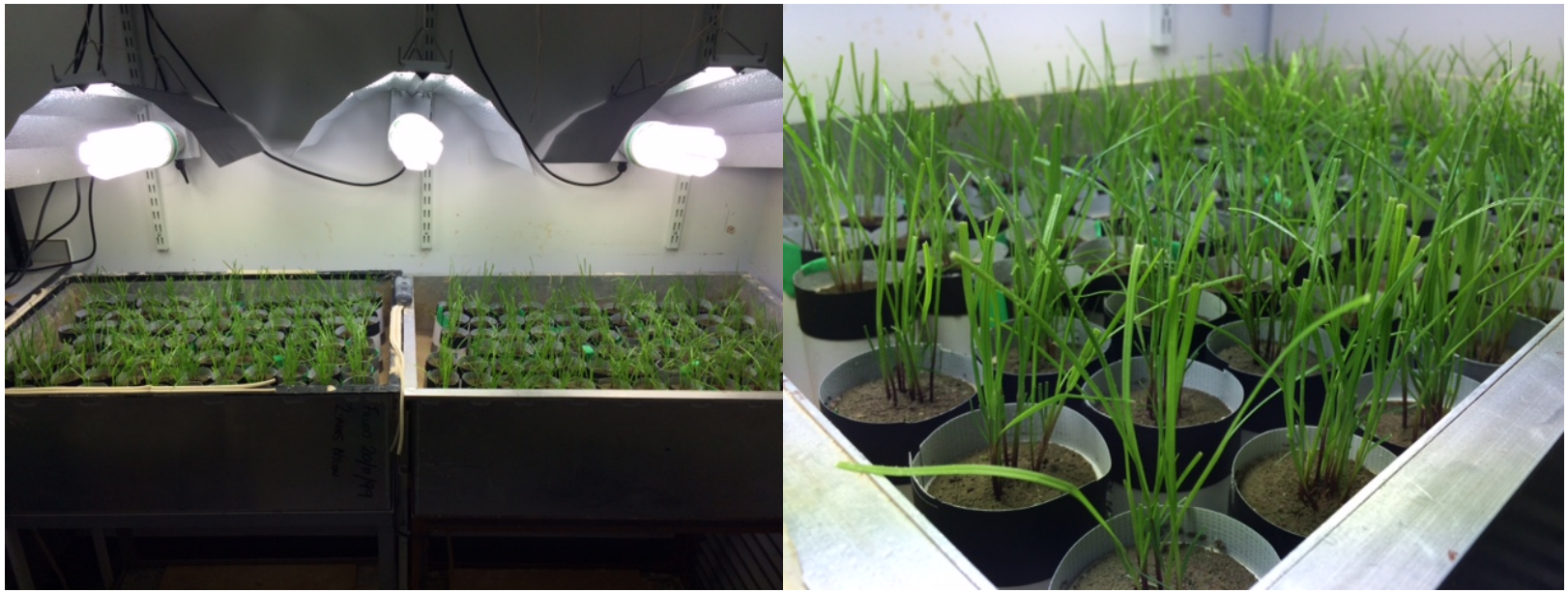


Figure 5.1. Grass mesocosms on sand tension tables set at -6kPa. Grasses were grown under cool white light that provides a light wavelength within the blue light spectrum. Samples were maintained at 20^{o}C for 24 Days after fertiliser application. As grass roots and the transpiration from grass leaves will have depleted water volume, the constant head (Jerry Can) container was maintained at a height between the middle of the soil columns and the top of the water level of 60 cm H_2O every day.

5.2.4 Fertiliser treatments

Fertiliser applications were applied at a rate consistent to accepted field application. Urine and slurry were applied at rates of 5 L m^{-2}, a typical application rate for urine deposition on grassland (Misslebrook et al. 2014) and slurry was applied at a rate of 50 m^3 ha^{-1} as suggested by DEFRA (RB209 Handbook). Therefore, using the calculations below the concentrations of fertiliser were calculated;

$$\text{Radius of soil PVC tube } \pi \cdot 2.5 \text{ cm}^2 = 19.64 \text{ cm}^2$$

$$\equiv 0.01964 \text{ m}^2$$

$$\equiv 0.0000001964 \text{ hectares}$$

As an application rate for urine of 5 L m^2, which is equivalent to 0.5 mL cm^{-2}, the following calculation was used to determine the concentrations, in liquid form, should be applied to each soil sample surface inside the universal tubes;

$$0.5 \text{ mL cm}^{-2} \cdot 19.6 \text{ cm}^2 = 9.8 \text{ mL per soil column (10 mL)}$$

For slurry the application rate was calculated as;

As 50 m^3 ha^{-1} is equivalent to 50,000 L ha^{-1}, the formula equals;

$$50{,}000 \text{ (L)} \cdot 0.0000001964 = 0.00982 \text{ (L)} = 9.82 \text{ mL (10 mL)}$$

In order to provide a weight of slurry 10 mL was weighed on a weighing balance to determine the weight equivalent of liquid slurry that was being applied. It was measured that 10 mL of slurry was equivalent to ten (10) g solid weight, typically the rate found for most liquid solutions.

5.2.5 DCD application

In order to replicate field conditions an application rate of 10 kg ha^{-1} DCD was applied to each sample receiving DCD treatments. The following calculation details the concentration of DCD applied to each sample receiving DCD treatment;

$$10 \text{ kg} \cdot 0.0000001964 = 0.000001964 \text{ (kg)} = 0.001964 \text{ (g)} = 1.964 \text{ (mg)}$$

This was rounded up to 2.0 mg per sample and was applied at a volume of 2 mL (1 mg DCD mL^{-1}).

5.2.6 Ammonium analysis

Using the method of Kandeler. (1996), Ten (10) g of fresh soil were weighed into a 50 mL centrifuge tube (Fisher Scientific, UK) to which 20 mL of 2M potassium chloride (KCl) was added. Samples were shaken for 2 hours on an orbital shaker. Samples were then centrifuged for 10 mins at 4500 r.p.m and filtered through a Merck Millipore syringe filter (0.22µm) (Merck, UK) into a 25 mL universal tube. Once filtered, 2.5 mL of sample was pipetted into a 4 mL cuvette which contained a 1.2 mL of a solution of 0.3M sodium hydroxide (NaOH), 1.06 sodium salicylate solution and de-ionised H_2O. Finally, 0.8 mL (800 µl) of sodium dichloroisocyanurate was added

to bring about colour development. Samples were left to stand for 30 mins at room temperature before being measured on a Hitachi U-2010 spectrophotometer at 660 nm.

5.2.7 Total carbon (TC), dissolved organic carbon (DOC), inorganic carbon (IC) and total nitrogen (TN)

Five (5) g of fresh soil were weighed into a 50 mL centrifuge tube and 25 mL of potassium sulphate solution (K_2SO_4) was added. The resultant soil slurry was then shaken at 300 r.p.m for 30 mins at room temperature to extract total carbon and total nitrogen. The soil slurries were then centrifuged at 4500 r.p.m for 10 minutes to separate the soil and extractant. After centrifugation, the samples were filtered through a Millipore syringe filter (0.22µm) (Merck, UK), in order to remove particulate organic carbon which are above 0.22µm, allowing the estimation of the dissolved organic fraction within the soil sample. Samples were diluted 1:10 with MilliQ de-ionised water into 30 mL glass test tubes and measured for TOC (DOC), TC, IC and TN on a Shimadzu TOC:TN analyser.

5.2.8 Soil potential nitrification activity

Using the method developed by Kandeler (1996), five (5) g soil were suspended in 20 ml of a 2 mM ammonium sulphate $(NH4)_2SO_4$ and 10 mM sodium chlorate $NaClO_3$ (an inhibitor of nitrite oxidation) solution. Samples were shaken for 5 hrs at 200 r.p.m at 25°C. After the 5 hr incubation, soil sample slurries and controls were extracted with 2 M potassium chloride (KCl) (Fisher Scientific, UK) by shaking and then centrifuged at 4500 r.p.m. for 5 min and filtered through a Millipore syringe filter (0.22µm) (Merck, UK). Sample supernatants were analysed for nitrite using the Griess colorimetric (Shinn. 1941) procedure. Briefly, 2.35 mL of sample was mixed with 1.2

mL colour reagent, 2 g of sulphanilamide ($C_6H_8N_2O_2S$) (Sigma-Aldrich) and 0.1 g N-(1-naphthyl)-ethylenediamine hydrochloride ($C_{10}H_7NHCH_2NH_2 \cdot 2HCl$) (Sigma-Aldrich, UK) in 150 ml distilled water and 20 ml concentrated phosphoric acid (H_3PO_4), 480 µl of 0.1% sodium dichloroisocyanurate ($C_3Cl_2N_3NaO_3$) (Sigma-Aldrich, UK) was then added. Samples were left to stand at room temperature for 30 mins, in order to allow full colour development and analysed spectrophotometrically at 520 nm using a Hitachi U-2010 spectrophotometer. The colour for the assay, once developed, was stable for 4 hours.

5.2.9 Soil Nitrate Reductase Activity

Using the method developed by Kandeler (1996), 5 (g) of fresh soil weighed into a 50 mL falcon tube. Four (4) mL of 2,4 Dinitrophenol with a concentration of 10 µg per mL, (40 µg in total) (concentration determined by a preliminary experiment; Appendix, Fig. 7.4) was added to inhibit the reduction of nitrite (NO_2^-) to nitric oxide (NO) by the denitrifying nitrite reductase (*nir*S/K) enzyme. The soil was then pre-incubated for 16h at 25ºC. After pre-incubation, soil samples were supplemented with 1 mL substrate solution (25mM potassium nitrate (KNO_3)) and 5 mL of de-ionised H_2O.

After incubation 10 mL of 4M potassium chloride (KCl solution was added to each of the samples. These were mixed briefly and centrifuged at 5000 r.p.m and filtered through a Millipore syringe filter (0.22µm) (Merck, UK). Five (5) mL of supernatant was mixed with 3 mL ammonium buffer and 2 mL colour reagent. Nitrite concentrations were analysed spectrophotometrically at 520 nm.

5.2.10 Gas analysis

The following calculation was used to determine the rate of gas flux μg m^{-2} h^{-1} and amount of N_2O-N;

$$N_2O\ (\mu g\ mL^{-1}) \cdot 137.5\ mL = \text{Mass of } N_2O\ (\mu g\ \text{volume}^{-1}\ \text{headspace})$$

Where N_2O (μg mL^{-1}) is the measured concentration of N_2O taken from the GC and 137.5 mL is equal to the headspace in the sampling chamber. Gas flux was then calculated using the following formula;

$$\text{Gas Flux (mg m}^2\ \text{h}^{-1}) = \frac{\delta c}{\delta t} \cdot \frac{N_2O\ (\mu g) \cdot \rho \cdot Mw(N_2O-N)}{R \cdot T \cdot A}$$

Where ρ = atmospheric air pressure (101.3000k Pa), C = concentration of N_2O in the sample (μg), Mw = molecular weight of N_2O-N = 44.013 g mol^{-1} · 0.6365 (0.6365 is the percentage of N in N_2O which is equal to 28.0143 g mol^{-1}), R = universal gas coefficient (8.31 J mol^{-1} K^{-1}), T = Temperature at which gas samples were taken in Kelvin (273.15°K + 20°C) and A = soil microcosm surface area (m^2). Samples were analysed at (t) = 0 (background) and (t) = 60 (hourly N_2O flux) minuetes.

5.2.11 Water-filled pore space

Water-filled pore space was calculated as;

WFPS = (%MC (θg) · Dry Bulk Density (ρ))/ (Dry Bulk Density (ρ)/(Particle Density (P_D)) (ρ)), Effectively;

WFPS (ϕ_{WF}) = Volumetric Water Content (θ_w) /Total Porosity (ϕ);

$$\phi_{WF} = \theta_w/\phi$$

5.2.12 Soil pH

Five (5) g of fresh soil sample were weighed into a 50 mL centrifuge tube (Fisher Scientific) which had 12.5 mL de-ionised H_2O added (1:2.5 ratio). Samples were shaken on an orbital shaker at 200 r.p.m for 30 minutes. Soil pH was then measured using a benchtop pH meter and probe (HANNA instruments) calibrated with pH calibration standards (pH 7.01 and 4.0).

5.2.13 Statistical analysis

Data were analysed using Genstat 17th edition (VSN International, Hemel Hempstead, UK). For gas fluxes data were analysed using fertiliser, bulk density, DCD and Day as factors in a four-way ANOVA, sample depth was not included in this particular ANOVA model as sample gas fluxes were taken from the whole column sample.

All other variables (NH_4^+, NO_3^-, total dissolved carbon, dissolved organic carbon, dissolved inorganic carbon, total nitrogen, %WFPS, pH, potential nitrification activity and nitrate reductase activity) were analysed in a four-way ANOVA using bulk density, DCD treatment, sampling depth and Day as factors. As the data set was quite large, a one-way ANOVA was used to discern the differences between urine and slurry treatments. The aforementioned four-way ANOVA was then performed on urine and slurry treatments separately. LSDs were used to distinguish differences between mean values between treatments. Where appropriate (N_2O fluxes), 2 times the standard error

of the mean (SEM) was used to distinguish significant differences between sample mean values (Cumming et al. 2007).

5.3. RESULTS

5.3.1 Nitrous oxide (N_2O) flux

Soil nitrous oxide fluxes (N_2O-N) varied with both fertiliser ($P < 0.001$), Bulk Density ($P = 0.013$) and Day ($P = 0.016$) (Table 5.2) with greater fluxes observed in the urine treated soils. Variation in soil N_2O fluxes related to bulk density were observed at the start of the experiment (Day 0) before urine and slurry treatments were added with the 1.3 g cm^3 generally higher than the 1.1 g cm^3 (Table. 5.1). Urine produced significantly higher fluxes of N_2O in the urine treated soils than the slurry treated soils (Table 5.1; Table 5.2), with peak fluxes occurring on Day 4. Highest peak fluxes were observed in the 1.3 g cm^3 Bulk Density treatment for urine treated samples, with no significant variation between DCD treatments. A large flux of N_2O-N was observed for the 1.3 g cm^3 Urine + DCD treatment (363.1 N_2O-N mg m^{-2} h^{-1}), however, this value was 5.5 times higher than the average values observed for the 1.3 g cm^3 Urine + DCD treatment and therefore considered an outlier with the data and excluded from the mean data set for Day 4. Significant differences between cumulative N_2O fluxes Urine and Slurry treatment for both no-DCD and DCD treated soils were observed (Table 5.2. $P < 0.001$; Fig 5.2, $P \leq 0.05$).

Table 5.1. N_2O fluxes from soils treated with Urine and Slurry during the five day sampling period (0, 4, 8, 16 and 24 Days). Sample mean values are displayed with ± standard error of the mean (SEM). n = (4). Outlier for Urine + DCD 1.3 g cm^3 363.1 N_2O-N mg m^{-2} h^{-1}.

Soil Treatment & Day	Urine N_2O-N (mg m^{-2} h^{-1})					Slurry N_2O-N (mg m^{-2} h^{-1})				
	0	4	8	16	24	0	4	8	16	24
1.1. g cm^3 No DCD	20.2 ± 9.0	25.0 ± 3.7	33.9 ± 7.5	6.7 ± 0.4	46.9 ± 15.0	0.9 ± 0.2	4.2 ± 0.9	0.7 ± 0.5	0.5 ± 0.2	0.4 ± 0.6
1.3. g cm^3 No DCD	6.0 ± 0.4	54.0 ± 9.1	26.8 ± 3.8	31.0 ± 4.4	21.7 ± 2.9	6.1 ± 0.4	11.3 ± 1.5	5.3 ± 0.8	4.2 ± 1.1	0.2 ± 0.1
1.1 g cm^3 DCD	8.0 ± 1.8	14.4 ± 3.7	15.5 ± 4.0	3.1 ± 0.5	-3.8 ± 2.9	-0.3 ± 0.2	1.6 ± 0.4	0.9 ± 0.3	-3.5 ± 1.8	0.5 ± 0.5
1.3 g cm^3 DCD	10.7 ± 1.7	59.0 ± 9.2	53.0 ± 16.2	2.6 ± 0.7	3.4 ± 0.8	17.2 ± 2.7	5.5 ± 0.3	3.1 ± 0.6	-0.3 ± 2.6	0.1 ± 0.5

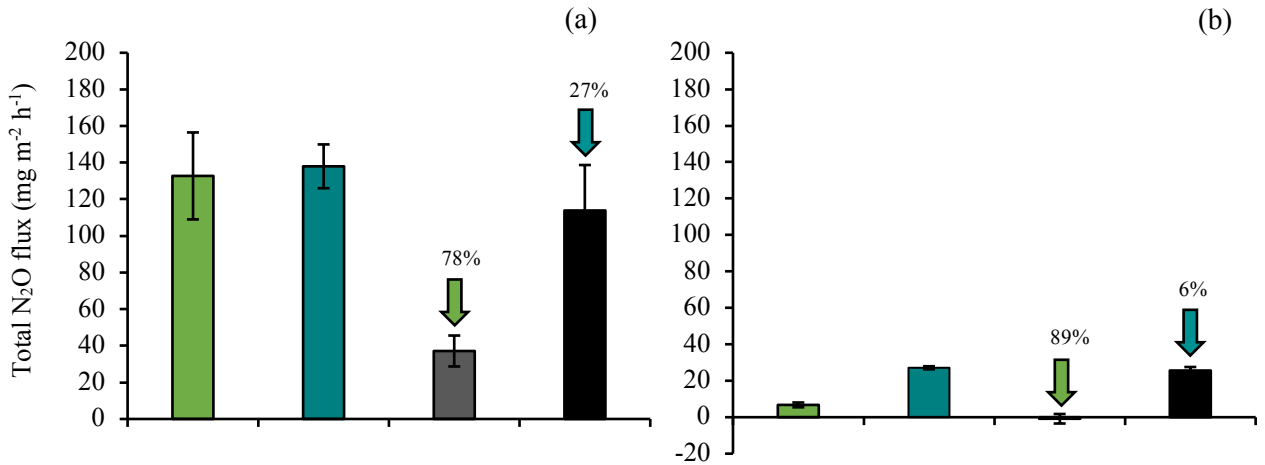


Figure 5.2. Graphs for cumulative N_2O fluxes 1.1 g cm^3 + No DCD (green bars), 1.3 g cm^3 + No DCD (teal bars), 1.1 g cm^3 + DCD (grey bars) and 1.3 g cm^3 + DCD (black bars). Arrows indicate increases due to DCD treatment when compared to respective bulk density treatments. Error bars represent standard error of means (SEM).. n = (4).

Table 5.2. ANOVA output for N_2O and cumulative N_2O emissions. Missing values for cumulative N_2O are due to the elimination of Day from the ANOVA factorial model, as cumulative flux analysis was only conducted on samples for Day 24. Significant values $P \leq 0.05$ and higher are highlighted in bold.

Effect	N_2O ($mg\ m^{-2}\ h^{-1}$)		Cumulative N_2O ($mg\ m^{-2}\ h^{-1}$)	
	F	*P*	F	*P*
Fertiliser	22.62	**< 0.001**	10.47	**< 0.001**
Bulk Density	6.55	**0.013**	0.19	0.099
DCD	0.40	0.530	0.00	0.099
Day	3.31	**0.016**	-	-
Fertiliser x Bulk Density	2.34	0.132	0.33	0.567
Fertiliser x DCD	0.20	0.652	0.15	0.705
Bulk Density x DCD	2.49	0.120	1.01	0.321
Fertiliser x Day	2.39	0.060	-	-
Bulk Density x Day	2.50	0.052	-	-
DCD x Day	1.18	0.331	-	-
Fertiliser x Bulk Density x DCD	2.07	0.156	0.06	0.807
Fertiliser x Bulk Density x Day	2.29	0.070	-	-
Fertiliser x DCD x Day	1.52	0.209	-	-
Bulk Density x DCD x Day	0.96	0.438	-	-
Fertiliser x Bulk Density x DCD x Day	1.29	0.286	-	-

5.3.2 Carbon dioxide (CO_2) Fluxes

Soil CO_2 fluxes demonstrated significant variations in relation to interactions between Bulk Density x DCD and DCD x Day (Table 5.3, $P = 0.014$ and $P = 0.028$ respectively). DCD significantly increased CO_2 fluxes between Bulk Density treatments (1.1 g cm^3 and 1.3 g cm^3) on Day 24 for urine treated samples. (Fig.5.3. (a-b); $P \leq 0.05$). No significant variation was observed for Slurry treated samples. The most notable response was the variations between both Bulk Density and DCD treatments for cumulative CO_2 fluxes. Both Bulk Density and Day significantly increased sample CO_2 fluxes (Table 5.3, $P < 0.001$ and $P < 0.001$ respectively; Fig. 5.3. (c-d), $P \leq 0.05$).

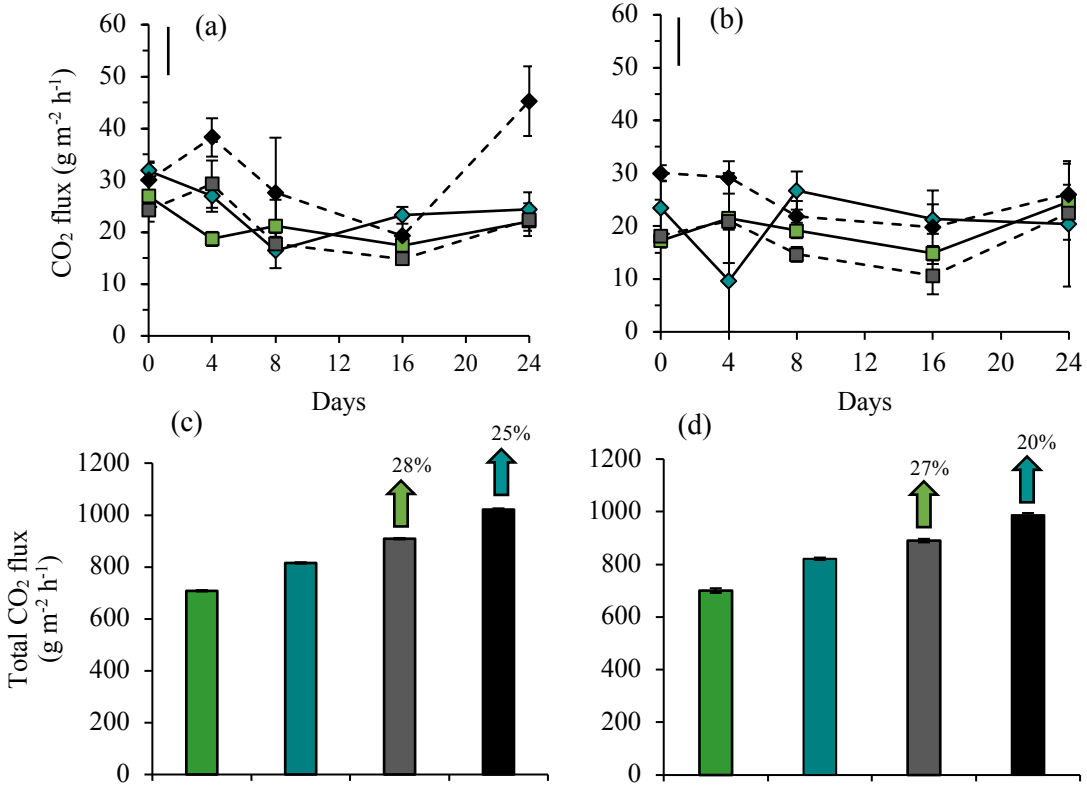


Figure 5.3. Graphs for CO_2 flux for Urine treatment (a) and Slurry treatment (b). Total CO_2 fluxes are shown in graphs (c) and (d) 1.1 g cm^3 + No DCD (green squares with solid lines), 1.3 g cm^3 + No DCD (teal diamonds with solid lines), 1.1 g cm^3 + DCD (grey squares with dashed lines) and 1.3 g cm^3 + DCD (black diamonds with dashed lines). For cumulative fluxes 1.1 g cm^3 + No DCD (green bars), 1.3 g cm^3 + No DCD (teal bars), 1.1 g cm^3 + DCD (grey bars) and 1.3 g cm^3 + DCD (black bars). Arrows indicate increases due to DCD treatment when compared to respective bulk density treatments. Error bars represent standard error of means (SEM). LSD bars represent 95% confidence ($P < 0.05$) and demonstrate the difference for each variable for each N treatment at both soil depth treatments (i.e. LSD show the difference between 0-5 and 5-10 cm sample measurements). n = (4)

Table 5.3. ANOVA output for CO_2 and cumulative CO_2 emissions. Missing values for cumulative CO_2 are due to the elimination of Day from the ANOVA factorial model, as cumulative flux analysis was only conducted on samples for Day 24. Significant values $P \leq 0.05$ and higher are highlighted in bold.

Effect	CO_2 ($g\ m^{-2}\ h^{-1}$)		Cumulative CO_2 ($g\ m^{-2}\ h^{-1}$)	
	F	*P*	F	*P*
Fertiliser	8.76	**0.004**	2.25	0.140
Bulk Density	15.54	**< 0.001**	82.02	**< 0.001**
DCD	3.64	0.059	221.11	**< 0.001**
Day	4.81	0.001	-	-
Fertiliser x Bulk Density	0.72	0.399	0.01	0.917
Fertiliser x DCD	0.75	0.387	0.58	0.452
Bulk Density x DCD	6.20	**0.014**	0.06	0.809
Fertiliser x Day	0.97	0.427	0.32	-
Bulk Density x Day	0.21	0.932	-	-
DCD x Day	2.82	**0.028**	-	-
Fertiliser x Bulk Density x DCD	0.00	0.986	0.22	0.644
Fertiliser x Bulk Density x Day	1.77	0.139	-	-
Fertiliser x DCD x Day	0.97	0.427	-	-
Bulk Density x DCD x Day	0.72	0.582	-	-
Fertiliser x Bulk Density x DCD x Day	1.23	0.303	-	-

Table 5.4. One-way ANOVA output for variation between urine and slurry treatments on soil variables at different soil sampling depths. Significant values of $P \leq 0.05$ and higher are highlighted in bold.

Source	N_2O	CO_2	NA	NR	NH_4^+	NO_3^-	DOC	IC	TN	WFPS	pH
Fertiliser Treatment (0-5 cm)	**< 0.001**	0.361	**0.011**	**< 0.001**	**< 0.001**	**< 0.001**	0.450	0.538	**< 0.001**	**< 0.001**	**0.003**
Fertiliser Treatment (5-10 cm)			0.358	**< 0.001**	**0.003**	**0.008**	0.565	**0.003**	**< 0.001**	**< 0.001**	**0.022**

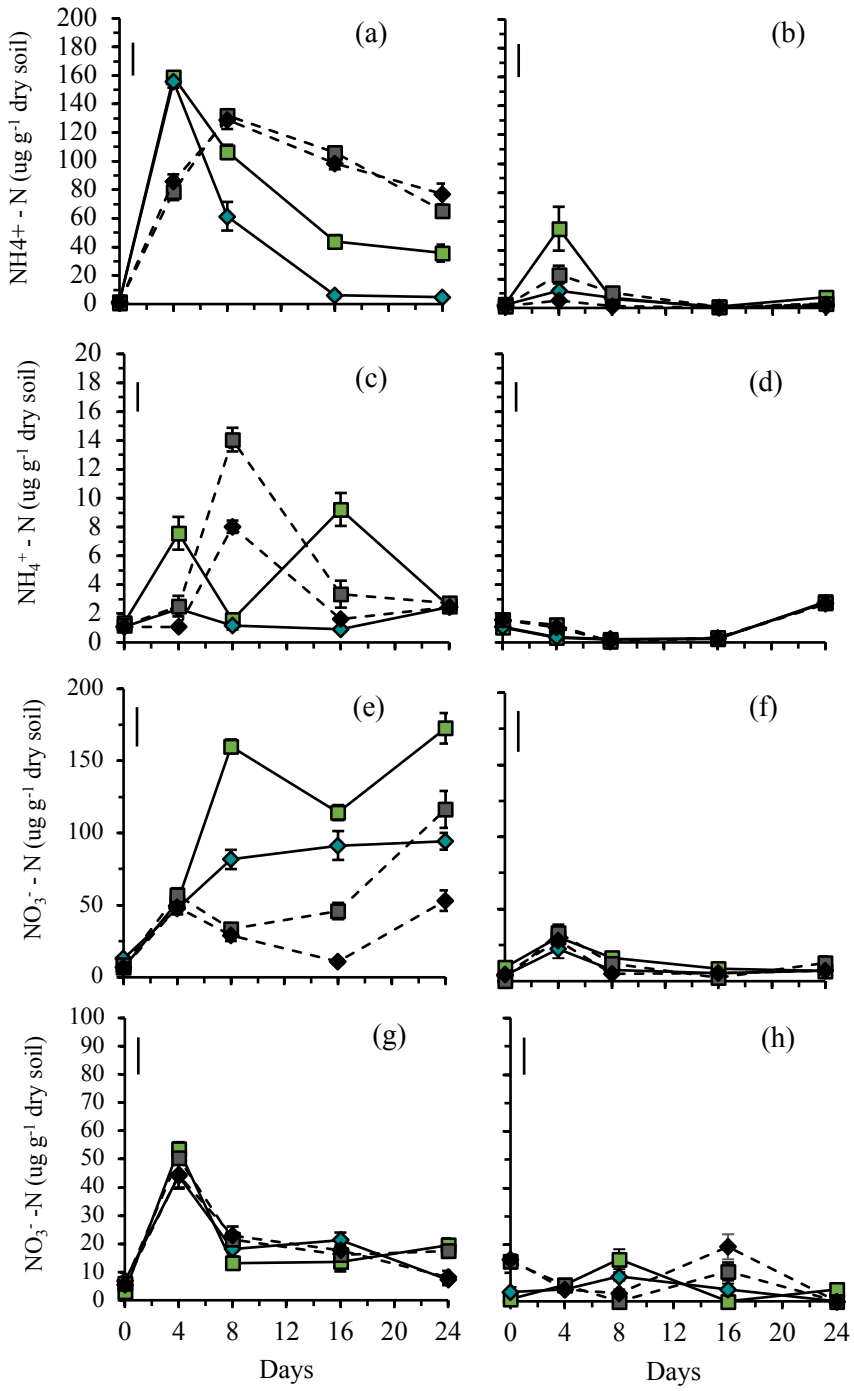


Figure 5.4. Graphs for NH_4^+ for urine treatment 0-5 cm (a); 5-10 cm (b) and slurry treatment 0-5 cm (c); 5-10 cm (d). Graphs for NO_3^- for urine treatment 0-5 (e); 5-10 (f) and slurry treatment 0-5 cm (g); 5-10 (h). 1.1 g cm^3 + No DCD (green squares with solid lines), 1.3 g cm^3 + No DCD (teal diamonds with solid lines), 1.1 g cm^3 + DCD (grey squares with dashed lines) and 1.3 g cm^3 + DCD (black diamonds with dashed lines). Error bars represent standard error of means (SEM). LSD bars represent 95% confidence ($P < 0.05$) and demonstrate the difference for each variable for each N treatment at both soil depth treatments (i.e. LSD show the difference between 0-5 and 5-10 cm sample measurements). n = (4). Values for mean data points are presented below in Tables 5.6, 5.7, 5.8 and 5.9.

5.3.3 Ammonium (NH_4^+)

5.3.3.1 Urine treated samples

Soil ammonium concentrations demonstrated significant increases between urine and slurry treatements (Table 5.4, $P < 0.001$; Fig. 5.4). Similar increases in NH_4^+ were observed for the urine 1.1 and 1.3 g cm^3 + No DCD treatment for the 0-5 cm layer samples on Day 4; 159.1 (± SE 1.9; Table. 5.6) µg g^{-1} dry soil and 155.9 (± SE 4.3; Table. 5.6) µg g^{-1} dry soil for the 1.1 and 1.3 g cm^3 treatments respectively. Significant variations in soil NH_4^+ were observed in the 0-5 cm layer samples between the 1.1 g cm^3 and 1.3 g cm^3 + No DCD treatments from Day 8 until Day 24 (Fig. 5.4 (a); $P \leq 0.05$) as demonstrated by the interaction between Bulk Density x Depth x Day ($P = 0.020$). NH_4^+ concentrations in the 5-10 cm layer samples also demonstrated peaks in on Day 4, but with concentrations significantly lower than their respective treatments in the 0-5 cm layer soils (Fig 5.4 (a) and (b); $P \leq 0.05$). Reductions in NH_4^+ were observed for both bulk densities after Day 4. NH_4^+ concentrations declined by 67% for the 1.1 g cm^3 + No DCD treatment, from 159.1 (± SE 1.9) to 106. 6 (± SE 5.0) (Table 5.6) µg g^{-1} dry soil in the 0-5 layer. Sample NH_4^+ concentrations were observed to decrease faster for the 1.3 g cm^3 treatment by 2.5 times, from 155.9 µg g^{-1} dry soil (± SE 4.3) to 61.5 (± SE 10.0) µg g^{-1} dry soil than the 1.1 g cm^3 samples. After Day 8, NH_4^+ concentrations significantly reduced for both bulk density treatments for the urine + No DCD treatment (Fig. 5.4; $P \leq 0.05$) until the end of the experiment (Day 24), with the 1.3 g cm^3 bulk density for the urine + no DCD returning to concentrations similar to what were measured on Day 0 of the experiment (4.7 ± SE 1.5 µg g^{-1} dry soil).

A significant interaction between Bulk Density x DCD (Table 5.5, $P = 0.007$) and DCD x Depth x Day (Table 5.5, $P < 0.001$) were observed for NH_4^+ concentrations in

the urine treated samples in the 0-5 cm layer (Fig. 5.4 (a); $P \leq 0.05$). The application of DCD eliminated the impact of Bulk Density on the variability of NH_4^+ concentrations as demonstrated by the significant interaction between Bulk Density x DCD ($P = 0.007$; Table. 5.5) with no observed significant variation between the two bulk density treatments when DCD was applied in both the 0-5 cm and 5-10 cm layer (Fig. 5.4). With the exception of the 1.1 g cm^3 + DCD on Day 8, all DCD treated samples demonstrated significant variations in soil NH_4^+ concentrations when compared to non-DCD treated samples for the 0-5 cm layer samples. 50% reductions (79.0 ± SE 6.3 μg g^{-1} dry soil) were observed for the 1.1 g cm^3 treatment and 45% reductions (86.1 ± SE 4.7 μg g^{-1} dry soil) observed for the 1.3 g cm^3 treatment on Day 4 of the experiment when compared to non-DCD treated samples (Fig 5.4 (a); $P \leq 0.05$). From Day 8 onwards DCD treatment significantly ($P \leq 0.05$; Fig. 5.4 (a)) increased the NH_4^+ concentrations in urine treated samples in comparison to non-DCD treated samples until the end of the experiment.

5.3.3.2. Slurry Treated Samples

NH_4^+ concentrations in the slurry treated samples were significantly lower than those observed in the urine treated samples (Table 5.5, $P < 0.001$; Fig 5.4), with NH_4^+ concentrations 20 times lower on Day 4 for 1.1 g cm^3 no DCD treated soils and > 60 times lower than the 1.3 g cm^3 30 times lower for all the other treatments than urine treated soils. Interactions between Bulk Density x DCD x Depth x Day (Table 5.5, $P = 0.001$) were observed in the 0-5 cm samples (Fig 5.4 (c)) with no significant changes in soil NH_4^+ concentrations were observed in the 5-10 cm layer. Sample NH_4^+ concentrations fluctuated in the 1.1 g cm^3 + No DCD producing significantly higher concentrations of NH_4^+ than all other treatments on Days 4 and 16 (Fig. 5.4; $P \leq 0.05$).

Highest mean concentrations of NH_4^+ on Day 8 were observed for DCD treated samples for both bulk density treatments (Fig. 5.4; $P \leq 0.05$). By Day 24 NH_4^+ concentrations intersected for all sample treatments in the 0-5 cm layer.

Table 5.5. ANOVA output for ammonium (NH_4^+), Nitrate (NO_3^-) and total nitrogen. Significant values of $P \leq 0.05$ and higher are highlighted in bold.

Source		NH_4^+				NO_3^-				Total Nitrogen			
		Urine		Slurry		Urine		Slurry		Urine		Slurry	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
BD	1	12.87	**< 0.001**	26.69	**< 0.001**	26.27	**< 0.001**	0.18	0.675	35.12	**< 0.001**	6.70	**0.012**
DCD	1	8.46	**0.005**	4.67	**0.035**	46.05	**< 0.001**	1.45	0.233	1.84	0.180	0.69	0.411
Depth	1	478.36	**< 0.001**	106.81	**< 0.001**	287.16	**< 0.001**	24.33	**< 0.001**	631.72	**< 0.001**	283.04	**< 0.001**
Day	4	89.75	**< 0.001**	7.82	**< 0.001**	28.87	**< 0.001**	0.08	0.780	66.81	**< 0.001**	8.89	**< 0.001**
BD x DCD	1	1.08	**0.007**	0.90	0.347	1.59	0.213	2.28	0.071	0.30	0.589	0.43	0.513
BD x Depth	1	0.32	0.576	26.97	**< 0.001**	18.34	**< 0.001**	0.61	0.437	2.49	0.120	3.75	0.058
DCD x Depth	1	19.58	**< 0.001**	1.58	0.214	48.92	**< 0.001**	0.27	0.608	0.20	0.655	0.10	0.753
BD x Day	4	1.08	0.375	4.22	**0.005**	4.37	**0.004**	2.28	0.071	2.60	**0.045**	1.71	0.160
DCD x Day	4	33.60	**< 0.001**	22.70	**< 0.001**	9.41	**< 0.001**	1.68	0.166	1.55	0.201	1.06	0.382
Depth x Day	4	45.61	**< 0.001**	26.48	**< 0.001**	32.12	**< 0.001**	25.43	**< 0.001**	24.45	**< 0.001**	10.43	**< 0.001**
BD x DCD x Depth	1	3.58	0.063	1.21	0.276	0.70	0.406	0.63	0.430	0.47	0.497	0.53	0.469
BD x DCD x Day	4	0.58	0.679	4.79	**0.002**	1.41	0.241	0.17	0.954	0.98	0.426	0.84	0.503
BD x Depth x Day	4	3.15	**0.020**	4.40	**0.004**	4.71	**0.002**	0.61	0.657	1.17	0.332	2.37	0.063
DCD x Depth x Day	4	18.21	**< 0.001**	27.81	**< 0.001**	9.09	**< 0.001**	3.23	**0.018**	1.80	0.140	0.63	0.642
BD x DCD x Depth x Day	4	1.26	0.296	5.18	**0.001**	2.07	0.096	0.24	0.913	0.65	0.630	0.85	0.502
Total	157												

5.3.4.Nitrate (NO_3^-)

5.3.4.1 Urine treated samples

As observed for the NH_4^+ concentrations, significant interactions between Bulk Density x Depth x Day and DCD x Depth x Day were observed for nitrate concentrations in urine treated samples (Table 5.5, $P = 0.002$ and $P < 0.001$, respectively). Both 1.1 g cm^3 + No DCD and 1.1 g cm^3 + DCD demonstrated significantly higher (Fig. 5.4 (e); $P \leq 0.05$) increases in soil NO_3^- concentrations when compared to their 1.3 g cm^3 counterparts. By Day 8, nitrate concentrations reached 160.1 (± SE 4.8) μg g^{-1} dry soil, 2 times greater than the 1.3 g cm^3 bulk density. With the exception of Day 16, NO_3^- concentrations remained similar (Fig. 5.4 (e); $P > 0.05$) for the duration of the experiment for the 1.1 g cm^3 + No DCD treatment, however, with 1.3 g cm^3 + No DCD treated samples, nitrate concentrations started to plateau after Day 8 (Fig. 5.4 (e); $P \leq 0.05$) DCD had no effect on reducing differences in NO_3^- concentrations between bulk density treatments with bulk density treatment producing similar patterned differences as observed for non-DCD treated samples (Fig 5.4). DCD treatment reduced NO_3^- concentrations by 4.8 times in the 1.1 g cm^3 treatment ($P \leq 0.05$) and by 2.8 times in the 1.3 g cm^3 bulk density ($P \leq 0.05$) when comparing NO_3^- levels between sample treatments on Day 8.

NO_3^- concentrations were significantly lower in the 5-10 cm layer in comparison to the 0-5 cm soil ($P \leq 0.05$) on Day 8 for both sample Bulk Density treatments in the non-DCD treated samples (Fig. 5.4 (e) and (f)). Significant increases of ammonium concentrations were only observed on Days 16 and 24 for the 1.1 g cm^3 + DCD treated samples in the 0-5 cm soils

(Fig 5.4; $P \leq 0.05$) when compared to all sample treatments in the 5-10 cm layer. Unlike NH_4^+ DCD had no effect in reducing variation in soil NO_3^- concentrations as a result of soil bulk density treatment.

5.3.4.2. Slurry treated samples

Slurry treated soil NO_3^- concentrations differed significantly when compared to urine treated samples (Table 5.4; $P < 0.001$). Peak increases in NO_3^- concentrations were mainly observed on Day 4 for all sample treatments (Fig. 6.3), and for all sample treatments, which demonstrated similar concentrations to those observed in the urine treated samples (Fig. 5.4 (e) and (g)). Although a significant interaction between DCD x Depth x Day (Table 5.5, $P = 0.018$) was seen, which could explain the some of the variations in NO_3^- concentrations on Days 4 and 8 between the 0-5 cm and 5-10 cm samples, it was observed in the ANOVA result, it was mainly shown that Depth x Day (Table 5.5, $P < 0.001$) was the overriding factorial interaction that explained the variations in NO_3^- concentrations. Sample bulk density had no effect on NO_3^- concentrations in the slurry treated samples (Fig. 5.4 (g) and (e); $P > 0.05$).

5.3.5. Total Nitrogen

5.3.5.1. Urine treated samples

Urine treatment increased total nitrogen concentrations by over 1000 µg g^{-1} dry soil for all sample treatments (Fig 5.5). Urine treated soils produced significantly higher concentrations of total nitrogen in comparison to Slurry treated samples (Table 5.5; Fig 5.5). Both Bulk Density and Depth both had

a significant impact on total nitrogen concentrations, although this significantly decreased after the first measurement on Day 4 in the 0-5 cm soils (Table 5.5; Bulk Density x Day and Depth x Day; $P = 0.045$ and $P < 0.001$ respectively). 1.1 g cm^3 ($P \leq 0.05$), on Days 8 and 24 and Day 16 for non-DCD and DCD treated samples respectively. Total nitrogen concentrations demonstrated significant variations in in both 0-5 and 5-10 cm samples, however, this was only the case between the 1.1 g cm^3 + DCD treated samples on Day 4 for the 5-10 cm layer. Observed total nitrogen concentrations were generally significantly ($P \leq 0.05$) greater in the 0-5 cm soils and demonstrated significant variations between sample treatments during the course of the experiment (Fig. 5.5)

5.3.5.2. Slurry treated samples

Total nitrogen concentrations in the slurry treated sampled were 2.5 -3 times lower than urine treated samples (Fig. 5.5). Significant interactions between Depth x Day (Table. 5.5, $P < 0.001$) explained the variation in total nitrogen concentrations for Slurry treated samples, with total nitrogen concentrations demonstrating significant increases for both DCD treatments in the 1.1 g cm^3 treated samples. Total nitrogen was significantly reduced by the end of the experiment. No significant variation in total nitrogen was observed throughout the experiment in the 5-10 cm layer.

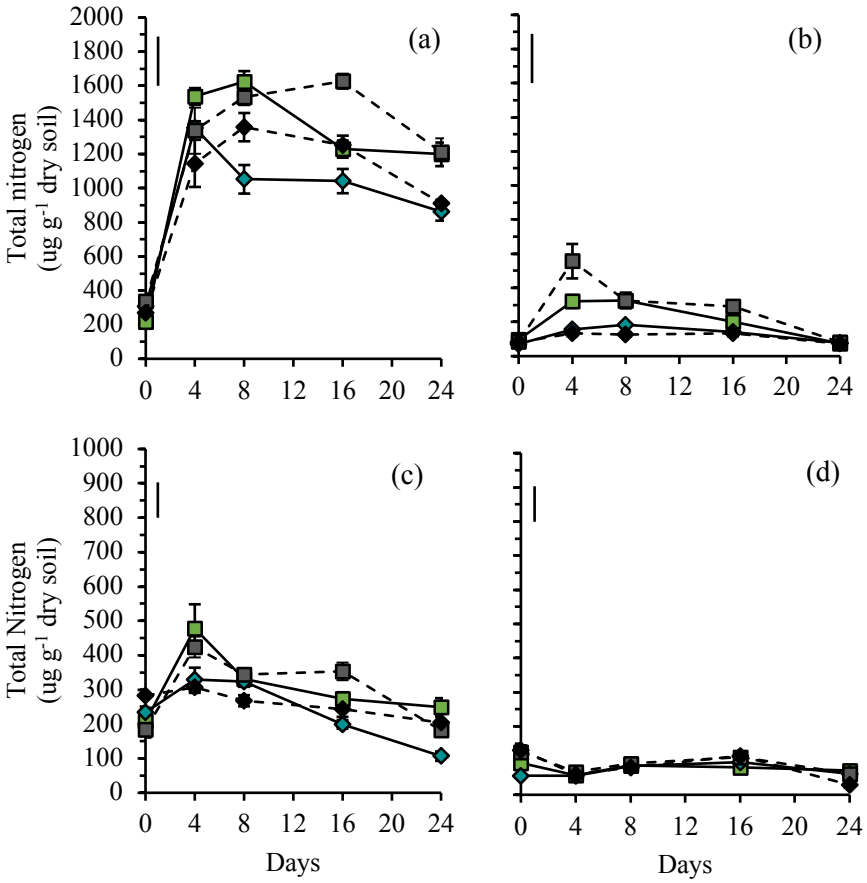


Figure 5.5. Graphs for total nitrogen for urine treatment 0-5 cm (a); 5-10 cm (b) and slurry treatment 0-5 cm (c); 5-10 cm (d). 1.1 g cm^3 + No DCD (green squares with solid lines), 1.3 g cm^3 + No DCD (teal diamonds with solid lines), 1.1 g cm^3 + DCD (grey squares with dashed lines) and 1.3 g cm^3 + DCD (black diamonds with dashed lines). Error bars represent standard error of means (SEM). LSD bars represent 95% confidence ($P < 0.05$) and demonstrate the difference for each variable for each N treatment at both soil depth treatments (i.e. LSD show the difference between 0-5 and 5-10 cm sample measurements). n = (4). Values for mean data points are presented below in Tables 5.6, 5.7, 5.8 and 5.9.

Table 5.6. Summary table for NH_4^+, NO_3^- and Total Nitrogen for urine treated soils in the 0-5 cm section of the soil columns. n = (4)

	NH_4^+ (µg g^{-1} dry soil)					**NO_3^- (µg g^{-1} dry soil)**					**Total Nitrogen (µg g^{-1} dry soil)**				
	Day					Day					Day				
Soil Treatments 0-5 cm	0	4	8	16	24	0	4	8	16	24	0	4	8	16	24
1.1 g cm^3 No DCD	1.9 ± 0.05	159.1 ± 1.9	106.6 ± 5.0	44.0 ± 4.4	35.7 ± 6.0	6.7 ± 1.6	52.2 ± 3.8	160.1 ± 4.8	114.2 ± 5.2	172.6 ± 10.6	218.2 ± 9.4	1538.1 ± 48.4	1624.4 ± 61.7	1229.1 ± 50.1	1198.5 ± 68.9
1.3 g cm^3 No DCD	1.9 ± 0.02	155.9 ± 4.3	61.5 ± 10.0	6.0 ± 1.8	4.7 ± 1.5	12.9 ± 1.1	48.0 ± 4.1	81.7 ± 6.7	91.4 ± 10.0	94.3 ± 5.8	308.8 ± 6.0	1353.4 ± 38.6	1052.1 ± 83.8	1041.6 ± 71.1	861.6 ± 52.1
1.1 g cm^3 DCD	1.0 ± 0.1	79.0 ± 6.3	132.0 ± 3.3	106 ± 4.0	65.4 ± 4.0	8.4 ± 1.0	57.1 ± 4.6	33.3 ± 3.6	46.0 ± 5.7	116.4 ± 12.8	336.7 ± 28.2	1337.0 ± 135.4	1533.7 ± 47.5	1626.6 ± 44.6	1.9 ± 0.02
1.3 g cm^3 DCD	1.0 ± 0.02	86.1 ± 4.7	129.0 ± 6.5	98.5 ± 4.1	77.1 ± 7.2	6.6 ± 1.9	48.7 ± 3.1	29.0 ± 3.8	10.9 ± 1.7	53.2 ± 7.1	268.6 ± 16.4	1145.4 ± 138.3	1357.7 ± 82.9	1253.7 ± 82.0	912.6 ± 19.3

Table 5.7. Summary table for NH_4^+, NO_3^- and Total Nitrogen for slurry treated soils in the 0-5 cm section of the soil columns. n = (4)

	NH_4^+ (µg g^{-1} dry soil)					**NO_3^- (µg g^{-1} dry soil)**					**Total Nitrogen (µg g^{-1} dry soil)**				
	Day					Day					Day				
Soil Treatments 0-5 cm	0	4	8	16	24	0	4	8	16	24	0	4	8	16	24
1.1 g cm^3 No DCD	1.4 ± 0.2	7.6 ± 1.1	1.6 ± 0.4	9.3 ± 1.1	2.5 ± 0.0	3.0 ± 0.6	53.4 ± 2.6	13.1 ± 2.3	13.6 ± 3.4	19.5 ± 2.5	207.2 ± 13.0	477.9 ± 70.3	331.0 ± 14.7	272.4 ± 20.1	248.2 ± 27.0
1.3 g cm^3 No DCD	1.1 ± 0.1	2.3 ± 0.1	1.2 ± 0.3	0.9 ± 0.1	2.5 ± 0.0	7.0 ± 0.9	44.0 ± 4.5	18.2 ± 2.9	21.4 ± 2.6	7.2 ± 1.8	234.1 ± 13.1	329.4 ± 34.5	323.9 ± 16.0	198.9 ± 12.2	106.6 ± 14.0
1.1 g cm^3 DCD	1.2 ± 0.1	2.5 ± 1.0	14.1 ± 1.0	3.4 ± 1.0	2.7 ± 0.1	5.9 ± 1.2	50.3 ± 2.3	21.6 ± 2.1	16.0 ± 3.4	17.5 ± 2.5	183.5 ± 24.0	424.0 ± 30.3	343.8 ± 17.2	353.1 ± 25.7	181.6 ± 17.4
1.3 g cm^3 DCD	1.1 ± 0.0	1.1 ± 0.0	8.0 ± 0.4	1.6 ± 0.1	2.5 ± 0.0	5.4 ± 0.5	44.3 ± 4.4	22.8 ± 3.3	17.8 ± 4.2	8.1 ± 2.3	282.3 ± 5.6	306.9 ± 16.2	267.7 ± 16.1	242.8 ± 21.9	203.8 ± 18.0

Table 5.8. Summary table for NH_4^+, NO_3^- and Total Nitrogen for Urine treated soils in the 5-10 cm section of the soil columns. n = (4). ND = not detected.

Soil Treatments 5-10 cm	**NH_4^+ (µg g^{-1} dry soil)**					**NO_3^- (µg g^{-1} dry soil)**					**Total Nitrogen (µg g^{-1} dry soil)**				
	Day					Day					Day				
	0	4	8	16	24	0	4	8	16	24	0	4	8	16	24
1.1 g cm^3 No DCD	2.6 ± 0.1	54.9 ± 15.2	6.2 ± 1.7	1.0 ± 0.1	7.7 ± 1.0	9.4 ± 2.7	30.5 ± 0.1	16.3 ± 3.8	8.4 ± 1.7	7.1 ± 2.1	94.5 ± 3.2	320.3 ± 37.5	326.1 ± 46.2	202.3 ± 13.0	74.2 ± 3.0
1.3 g cm^3 No DCD	2.1 ± 0.0	12.0 ± 1.7	7.6 ± 2.0	0.3 ± 0.0	1.4 ± 0.2	3.4 ± 2.0	22.3 ± 6.3	7.8 ± 1.8	5.6 ± 0.6	7.3 ± 2.1	71.1 ± 3.2	157.6 ± 5.1	184.0 ± 18.2	142.9 ± 5.6	76.0 ± 3.2
1.1 g cm^3 DCD	1.2 ± 0.0	23.2 ± 6.1	10.7 ± 2.2	0.8 ± 0.2	2.9 ± 0.0	ND	33.7 ± 2.4	12.4 ± 2.0	2.2 ± 0.7	12.7 ± 4.4	83.4 ± 0.3	556.0 ± 100.9	323.7 ± 35.4	292.4 ± 18.7	81.1 ± 2.5
1.3 g cm^3 DCD	1.1 ± 0.0	5.1 ± 1.3	1.1 ± 0.4	0.2 ± 0.0	2.7 ± 0.0	4.9 ± 2.4	28.1 ± 6.4	5.3 ± 0.4	5.6 ± 1.4	7.4 ± 2.1	81.1 ± 3.3	133.6 ± 16.0	127.5 ± 12.3	133.7 ± 17.0	73.1 ± 3.7

Table 5.9. Summary table for NH_4^+, NO_3^- and Total Nitrogen for Slurry treated soils in the 5-10 cm section of the soil columns. n = (4). ND = not detected.

Soil Treatments 5-10 cm	**NH_4^+ (µg g^{-1} dry soil)**					**NO_3^- (µg g^{-1} dry soil)**					**Total Nitrogen (µg g^{-1} dry soil)**				
	Day					Day					Day				
	0	4	8	16	24	0	4	8	16	24	0	4	8	16	24
1.1 g cm^3 No DCD	1.0 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	2.8 ± 0.0	0.9 ± 0.5	5.7 ± 0.0	14.9 ± 3.5	ND	4.6 ± 2.1	93.2 ± 13.1	57.0 ± 3.1	85.7 ± 4.0	80.6 ± 2.8	70.3 ± 5.2
1.3 g cm^3 No DCD	1.1 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	2.7 ± 0.1	3.4 ± 1.7	4.1 ± 0.3	8,8 ± 1.5	4.2 ± 2.1	ND	56.1 ± 3.0	56.0 ± 3.1	83.2 ± 6.3	95.5 ± 7.0	62.3 ± 4.3
1.1 g cm^3 DCD	1.6 ± 0.1	1.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	2.7 ± 0.0	14.1 ± 2.4	5.7 ± 0.4	ND	10.6 ± 3.1	ND	126.1 ± 18.5	67.8 ± 5.6	91.1 ± 1.0	110.9 ± 2.6	61.7 ± 2.5
1.3 g cm^3 DCD	1.6 ± 0.0	1.1 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	2.7 ± 0.0	14.8 ± 1.0	4.3 ± 0.4	2.9 ± 1.4	19.2 ± 4.4	ND	131.6 ± 3.4	60.0 ± 2.5	80.5 ± 3.6	114.6 ± 3.7	29.0 ± 8.4

Table 5.10. Summary table for dissolved organic carbon and inorganic carbon for Urine treated soils in the 0-5 cm section of the soil columns. n =(4)

Soil Treatments 0-5 cm	**Dissolved Organic Carbon (µg g^{-1} dry soil)**					**Inorganic Carbon (µg g^{-1} dry soil)**				
	Day					Day				
	0	4	8	16	24	0	4	8	16	24
1.1 g cm^3 No DCD	76.6 ± 0.2	164.9 ± 9.6	90.4 ± 2.2	114.0 ± 3.8	121.3 ± 2.0	13.3 ± 1.5	15.7 ± 0.8	10.0 ± 0.4	10.4 ± 0.3	12.8 ± 0.5
1.3 g cm^3 No DCD	86.7 ± 6.2	133.2 ± 4.0	93.0 ± 1.1	136.6 ± 3.5	126.4 ± 1.6	9.1 ± 1.0	15.7 ± 1.1	7.9 ± 0.4	6.7 ± 0.4	10.0 ± 0.5
1.1 g cm^3 DCD	86.0 ± 1.2	135.1 ± 7.7	96.0 ± 1.5	134.0 ± 3.7	123.7 ± 1.5	9.2 ± 1.0	15.7 ± 1.0	9.8 ± 0.5	9.0 ± 0.3	9.9 ± 0.2
1.3 g cm^3 DCD	76.1 ± 1.0	140.1 ± 8.5	107.5 ± 1.0	131.1 ± 1.0	136.6 ± 1.0	9.7 ± 1.0	16.6 ± 1.1	11.2 ± 0.3	10.7 ± 0.3	9.7 ± 0.2

Table 5.11. Summary table for dissolved organic carbon and inorganic carbon for Slurry treated soils in the 0-5 cm section of the soil columns. n = (4)

Soil Treatments 0-5 cm	**Dissolved Organic Carbon (µg g^{-1} dry soil)**					**Inorganic Carbon (µg g^{-1} dry soil)**				
	Day					Day				
	0	4	8	16	24	0	4	8	16	24
1.1 g cm^3 No DCD	79.3 ± 5.8	193.2 ± 14.5	77.9 ± 0.8	120.9 ± 3.0	136.0 ± 2.6	15.5 ± 2.3	15.5 ± 1.8	10.2 ± 0.4	10.0 ± 0.3	9.9 ± 0.6
1.3 g cm^3 No DCD	66.5 ± 4.0	117.2 ± 3.8	79.9 ± 1.8	126.1 ± 4.7	131.2 ± 3.8	10.5 ± 0.3	9.3 ± 0.1	11.3 ± 0.2	12.1 ± 0.7	11.9 ± 0.2
1.1 g cm^3 DCD	74.4 ± 5.0	129.2 ± 12.4	80.6 ± 1.0	119.4 ± 1.8	147.1 ± 2.7	11.6 ± 0.9	11.5 ± 0.8	11.9 ± 0.1	11.4 ± 0.4	11.3 ± 0.2
1.3 g cm^3 DCD	85.9 ± 3.2	126.2 ± 8.2	81.0 ± 1.0	121.8 ± 1.8	158.7 ± 4.5	14.0 ± 0.7	8.7 ± 0.3	11.1 ± 0.2	12.5 ± 0.3	11.2 ± 0.1

Table 5.12. Summary table for dissolved organic carbon and inorganic carbon for Urine treated soils in the 5-10 cm section of the soil columns. n = (4)

Soil Treatments 5-10 cm	**Dissolved Organic Carbon (µg g^{-1} dry soil)**					**Inorganic Carbon (µg g^{-1} dry soil)**				
	Day					Day				
	0	4	8	16	24	0	4	8	16	24
1.1 g cm^3 No DCD	96.3 ± 5.5	106.3 ± 7.2	69.4 ± 0.6	91.0 ± 1.5	95.0 ± 2.9	15.6 ± 0.4	16.4 ± 1.1	10.8 ± 0.1	10.5 ± 0.1	11.9 ± 0.3
1.3 g cm^3 No DCD	67.3 ± 1.1	82.2 ± 3.0	72.0 ± 0.5	90.4 ± 1.1	90.2 ± 1.4	8.4 ± 0.2	12.5 ± 0.3	8.4 ± 0.2	6.8 ± 0.3	9.6 ± 0.5
1.1 g cm^3 DCD	76.9 ± 2.5	102.6 ± 3.7	74.0 ± 1.1	97.8 ± 1.4	96.2 ± 2.0	10.4 ± 0.1	16.8 ± 1.0	8.3 ± 0.3	8.6 ± 0.3	8.4 ± 0.4
1.3 g cm^3 DCD	74.1 ± 0.9	84.0 ± 5.4	72.0 ± 0.8	91.0 ± 1.5	95.8 ± 0.6	10.1 ± 1.1	12.1 ± 0.5	8.9 ± 0.3	8.5 ± 0.2	8.6 ± 0.4

Table 5.13. Summary table for dissolved organic carbon and inorganic carbon for Slurry treated soils in the 5-10 cm section of the soil columns. n = (4)

Soil Treatments 5-10 cm	**Dissolved Organic Carbon (µg g^{-1} dry soil)**					**Inorganic Carbon (µg g^{-1} dry soil)**				
	Day					Day				
	0	4	8	16	24	0	4	8	16	24
1.1 g cm^3 No DCD	56.0 ± 2.3	72.4 ± 4.0	69.4 ± 0.6	94.2 ± 1.7	114.8 ± 3.8	11.7 ± 0.8	10.5 ± 0.5	11.4 ± 0.3	10.5 ± 0.4	11.6 ± 0.3
1.3 g cm^3 No DCD	55.9 ± 2.9	76.5 ± 5.2	64.2 ± 0.9	93.0 ± 2.1	106.7 ± 2.0	13.3 ± 1.0	10.9 ± 0.8	12.1 ± 0.2	11.9 ± 0.3	12.7 ± 0.4
1.1 g cm^3 DCD	74.1 ± 5.9	79.0 ± 6.3	68.5 ± 0.2	98.9 ± 1.4	124.4 ± 1.6	14.9 ± 1.2	12.7 ± 0.9	11.7 ± 0.3	12.2 ± 0.0	12.9 ± 0.3
1.3 g cm^3 DCD	70.9 ± 2.1	86.1 ± 4.7	68.4 ± 1.3	95.5 ± 2.1	137.5 ± 5.7	13.8 ± 0.4	9.2 ± 0.2	12.1 ± 0.2	12.5 ± 0.3	11.4 ± 0.1

5.3.6. Dissolved Organic Carbon

Dissolved organic carbon (DOC) concentrations were affected by combinations of Bulk Density x Depth, Bulk Density x Day and Depth x Day (Table 5.14, $P = 0.022$, $P = 0.009$ and $P < 0.001$ respectively) for urine treated samples. In the slurry treated samples the dissolved organic carbon concentrations were seen to have significant interactions with DCD x Day and Depth x Day and Bulk Density x DCD x Depth x Day (Table 5.14, $P = 0.039$, $P < 0.001$ and $P = 0.046$).

DOC peaked on Day 4 for both the urine and slurry treated soils, with the 1.1 g cm^3 + no DCD treatment in the slurry amended samples demonstrating the highest increases in dissolved organic carbon. Significant reductions in dissolved organic carbon were observed for the 1.1 g cm^3 samples in both urine and slurry treated soils (Fig. 5.6 (a) and (c); $P \leq 0.05$) for the 0-5 cm layer. Dissolved organic carbon concentrations were significantly lower in the 5-10 cm layer than in the top 0-5 cm for all treatments with urine additions.

5.3.7. Inorganic Carbon

Urine and Slurry treatments both increased inorganic carbon concentrations (IC) (Table 5.4; Fig. 5.6) with significant interactions observed in the urine treated samples between sample treatments, Bulk Density x DCD and DCD x Day ($P < 0.001$ and $P = 0$ 0.031 respectively). Significant increases in inorganic carbon were noted for all treatments except for 1.1 g cm^3 + No DCD on Day 4 of the experiment in the urine treated

samples. In both 0-5 cm soils and 5-10 cm layer IC was observed to significantly decreases after Day 4 and remained relatively unchanged for the remainder of the experiment (Fig. 5.6; $P > 0.05$). The only observed significant change for the Slurry treated samples was the reduction in inorganic carbon for 1.1 g cm^3 + No DCD treatment were apparent after Day 4 (Fig. 5.6; $P \leq 0.05$).

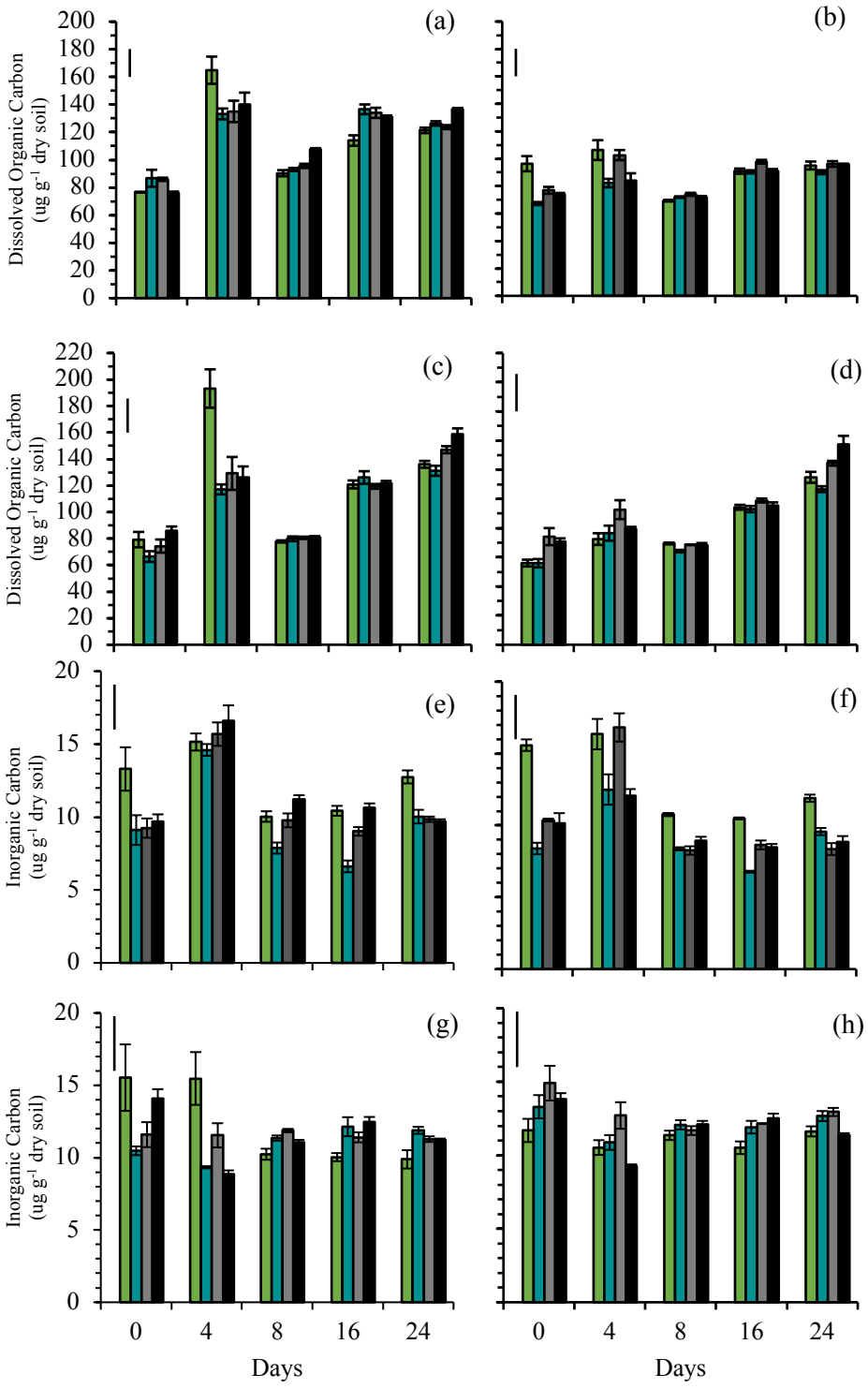


Figure 5.6. Graphs for dissolved organic carbon for urine treatment 0-5 cm (a); 5-10 cm (b) and slurry treatment 0-5 cm (c); 5-10 cm (d). Graphs for dissovled inorganic carbon for urine treatment 0-5 (e); 5-10 (f) and slurry treatment 0-5 cm (g); 5-10 (f). 1.1 g cm^3 + No DCD (green bars), 1.3 g cm^3 + No DCD (teal bars), 1.1 g cm^3 + DCD (grey bars) and 1.3 g cm^3 + DCD (black bars). Error bars represent standard error of means (SEM). LSD bars represent 95% confidence ($P < 0.05$) and demonstrate the difference for each variable for each N treatment at both soil depth treatments (i.e. LSD show the difference between 0-5 and 5-10 cm sample measurements). n = (4). Values for mean data points are presented above in Tables 5.10, 5.11, 5.12 and 5.13.

Table 5.14. ANOVA output for dissolved organic carbon and inorganic carbon. Significant values of $P \leq 0.05$ and higher are highlighted in bold.

Source		Dissolved Organic Carbon				Inorganic Carbon			
		Urine		Slurry		Urine		Slurry	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
BD	1	1.96	0.167	1.91	0.172	23.23	**< 0.001**	0.59	0.447
DCD	1	0.38	0.538	3.09	0.084	1.49	**< 0.001**	0.54	0.467
Depth	1	150.75	**< 0.001**	88.99	**< 0.001**	1.83	**< 0.001**	0.84	**0**.364
Day	4	51.50	**< 0.001**	64.62	**< 0.001**	41.09	**< 0.001**	3.16	**0.020**
BD x DCD	1	0.58	0.448	3.71	0.059	24.13	**< 0.001**	2.28	0.071
BD x Depth	1	5.57	**0.022**	0.65	0.424	3.97	0.051	0.41	0.527
DCD x Depth	1	0.15	0.698	3.77	0.057	2.48	0.121	0.76	0.386
BD x Day	4	3.70	**0.009**	2.70	0.060	1.23	0.307	3.19	0.019
DCD x Day	4	1.39	0.247	2.70	**0.039**	2.87	**0.031**	0.73	0.574
Depth x Day	4	10.60	**< 0.001**	9.94	**< 0.001**	0.85	0.500	0.33	0.855
BD x DCD x Depth	1	0.12	0.727	3.41	0.070	0.09	0.762	3.39	0.071
BD x DCD x Day	4	1.84	0.134	0.90	0.473	1.86	0.129	0.97	0.433
BD x Depth x Day	4	0.13	0.973	1.80	0.142	1.41	0.241	0.69	0.600
DCD x Depth x Day	4	0.45	0.772	1.59	0.189	0.36	0.839	0.47	0.755
BD x DCD x Depth x Day	4	2.00	0.107	2.58	**0.046**	0.34	0.853	1.66	0.171
Total	157								

5.3.8. Water-filled pore space

The Urine treated soils demonstrated significantly higher %WFPS values than observed for the slurry treated samples (Table 5.4; $P < 0.001$; Fig. 5.7) with significnat interactions between Depth x Day and Bulk Density x Depth for Urine treated samples (Table 5.15; $P = 0.022$ and $P = 0.018$ respectively). Bulk Density x DCD x Depth ($P = 0.028$) also demonstrated a significant but unexpected interaction, however, as DCD would not have had a significant effect on the %WFPS and coul be as a result of these samples reatining more water in their section of the tension table (Table. 5.15.). Soil samples from the lower section of the sample columns overall had higher %WFPS than the samples from the 0-5 cm layer in both Urine and Slurry treatments, with the 1.3 g cm^3 bulk density demonstrating an overall trend in increasing %WFPS when compared to the lower 1.1 g cm^3 bulk density (Table 5.15; Fig 5.7).

5.3.9. pH

Urine treated samples were observed to increase pH significantly more than slurry treated samples (Table 5.4; $P < 0.001$, Fig 5.7) after Day 0 when the fertiliser treatments were applied. pH variations were associated with to the interactions between Bulk Density x DCD x Depth x Day (Table 5.15, $P < 0.001$; Fig 5.7).

Bulk density did have an effect on pH (Table 5.15, $P = 0.005$; Fig 5.7 (e)), however, this was only obsereved on Day 4. Highest pH values were measured on Day 4 for non DCD treated soils, with no differences observed between sample bulk density as these were significantly higher than all other sample treatments for the duration of the experiment. Sample pH had significantly decreased by Day 8 for urine + no DCD treated soils, which then steadily increased towards the end of the experiment, with the

exception of the urine 1.1 g cm^3 + DCD treated samples which demonstrated signifcant increases, as all the other sample treatments demonstrated significant decreases in soil pH on Day 8. Urine ammended soils not treated with DCD showed that pH slowly increased until the end of the experiment, but remained significantly lower than what was observed on Day 0. Urine + DCD treated samples demonstrated no change in pH for Days 8 and 16, with only a signifcant drop in pH observed on Day 24, but still remained higher than what was observed at the beginning of the experiment (Fig 5.7).

Slurry treated samples were more stable than the in urine treated soils, however, significant interactions were only observered between Bulk density x DCD x Day and highly significant interactions between Depth x Day and DCD x Day (Table 5.15). Changes in pH in the slurry treatment were slight but consistant, with the most notable decline also observed, as with the Urine ammended soils on Day 8 in the 5-10 cm layer.

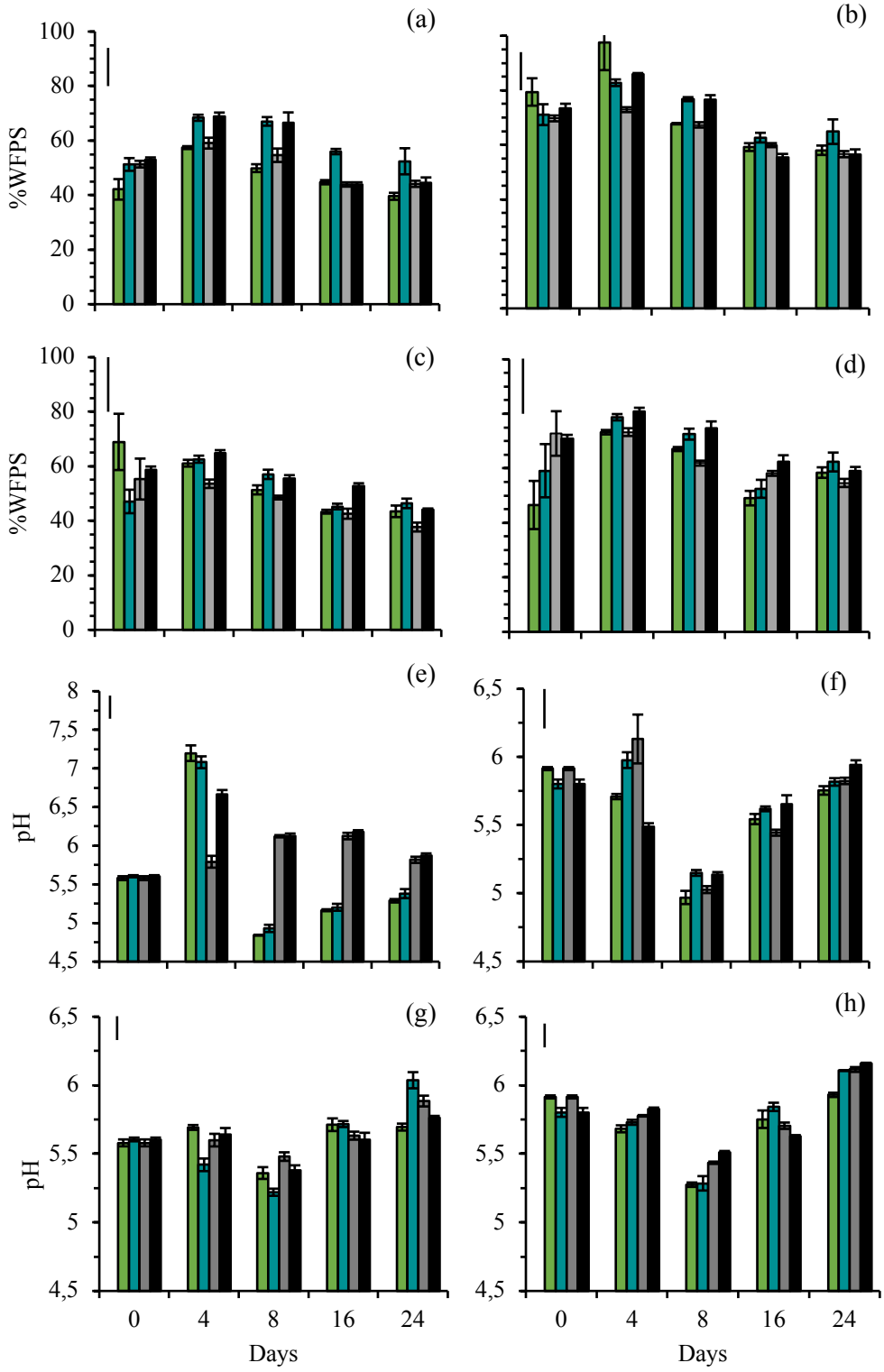


Figure 5.7. Graphs for %WFPS for urine treatment 0-5 cm (a); 5-10 cm (b) and slurry treatment 0-5 cm (c); 5-10 cm (d). Graphs for pH for urine treatment 0-5 (e); 5-10 (f) and slurry treatment 0-5 cm (g); 5-10 (h). 1.1 g cm^3 + No DCD (green bars), 1.3 g cm^3 + No DCD (teal bars), 1.1 g cm^3 + DCD (grey bars) and 1.3 g cm^3 + DCD (black bars). Error bars represent standard error of means (SEM). LSD bars represent 95% confidence ($P < 0.05$) and demonstrate the difference for each variable for each N treatment at both soil depth treatments (i.e. LSD show the difference between 0-5 and 5-10 cm sample measurements). n = (4). (Note the different scale axis for pH.

Table 5.15. ANOVA output for %WFPS and pH. Significant values of $P \leq 0.05$ and higher are highlighted in bold.

Source		%WFPS				pH			
		Urine		Slurry		Urine		Slurry	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
BD	1	7.10	**0.010**	5.19	**0.026**	8.52	**0.005**	0.01	0.920
DCD	1	1.38	0.245	0.90	0.345	71.13	**< 0.001**	3.07	0.085
Depth	1	144.53	**< 0.001**	22.39	**< 0.001**	18.11	**< 0.001**	60.11	**< 0.001**
Day	4	18.23	**< 0.001**	12.82	**< 0.001**	198.53	**< 0.001**	94.98	**< 0.001**
BD x DCD	1	0.11	0.742	1.37	0.247	0.04	0.836	0.96	0.330
BD x Depth	1	5.88	**0.018**	0.32	0.572	1.41	0.239	1.18	0.283
DCD x Depth	1	2.86	0.096	1.40	0.242	17.93	**< 0.001**	1.20	0.278
BD x Day	4	0.89	0.478	0.77	0.548	1.52	0.209	2.21	0.079
DCD x Day	4	0.47	0.756	1.76	0.150	77.91	**< 0.001**	5.66	**< 0.001**
Depth x Day	4	3.10	**0.022**	0.74	0.569	26.49	**< 0.001**	6.12	**< 0.001**
BD x DCD x Depth	1	5.06	**0.028**	0.97	0.328	4.10	**0.047**	0.05	0.821
BD x DCD x Day	4	1.24	0.303	0.03	0.998	0.32	0.866	3.97	**0.006**
BD x Depth x Day	4	0.23	0.921	0.33	0.858	2.24	0.076	2.15	0.086
DCD x Depth x Day	4	0.90	0.467	0.47	0.754	20.52	**< 0.001**	0.72	0.581
BD x DCD x Depth x Day	4	0.81	0.523	0.54	0.704	5.58	**< 0.001**	1.87	0.127
Total	157								

5.3.10. Soil enzyme activity

5.3.10.1. Soil nitrification potential

Urine fertiliser increased soil potential nitrification activity more than the slurry fertilised soils (Table. 5.4; $P < 0.001$) with peaks of potential nitrification activity were observed on Day 4 of the experiment in the 0-5 cm layer, which steadily declined thereafter (Fig. 6.8 (a)). Variations in soil nitrification potential for urine treated samples were mainly explained by the significant interaction between Bulk density x DCD x Depth (Table 5.16, $P = 0.031$; Fig. 5.8 (a-b)), with potential nitrification activity significantly higher in 1.1 g cm^3 bulk density treatment, which was mainly seen in the 0-5 cm layer (Table 5.16; Depth = $P < 0.001$) and significantly reduced by the application of DCD (Table 5.16; Depth = $P < 0.001$)

Slight, but significant differences ($P \leq 0.05$) in potential nitrification activities that were observed between the 1.1 g cm^3 and 1.3 g cm^3 bulk densities with increases on Days 4 and 8 between the two bulk densities (Fig. 5.8 (a-b)), and on Day 0 and 24 for the 5-10 cm layers as explained by the interaction between Bulk Density x Depth x Day (Table 5.16; $P = 0.002$).

Not only did DCD reduce the increases in potential nitrification activity, which was observed on Days 4, 8 and 16 in the 0-5 cm samples (Table 5.16; DCD = $P < 0.001$; Fig. 5.8; $P \leq 0.05$), but the effect of Bulk Density was eliminated by the application of DCD (Fig. 6.8 (a); Table 5.16; Bulk Density x DCD = $P < 0.001$)), with the exception of Day 16 in the 0-5 cm samples. The significant reduction ($P \leq 0.05$) in potential nitrification due to DCD was only observed in the 0-5 cm samples during Days 4, 8 and 16 (DCD x Depth x Day; Table. 5.16, $P < 0.001$).

Slurry treated samples demonstrated variation between bulk density samples, however, only on Days 4 and 16 for the 0-5 cm soils (Bulk Density x Day; Table 5.16, $P = 0.010$; Fig. 5.8, $P \leq 0.05$). Most differences for potential nitrification activity were as a result of the interaction between DCD x Depth x Day ($P = 0.011$) with potential nitrification activity reduced by the application of DCD in the 0-5 cm sections of the soil on Days 0, 16 and 24.

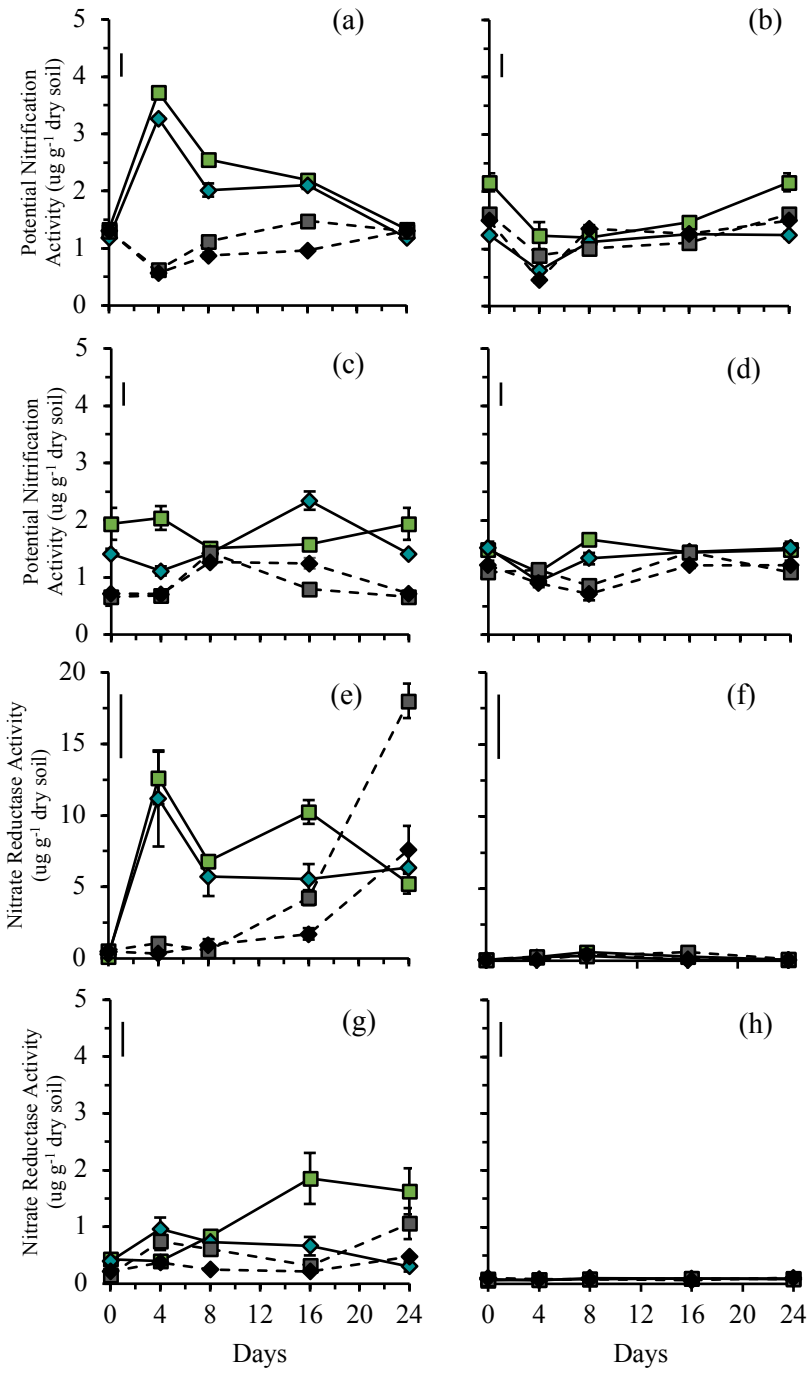


Figure 5.8. Graphs for potential nitrification for urine treatment 0-5 cm (a); 5-10 cm (b) and slurry treatment 0-5 cm (c); 5-10 cm (d). Graphs for nitrate reductase activity for urine treatment 0-5 (e); 5-10 (f) and slurry treatment 0-5 cm (g); 5-10 (h). 1.1 g cm^3 + No DCD (green squares with solid lines), 1.3 g cm^3 + No DCD (teal diamonds with solid lines), 1.1 g cm^3 + DCD (grey squares with dashed lines) and 1.3 g cm^3 + DCD (black diamonds with dashed lines). Error bars represent standard error of means (± SEM). LSD bars represent 95% confidence ($P \leq 0.05$) and demonstrate the difference for each variable for each N treatment at both soil depth treatments (i.e. LSD show the difference between 0-5 and 5-10 cm sample measurements). n = (4). Note, if ± SEM bars are not visible it is due to ± SEM being smaller than sample mean symbols.

5.3.10.2. Nitrate Reductase Activity

Soil nitrate reductase activity was significantly higher in urine treated soils than slurry treated soils (Table 5.4; Fig. 5.8 (e-h). However, the significant variation between treatments in urine fertilised samples were attributed to the significant interaction between DCD x Depth x Day (Table 5.16; $P < 0.001$). Significant changes in nitrate reductase activity were only observed in the 0-5 cm layer not the 5-10 cm soils throughout the experiment in Urine treatment. These were significantly reduced by DCD application for Days 4 and 8 for both 1.1 g cm^3 and 1.3 g cm^3 bulk densities, and on Day 16 for 1.1 g cm^3 (Fig. 5.8; $P \leq 0.05$). Nitrate reductase activity peaked on Day 4 for the non-DCD treated soils, with only significant variances observed for the 1.1 g cm^3 soil on Days 4 and 24 (Fig. 5.8; $P \leq 0.05$). Bulk density alone demonstrated significant differences (Table. 5.16; $P = 0.049$), which were observed on Days 16 and 24 for non-DCD and DCD treated samples respectively (Fig. 5.8).

Slurry fertilised soils demonstrated significant interactions between Bulk Density x Depth and DCD x Depth (Table 5.16; $P = 0.028$ and $P = 0.016$, respectively). Nitrate reductase activity was greater in the 0-5 cm than the 5-10 cm layers, as observed for the urine treated samples. Significant variations between bulk density treatments were observed on Days 16 and 24 of the experiment, with higher nitrate reductase activity measured in the 1.1 g cm^3 treatment for non-DCD treated samples. Any variation in nitrate reductase activity due to bulk density was eliminated by depth (Fig.5.8 (h)).

Table 5.16. ANOVA output for potential nitrification and nitrate reductase activity. Significant values of $P \leq 0.05$ and are highlighted in bold.

Source		Potential Nitrification				Nitrate Reductase			
		Urine		Slurry		Urine		Slurry	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
BD	1	29.55	**<0.001**	2.75	0.103	4.04	**0.049**	4.70	**0.034**
DCD	1	151.98	**< 0.001**	119.19	**< 0.001**	7.95	**0.007**	5.72	**0.020**
Depth	1	40.33	**< 0.001**	0.35	0.559	90.76	**< 0.001**	49.77	**< 0.001**
Day	4	0.31	0.869	5.35	**< 0.001**	8.48	**< 0.001**	1.52	0.207
BD x DCD	1	11.58	**0.001**	3.10	0.083	0.57	0.452	0.22	0.639
BD x Depth	1	0.61	0.436	0.00	0.947	3.24	0.077	5.07	**0.028**
DCD x Depth	1	90.49	**< 0.001**	11.05	**0.002**	8.17	**0.006**	6.12	**0.016**
BD x Day	4	1.04	0.396	3.63	**0.010**	0.88	0.484	1.63	0.178
DCD x Day	4	35.79	**< 0.001**	1.17	0.331	9.22	**< 0.001**	1.07	0.382
Depth x Day	4	43.03	**< 0.001**	1.26	0.295	8.87	**< 0.001**	1.52	0.207
BD x DCD x Depth	1	4.88	**0.031**	1.32	0.255	0.50	0.481	0.22	0.643
BD x DCD x Day	4	1.07	0.381	1.74	0.154	1.57	0.194	1.31	0.279
BD x Depth x Day	4	4.85	**0.002**	1.90	0.122	0.84	0.507	1.64	0.176
DCD x Depth x Day	4	34.07	**< 0.001**	3.59	**0.011**	9.31	**< 0.001**	0.97	0.429
BD x DCD x Depth x Day	4	1.07	0.381	0.63	0.642	1.62	0.182	1.38	0.252
Total	157								

Table 5.17. Spearman's rank correlation coefficients (ρ) between potential nitrfication activity and nitrate reductase activity with soil variables in 0-5 cm layer of soils for Urine treated samples (Total no. 39 $df = 37$)

Parameter	NR	NH_4^+	NO_3^-	DOC	IC	TN	WFPS	pH
Potential nitrification activity								
No DCD	0.560***	0.822***	0.104	0.425**	0.289	0.731***	0.438**	0.141
DCD	0.412**	-0.245	-0.057	-0.185	-0.685***	-0.141	-0.528***	-0.433**
Nitrate reductase activity (NR)								
No DCD	-	0.628***	0.501***	0.471**	0.107	0.722***	0.147	-0.113
DCD	-	-0.125*	0.299*	0.236*	-0.204**	0.013*	-0.546***	-0.293

Significance levels (P) from the Spearman's rank correlation.

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

Table 5.18. Spearman's rank correlation coefficients (ρ) between potential nitrfication activity and nitrate reductase activity with soil variables in 0-5 cm layer of soils for Slurry treated samples (Total no. 39 $df = 37$)

Parameter	NR	NH_4^+	NO_3^-	DOC	IC	TN	WFPS	pH
Potential nitrification activity								
No DCD	-0.125	-0.048	0.025	0.256	0.434**	-0.019	0.112	0.210
DCD	-0.025	0.458**	0.147	-0.212	0.324	0.098*	0.245	-0.258
Nitrate reductase activity								
No DCD		0.166	0.071	0.041	-0.424**	0.176	-0.237	-0.334**
DCD		0.311	0.223	0.277	-0.249**	0.314*	-0.333*	0.070

Significance levels (P) from the Spearman's rank correlation.

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

5.4. DISCUSSION

5.4.1 *Influence of Urine and Slurry deposition on Soil NH_4^+, NO_3^-, DOC, IC, pH and WFPS*

5.4.1.1 NH_4^+

Urine significantly ($P < 0.001$; Table 5.1) increased total nitrogen, NH_4^+ and NO_3^- in comparison to slurry application and was more dominant in the upper layer (0-5 cm) of the soil samples (Tables 5.5 and 5.6). This increase was due to the higher concentrations of total N and NH_4^+ between the two fertilisers (Appendix, Table 7.1). As urine is known to have up to 70% of its total N consisted of urea (Bristow et al. 1992) which is rapidly hydrolysed to NH_3 by the ubiquitous soil urease enzyme once deposited in soil (Boon et al. 2014), this would therefore lead to the significantly higher NH_4^+ concentrations in urine treated soils. DCD initially reduced soil NH_4^+ concentrations in comparison to non-DCD treated soils in the urine treatment on Day 4 of the experiment. These concentrations subsequently surpassed those in the non-DCD treatment by Day 8 and throughout the experiment. As NH_4^+ concentrations usually increase with DCD treatment, as observed in chapter 4, the significantly lower NH_4^+ concentrations are somewhat puzzling. Two possible reasons may account for this. First the apparent reduction may have simply been due to an increased uptake by the roots of the grass plants in the DCD treated columns. However, the same would have been noted for the non-DCD treatment also. Secondly, and much more theoretically, It has been recently demonstrated that *Nitrospira* spp., that have been demonstrated to be the most ubiquitous of all soil nitrifying bacteria (Daims et al. 2015), are known to possess genes that have the ability to hydrolyse urea (Koch et al.

2015). It is therefore possible that the reduction in NH_4^+ concentrations may be attributed to a reduction in growth of soil nitrifying bacteria containing urease enzyme coding genes, as the inhibition of ammonia monooxygenase activity by DCD (Amberger. 1989) reduces the ability to generate energy for cell growth gained from the oxidation of NH_4^+ to NO_2^- (Sayavedera-Soto et al. 1996). Reductions in growth of AOB in soils treated with DCD have commonly been observed in grassland soils (Di et al. 2009; Di et al. 2010; Dawson et al. 2014). More specifically, reductions in the growth of AOB with lineage to *Nitrospira* spp., or, *Nitrospira*-like groups in soils treated with urea and urea + DCD were found in a study by Ping et al. (2013). Therefore, the application of DCD could reduce the rate at which urea is hydrolysed in soil due to reduced growth of urease encoded AOB.

5.4.1.2. NO_3^-

Interestingly, NO_3^- concentrations in both Urine and Slurry treatments were observed to be relatively similar (Tables 5.5 and 5.6) in the 0-5 cm soil layer on Day 4. As NO_3^- concentrations were not measured in the urine or slurry fertilisers it is assumed that both urine and slurry contained similar amounts of NO_3^- as concentrations on Day 4 were so similar, as both Urine and Slurry contained a similar concentration of NH_4^+ (Appendix, Table 7.1) it is likely that when Urine and Slurry treatments were applied, the already present ammonium was rapidly converted to nitrate either in the soil. As the Slurry was more viscous and didn't infiltrate into the soil, it would have leached out from the liquid slurry, which would have been enhanced further when samples were sprayed with de-ionised water every morning and passed into the soil matrix. NO_3^- concentrations were then observed to increase in the urine treated soils as a result of increased soil potential nitrification (Fig. 5.8 (a)) and reduced

in the non-DCD treated soils due to the difference in NH_4^+ concentrations observed between the two fertiliser treatments.

5.4.1.3 Dissolved organic and inorganic carbon

Dissolved organic carbon and inorganic carbon concentrations were similar in both urine and slurry treatments (Fig. 5.7; Tables 5.9 and 5.10). Less DOC was measured in slurry than the urine fertiliser (Appendix, Table 7.1). In addition, as the slurry was more viscous, portions of the slurry were adhered to the leaves of grass. This would account for the lower concentrations of DOC found in the soil in relation to the measured concentrations in analysis of the slurry.

5.4.1.4 Soil WFPS and pH

Soil pH and WFPS were significantly variable between both fertilisers. The reason for the divergence in WFPS is simply due to the nature of the two liquids. As slurry was more viscous it did not pass through the soil matrix as easily as the urine. As urine is a solution and slurry is comprised of a mixture of solids and liquids, the density of the slurry would have meant that the volume of liquid applied would most likely have been less than the urine. The increases in pH, which were observed to significantly increase in urine treated soils in the 0-5 cm layer (Fig. 5.7) is due to the increased concentrations of NH_4^+ through urea hydrolysis which is known to increase soil pH Cabera et al. (1991). As DCD reduced the increase in NH_4^+, this would have reduced the increase in pH which was observed in the 1.1 g cm^3 Urine + DCD treatment (Fig. 5.7).

5.4.2. Influence of soil Urine and Slurry deposition, bulk density and DCD on N_2O and CO_2 emissions

Concentrations of N_2O were significantly greater in the urine treated soils than the slurry treated soils (Table. 5.1.; Table 5.2 & Table 5.4., $P < 0.001$ & $P < 0.001$ respectively). N_2O emission from urine treated soils were around 5-10 times higher than soils treated with cattle slurry for the 1.3 g cm^3 bulk density and 6 times higher for the 1.1 g cm^3 bulk density treatments. The increased amount of readily available N that is released by urine immediately, as discussed previously in this chapter, and subsequent increases in soil WFPS that were observed in the Urine treatment when compared to the Slurry treatment (Fig 5.7) increased WFPS is known to increase fluxes of N_2O (Ball et al. 2008), this in combination with the increased amount of N that is also known to a dominant factor which increases N_2O fluxes (Rees et al. 2013) would explain the dominance in N_2O fluxes in the Urine treatment. The significant increases of 16% and 21% in N_2O fluxes between the 1.3 g cm^3 + No DCD and 1.3 g cm^3 + DCD soil treatments respectively, from their respective counterparts in the 1.1 g cm^3 treatment ($P \leq 0.05$; Table 5.1, Day 4) clearly demonstrated that bulk density increased N_2O fluxes. As WFPS was also increased in the Urine treatment in the 1.3 g cm^3 bulk density this would explain the increased fluxes as has been demonstrated in many other studies (Lin and Doran. 1984; Skiba and Ball. 2002; Bateman and Baggs. 2005; Ruser et al. 2006; Ball et al. 2008; Hamonts et al. 2013) and also as observed in chapter 2. As WFPS increased to 68% (Fig. 5.7) this in combination with the increased N content in the Urine treated soils would explain the increased N_2O flux from the soil when compared to both Slurry treatment and the lower bulk density treatments. This suggests that the combination of cattle traffic in grasslands which induces increases in soil bulk

density (Bezkorowajnyj et al. 1993), followed by the subsequent deposition of urine creates optimal conditions that lead to the production of large quantities of N_2O.

DCD had mixed results on reducing soil N_2O fluxes the simulated grassland soil used in this study. DCD was observed to have no effect in reducing soil N_2O fluxes 4 Days after Urine fertiliser treatment in the 1.3 g cm^3. As DCD has generally been demonstrated to reduced N_2O fluxes in grassland soil type receiving urine inputs (Di and Cameron 2002; Di and Cameron. 2003; Di et al. 2007), the results from this study contradict these previous observations. High variations were observed in the 1.3 g cm^3 Urine + DCD treatment, with a flux of 363.1 N_2O-N mg m^{-2} h^{-1} occuring on Day 4 of the experiment which was subsequently removed, as it was thought that this was an erroneous data point. However, spatial variation in N_2O fluxes can occur due to the increase in anaerobic conditions, related to increased microbial respiration in soil which results from the addition of a decomposable source of organic matter (Christensen and Tiedje. 1998). This high peak of N_2O could be in relation to a 'hotspot' of decomposable C that may have been located in the sample at the time of gas sampling which in combination with the addition of a readily available N source, such as urine, resulted in an inceased N_2O production at a temporal and spacial scale.

In relation to the ineffectiveness of DCD in the urine treated soils, similar observations were made in the study by Barneze et al. (2015) who, as discussed previously in this chapter, demonstrated that a grassland soil receiving urine that had been treated with DCD demonstrated no reduction in N_2O fluxes, with DCD actually increasing the concentrations of N_2O emitted from the soil, and persisted longer than soils which had not been treated with DCD, once soil WFPS was increased to 80%. Similarly, fluxes of N_2O were observed to decrease at a much slower rate for the DCD treated soils in the urine treatment than that observed for the 1.3 g cm^3 + No DCD

treatment, which decreased significantly when comparing mean values on Day 4 (54 (± SE 9.1) mg N_2O-N m^{-2} h^{-1}) and Day 8 (26.8 (± SE 3.8) mg N_2O-N m^{-2} h^{-1}) (Table. 5.1) in the current study, corroborating with the observations by Barneze et al. (2015). Another study by Mazzetto et al. (2015) stated that DCD had no effect on reducing N_2O emissions from a Brazilian pasture soil. Both studies attributed the reduced efficacy of DCD due to the degradation that would have occurred in relation to atmospheric temperature and soil moisture based on observed relationships between DCD degradation and temperature. The study by Barneze et al. (2015) demonstrated that the half-life of DCD was between 7-10 Days during UK summer conditions, similar to the conditions in this study. Mazzetto et al. (2015), however, extrapolated the half-life of DCD using the equation produced by Kelliher et al. (2014) ($^1/_2$ life = 54 – 1.8*T; T being soil temperature). Using this equation, Mazzetto et al. (2015) estimated the half-life of DCD to be 1.8 days, thereby assuming that DCD had been fully degraded in the first couple of days. As potential nitrification rates were measured in this study, an estimation of the maximum potential activity of both the AMO (ammonia monooxygenase) and HAO (hydroxylamine oxidoreductase) enzymes and DCD reduces the activity specifically through the temporary inactivation of the AMO enzyme (Amberger. 1989), it is possible to use this enzyme assay as a proxy for the presence of DCD. Potential nitrification activity rates were similar to those observed on day 0 of the experiment, before urine or slurry treatments were applied, which were significantly lower than those observed for non-DCD treated soils (Fig. 5.8). Only by Day 16, were any increases in potential nitrification activity observed in the DCD treated samples (Fig. 5.8), therefore, this demonstrated that there must have been an adequate supply of DCD to inhibit increases in potential nitrification activity when peak N_2O fluxes occurred. Although soil temperature was not measured in this study,

it has been shown that the relationship between atmospheric and soil temperature at depths up to 10 cm is almost identical (Tsilingiridis and Papakostas. 2014), it is therefore possible to calculate the half-life of DCD using the formulation by Kelliher et al. (2014). Based on the average air temperature in this study (20^{o}C) the half-life of DCD would equate to 18 days. This estimation fits with the observed changes in potential nitrification activity of 16 days and would thereby indicate that the amount of DCD in the soil was effective in reducing the negative effects of nitrification on N_2O fluxes during observed peak and increased N_2O fluxes during the first 8 Days in this study.

In contrast to the discussion above, it was also observed that DCD demonstrated a trend, but not significantly, to reduce soil fluxes for samples at the lower bulk density, 1.1 g cm^3. Samples treated with DCD in the 1.1 g cm^3 soil demonstrated a 44% decrease in soil N_2O fluxes (25 mg N_2O-N m^2 h^{-1} (U + No DCD) and 14.4 mg N_2O-N m^2 h^{-1} (U + DCD)) on Day 4 of the experiment which occurred at 57% and 59% WFPS respectively. By Day 8 the difference between the 1.1 g cm^3 + No DCD and the 1.1 g cm^3 + DCD treatment were even more pronounced, with the non-DCD treated soils emitting 53% more than the DCD treated samples (34 mg N_2O-N m^2 h^{-1} (U + No DCD) and 16 mg N_2O-N m^2 h^{-1} (U + DCD) respectively), at WFPS's of 50% and 54% respectively.

The contribution of the different microbial groups at varying WFPSs were discussed in the study by Bateman and Baggs (2005), as previously discussed in chapter 2, have been observed to be a result of nitrifier denitrification at WFPS concentrations of 60% and denitrification at concentrations of 70%. Therefore, due to the decreases in N_2O emissions from the DCD treated soils at 59% to 50% WFPS are

most likely as a result of decreased nitrification activity in the soil, which would agree with the observed decreases in soil potential nitrification activities.

Significant increases in CO_2 fluxes were attributed to increased bulk density (Fig 5.3 (c) and (d)), and DCD treated soils during the experiment. CO_2 release from urine treated soils are attributed to urea hydrolysis or increased microbial respiration (Kelliher et al. 2007), this in combination with increases in WFPS from Day 0 to day 4 due to the addition of urine would have led to the increased CO_2 fluxes, as rewetting episodes are known to increase CO_2 flux from soil (Gelfand et al. 2015). However, similar increases were observed for slurry treated soils which neither had the same input of urea, or, increases in WFPS (Fig. 5.7). In addition, soil CO_2 fluxes increased as soils became drier towards the end of the experiment due to the increase in grass root density, there is a possibility of enhanced wetting of soil particles around the roots from the root exudates. As discussed in Walker. (2003), soils reach field capacity between -5 kPa and -10 kPa (Chaboud and Rougier. 1984). It has therefore been speculated that as the soils hydraulic potential decreases through drying, root exudates subsequently begin to lose water to the soil. Due to this the surface tension of the exudates decrease which increases their viscosity. Therefore, as the surface tension decreases, the ability of the exudates to wet the surround soil will become greater (Walker. 2003). If this process occurred in the soils in this study, it is possible that root exudation may have stimulated the surrounding soil microbial communities, leading to increased CO_2 production.

As CO_2 emissions have not generally been considered in DCD N_2O emissions studies, due to the focus of the studies on N_2O (Di & Cameron (2002); Di and Cameron 2003; Zaman et al. 2009 de Klein et al. 2011; de Klein et al. 2014; Misslebrook et al. (2014)), however a few studies have reported the effect of DCD on CO_2 fluxes from

DCD treated soils. A more recent study by Zhu et al. (2016) demonstrated significant increases in soil CO_2 emissions from slurry treated soils during an incubation experiment. Zhu et al. (2016) concluded that the increases in CO_2 may have been due to the degradation of DCD by microorganisms as discussed by Kelliher et al (2008). However, a study by Singh et al. (2008) in the same year demonstrated that cattle urine treated soils did not occur any increases in soil microbial biomass when DCD was used in combination with cattle urine. Increases in pH may have also resulted in an increase in soil CO_2 flux. A study by Monaghan and Barraclough. (1993) stated that due to the increase in pH provided by the application of urine to soil, solubilisation of soil organic carbon may be increased due to increased alkalinity, and the increased provision of labile carbon substrates could increase soil microbial metabolism. Although pH was increased with urine treated soils, and were stable with DCD treatment, similar increases were seen for the Slurry treated samples with no appreciable increases in soil pH in comparison to the Urine treated soils (Fig. 5.7 (e) and (g)), both with and without DCD. With no significant variation in cumulative CO_2 fluxes from Urine treated soils, a pH effect on increased CO_2 fluxes would rule out induced changes in CO_2 fluxes due to increases in pH, that increases CO_2 fluxes may have been due to microbial degradation of DCD. As both Urine and Slurry treated samples showed no difference in dissolved organic carbon concentrations, this would suggest that the addition of similar amounts of labile organic carbon, as has been observed in many other studies (Cleveland et al. 2006; Deng et al. 2017) would explain the similar increased fluxes of CO_2 from both treatments.

5.4.3. Understanding the sources of N_2O fluxes: Potential nitrification and nitrate reductase enzyme activities at different soil depths following Urine and Slurry deposition

5.4.3.1 Potential nitrification

As part of the study investigated the distribution of the potential enzyme activities for both potential nitrification and nitrate reductase activity in relation to depth of the soil system, with the sampling of both the top 0-5 cm and bottom 5-10 cm potentially serving as an indicator of the likely dominant areas of soil N_2O production. The majority of the increases in enzyme activities were related to the top 0-5 cm of the soil samples, indicating a spatial variation in the soils in this study. Variations in potential nitrification in this study was explained by the interactions between DCD x Depth x Day ($P < 0.001$) for both urine and slurry however, the effect was more evident in the urine treated samples treatments which displayed significant variations in nitrification potential when compared to slurry treated soils (Table 5.1). The effect of depth was attributed to the increased NH_4^+ concentrations that were present in the upper most part of the soil columns (0-5cm) as previously discussed. Variations between bulk density treatment would have been attributable to decreases in the level of oxygen in the soil due to decreases in air-filled porosity which was observed in chapter 2, which could have decreased the activity of the nitrifying community, however, a recent study by Thion et al. (2017) demonstrated that in grass planted soils at low pH had a higher average abundance of AOB in the bulk soil than AOA. However, as discussed by Prosser and Nicol. (2012), AOA have a higher affinity for NH_4^+ ($K_m < 0.02$ μm NH_3 + NH_4^+) than AOB (K_m = 6 - 11 μm NH_3), therefore, AOA are better suited to low

NH_4^+ environments and may have actually accounted for the nitrification potential in the 5-10 cm layers of the soil columns where ammonium concentrations were significantly lower than the 0-5 cm layers. Ammonium oxidizing archaea (AOA) have been shown to be oxygen insensitive (Park et al. 2010; French et al. 2012; Pett-Ridge et al. 2013), in comparison to AOB. Therefore, AOA would be a good candidate for the dominant nitrifying microbial group within the 5-10 cm layer of the soil in this study, as soils in the 5-10 cm layer demonstrated significantly higher water-filled pore spaces (80-90%; Fig. 5.8 (b) and (d) & Fig. 5.7 (b)), which would have resulted in lower concentrations of available oxygen. A study by Di et al. (2010) has also demonstrated that in the topsoil (0 – 20 cm) of three grassland sites AOB were more abundant, whereas AOA dominated subsoils (40 – 60 cm), which they attributed to the high-fertility of the topsoil whereas the subsoils had low-fertility. Di et al. (2010) also showed that increases in AOB gene abundance were in response to urine inputs, whereas, AOA only grew in the control soils without urine-N substrate attributed to the concentration of NH_3 that were present, the higher in urine treatment vs the lower concentration of NH_3 in the control treatments. It was observed in this experiment that on Day 4 in the 5-10 cm layer of the soil, the increased presence of ammonium, ≅ 60 ug g^{-1} dry soil, significantly reduced potential nitrification rates ($P \leq 0.05$; Fig. 5.8. (b)), which was in contrast to that observed in the upper layer of the samples (Fig.5.8. (a)). It is possible to suggest then that AOB/AOA ratios may have been similar in this grassland soil in relation to both the presence of NH_4^+ and the anoxic conditions that would have been present in the 5-10 cm layer that would likely have mimicked conditions that are typically observed deeper down in the soil profile. This may therefore highlight an artefact of sample preparation in the current experiment. Although attempts were made to reduce a layer effect by scraping the middle section

of the soil column before the addition of the final 0-5 cm layer, it may not have been enough to reduce the effect of layering due to the significant change in moisture content present in the 5-10 cm section of the soil mesocosm. AOA have also been demonstrated to produce N_2O. A recent study by Jung et al. (2014) demonstrated that AOA, associated with I.1a, I.1a associated and I.1b clades of *Thaumarchaeota*, produced N_2O concentrations comparable with those from AOB. ^{15}N labelling in their study allowed them to suggest that given that AOA dominate over AOB in most soils N_2O from both ammonia oxidation and nitrifier denitrification may be attributable to AOA. Therefore, due to the levels of potential levels of nitrification in the bottom section of the soil columns, AOA could have contributed to total N_2O production.

Potential nitrification activity rates coincided with N_2O fluxes, both with peak fluxes and the subsequent reductions. It has been previously demonstrated that N_2O fluxes from urine treated samples correlated well with AOB gene abundances, which in turn were well correlated with *nirK* abundance to which the authors concluded that due to the correlation between AOB and *nirK*, and that as DCD reduced the abundance of the *nirK* gene, *nirK* gene communities were probably ammonia oxidizers which are known to possess homologous genes for *nirK*. Therefore, the production of N_2O was due to the activities of AOB and that *nirK* abundances were related to the presence of *nirK* in AOB (Di et al. 2014). Shaw et al. (2006) has demonstrated the ability of *Nitrospira* spp., a nitrifying bacterium generally considered to be the most dominant nitrifying bacteria in soil (Daims et al. 2015), to produce N_2O with the addition of NO_2^- , a by-product of the activity of the ammonia-monooxygenase enzyme under oxygen limited conditions. As DCD was observed to decrease soil N_2O fluxes in the urine treatment for the lower bulk density soil (1.1 g cm^3), and given that the WFPS were maintained between the optimum ranges for autotrophic denitrification, as discussed

previously in this chapter, this could suggest that N_2O emissions in the lower bulk density soils were as a result of nitrifier denitrification, or, as a result of nitrifier coupled denitrification in the 1.3 g cm^3 bulk density soils, as nitrate reductase activity also coincided with peaks of potential nitrification. However, the subsequent inhibition of nitrification potential by DCD in the same upper section of the soils for the 1.3 g cm^3 treatments, which showed no reduction in N_2O fluxes, would therefore suggest that fluxes of N_2O were emitted from other microbial nitrogen cycling pathways in the DCD treated samples.

5.4.3.2 Nitrate reductase

NO_3^- is first reduced to NO_2^- by the nitrate reductase gene (*narG*) and is considered the first step in the pathway of N_2O production (Averill and Tiedje. 1982). Significant positive correlations between NO_3^- and nitrate reductase activity were observed for non-DCD treated samples treated with urine (Table 5.17, (ρ) = 0.501, $P < 0.01$). A study by Deiglmyar et al. (2004), looking into the effect of elevated CO_2 fluxes on the nitrate-reducing community in the rhizosphere of *Lolium perenne* and *Trifolium repens*, found that nitrate reductase activity was higher under high N (56 g N m^{-2} yr^{-1}) when compared to low N (14 g N m^{-2} yr^{-1}). The results in this study corroborate the findings made by Deiglmyar et al. (2004), that higher nitrogen concentrations favoured nitrate reductase activity. As this study also used *Lolium perenne*, it is possible to compare the activity rates from this study to that of Deiglmyar et al. (2004). Nitrate reductase activity was measured to range between 6 - 20 µg NO_2^- N g^{-1} dry soil $24h^{-1}$ (Fig 5.8 (e)), similar to the values observed by Deiglmayr et al. (2004), therefore, the effect of high nitrogen concentrations would seem to be a key regulator for the activity of soil denitrifying bacteria containing nitrate reducing

enzymes. It was also observed that nitrate reductase activity positively correlated with both NH_4^+ and nitrification potential (Table 5.17, (ρ) = 0.628, $P < 0.001$ and (ρ) = 0.560, $P < 0.001$ respectively) in the top 0-5 cm of the soil where activity rates were increased. In addition to the correlations between NO_3^- and NH_4^+, total nitrogen also correlated well with nitrate reductase activity in the urine treated samples (Table 5.17, (ρ) = 0.731, $P < 0.001$). In contrast to the urine treated soils, nitrate reductase activity in the slurry treated soils was significantly lower than observed for urine treated soils in the 0-5 cm layer (Table 5.3, $P < 0.001$; Fig. 5.8 (e) and (g)) and that no significant correlation between NO_3^-, NH_4^+ or total nitrogen was observed (Table 5.17).

A study by Marhan et al. (2011) also observed positive correlations between nitrate reductase activity and NH_4^+ and NO_3^- concentrations when studying the effect of temporal variations and soil depth alongside elevated atmospheric CO_2 concentrations. Wakelin et al. (2013) also observed a close coupling between nitrification and denitrification in urine amended pasture soils, as urine stimulated denitrifying microbial communities, elucidating that this was not a surprising interaction due to the flow of nitrate that proceeds nitrification, a key intermediate compound to denitrification (Wakelin et al. 2013). This interaction was highlighted more by the lack of nitrate reductase activity in the current study, when potential nitrification activity was inhibited by DCD during the incubation and that nitrate reductase activity started to increase only after potential nitrification rates increased (Fig. 5.8 (a) and (e)). This was further evident by the significant correlation between potential nitrification rates and nitrate reductase activity in DCD treated soil (Table 5.17). Therefore, the reductions in nitrate reductase activity that echoed reduced potential nitrification activity in the DCD treated soils and using observations by Marhan et al. (2011), it can be suggested that in the non-DCD treated soils nitrate

reductase activity was more active due to a symbiotic relationship with ammonia oxidiziers and would suggest that the activities of both AOB/AOA and nitrate reducers encoding *narG* and *napA* genes are spatially related. To the author's knowledge the interaction between nitrate reductase and DCD has not been previously observed in other studies. It is probable that N_2O fluxes were as a result of this spatial coexistence between ammonia oxidizers and nitrate reducers in the higher bulk density soils (1.3 g cm^3). As N_2O fluxes in the urine treated soils followed a similar trend to both peaks of potential nitrification activity and nitrate reductase activity in the non-DCD treatment, the sequential cycling of N from NH_4^+ to NO_3^- and then from NO_3^- to N_2O probably occurred as a result of nitrifier coupled denitrification. Interestingly, nitrate reductase activity was observed to be at its highest in the urine treated soils when WFPS volumes dropped to 52% WFPS (20 µg NO_2^-N g^{-1} dry soil $24h^{-1}$; Fig. 5.8 & Fig. 5.7). Kandeler et al. (2009) observed that nitrate reductase activity correlated with the *narG* carrying community and was higher in the surface soil in Spruce forest (between 0-5 cm) due to a higher soil water content which would have reduce oxygen concentration, thereby increasing anaerobic conditions that would have increased the activity of *narG*, thereby explaining the events that occurred in the 1.3 g cm^3 bulk density in the current study. The reduction of nitrate to nitrite is catalysed by two different types; a membrane-bound reductase (NAR) encoded by the *nar*G gene and a periplasmic nitrate reductase (NAP) encoded by the *nap*A gene (Kandeler et al. 2009). Nitrate reducers can carry either one or both nitrate reductase enzymes (Philippot & Hojberg, 1999). A study by Keil et al. (2010) studying the spatial distribution of N-cycling microorganisms in 6 grassland soils later demonstrated that nitrate reducers that contain *narG* and *napA* demonstrate spatial coexistence, therefore indicating that either both genes were present in the one group, or, that groups that contained one or

the other of the genes were localised. It is considered that the *nar* gene is the most active nitrate reductase, with *nap* expressed to help support the first step in aerobic denitrification (Potter et al. 2001). Both *nar* and *nap* are expressed differentially, with *nar* predominantly expressed under anaerobic when NO_3^- concentrations are in excess. *nap* is expressed preferentially under aerobic conditions and increased when carbon is limiting (Potter et al. 2001). As observed in this study nitrate reductase activity was most active when concentrations of NO_3^- were most abundant as discussed previously in this chapter (Fig. 5.4 (a) & Fig 5.8 (a)).

It has been suggested that NAP facilitates the switch from an aerobic stationary phase, to anaerobic nitrate respiration (Siddiqui et al. 1993). The reason for the apparent peaks in nitrate reductase activity are likely due to the activity of *napA* which is able to operate in aerobic conditions, then due to the methodology of the nitrate reductase assay (Kandeler. 1996), which uses anaerobic conditions under optimum levels of NO_3^- to express nitrate reductase activity. The apparent activation of the *narG* gene in the enzyme assay after soils were at an increased anaerobic state could indicate what would likely happen under rainfall events. Due to the ability of nitrate reducers to remain viable during aerobic conditions, subsequent rainfall events would induce nitrate respiration. Therefore, the efficacy of DCD is put further into question. As DCD initially inhibits the conversion of NH_4^+ to NO_3^- it may simply just produce a lag effect for N_2O fluxes. Once DCD has been degraded by microbial activity the ammonia monooxygenase gene is active once more and will also be stimulated under rainfall, inducing once again the potential ballet between nitrifiers and nitrate reducers.

The reduction in N_2O fluxes on Day 8 were perhaps due to the increased activities of the *nosZ* enzyme in reducing N_2O (N_2OR). Bergaust et al. (2010) demonstrated that pH of 7 was key to successful assemblage/folding of the N_2OR and due to the

sensitivity of genes located in the periplasm (Wilks and Slonczewski. 2007) where the *nosZ* gene is located. Bergaust also demonstrated low external pH, particularly pH 6, results in the unsuccessful assemblage/folding of the N_2OR resulting in increased N_2O concentrations. It has even been demonstrated that once the *nosZ* has been successfully initiated it can remain functional with subsequent decreases in pH (Liu et al.2014). Further to this, it has been demonstrated by Phillipot et al. (2001) that *narG* expression is halted once both *nirK* and *nosZ* have reached maximum expression, which was thought to be due to sequential induction of the denitrifying system as observed in *Pseudomonas fluorescens*. Therefore, as nitrate reductase activity was observed to drop on Day 8 when both NO_3^- concentrations and oxygen limitation would seem optimal for nitrate reductase to occur, it may be possible that this was due to a repression in *narG* activity due to the increased expression of *nirK* and *nosZ*, which would have been successfully active from Day 4 and potentially been able to remain fully functional during the reduction in pH to pH 6 on Day 8 (Fig. 5.7 (a)). However, it was proposed by Wakelin et al. (2013) that due to an increased ratio of *nirS*:*nosZ* in pasture soil after the addition of urine, in their study, indicated that a smaller proportion of the N in the denitrification pathway was likely to be reduced to N_2. The previous argument would seem more likely given that the conditions in the soil would seem optimal for N_2OR activity to take place, and that N_2O emissions had significantly reduced (Table 5.1, $P \leq 0.05$) in the 1.3 g cm^3 + No DCD treatment on Day 8 when compared to Day 4 (Table 5.1). It was originally planned to measure the activity of the *nosZ* gene through acetylene inhibition (Smith et al. 1978) thereby using the increased N_2O production that would serve as a proxy of N_2 fluxes, however, due to technical limitations this was not possible. This extra information would have helped elucidate as to whether this process was occurring.

As DCD halted both potential nitrification and nitrate reductase activity, the route for N_2O fluxes are not so clear. As both initial stages of nitrification and denitrification were inhibited it would indicate that an alternative pathway occurred due to this aforementioned inhibition.

5.4.4 Alternative routes for N_2O production

Eliminating the possible prokayotic routes for N_2O production in the DCD treated soils at 1.3 g cm^3 suggests that another group of organisms were responsible for the production of N_2O. Another propossed pathway for the production of N_2O in this system could have been due to the denitrification acitvities of soil fungi. The ability of fungi to dominate N_2O production in grassland soils has been increasingly noted of the last few decades (Laughlin and Stevens. 2002; Crenshaw et al. 2008; Laughlin et al. 2009; Chen et al. 2014; Maeda et al. 2015; Rex et al. 2018). A wide range of fungi are capable of producing N_2O (Shoun et al. 1992; Jirout et al. 2013) and has been observed to be a widespread trait in fungi (Maeda et al. 2015). Given the fact that soil N_2O fluxes still peaked on Day 4 when both nitrification potential and nitrate reductase activity were inhibitied by DCD suggest that fungi may have played a crucial role for N_2O production in the soil used in this study when conditions arise that induce the need for fungi to respire NO_3^- in soil. Zhou et al. (2001) demonstrated that the fungus *Fusarium oxysporum* required anoxic conditions to respire NO_3^-, unlike bacterial denitrifiers that require anaerobic conditions alone. A study by Ma et al. (2008) also identified that hypoxia over anaerobisis was essential for fungal reduction of ^{15}N-Labelled nitrate to $^{15}N_2O$ and soil formate regulated fungal N_2O production. Ma et al. (2008) also proposed that plant root exudates could affect N_2O production from fungi via their proposed formate-dependant pathway. Pendergast-Miller, Baggs and Johson (2011)

have also demonstrated the ability of ectomycorrhizal fungi to contribute to N_2O producution. N_2O producution in this study may have occurred in what could be considered hypoxic conditions, when %WFPS reached between 68-70% which may reflect the ideal hypoxic conditions conditions for fungal production of N_2O (Ma et al. 2008). The recent study by Rex et al. (2018) proposed that fungi, not bacteria, dominated the contributions to N_2O fluxes in a simulated urine amended pasture soil, through the inhibition of NH_4^+ - ^{15}N enrichments by cylcoheximide, concluding that fungi have a significant role in the production of N_2O when soils are affected by high rates of urea, as those observed in urine deposition in grasslands soils. In addition, fungi do not possess an N_2OR gene (Maeda et al. 2015). As diccussed earlier in this chapter, for the non-DCD treated soils which peaked on Day 4 as did DCD treated soils in the 1.3 g cm^3 soil treatment, fluxes were observed to decrease by Day 8. This was attibuted to the increased transcriptional activity of *nosZ* in soil denitrifying bacteria, however, in constrast to the Urine + No DCD treated soils Urine + DCD treated soils showed no significant reductions in N_2O fluxes on Day 8 which could further suggest, in combination with the inhibition of both soil potential nitrification and nitrate reductase activities, that fungi were contributing to the production of N_2O.

The regulations of fungal N_2O production have been studied. Chen et al. (2015) studied the effect of soil moisture and pH as factors regulating fungal and bacterial N_2O production. From their study it was observed that both bacteria and fungi preferred more anoxic and neutral, or slighlty alkaline conditions for producing N_2O. As peak N_2O fluxes were observed with Urine treated soils, as urine increased soil pH to ~7 and ~6 in the non-DCD and DCD soils respectively, and the soils were maintained at ~70% WFPS, these conditions would agree with the findings by Chen et al. (2015). *Lolium perenne* roots have been found to be commonly infected with

fungi such as *Penicillium* spp and *Fusarium oxysporum* (Skipp and Christensen. (1989), which have been shown to be more dominant in the production of fungal N_2O emissions (Jirout et al. 2013; Chen et al. 2015). As this study utilised columns planted with *Lolium perenne* grass, it is possible that the roots could have been invaded by these types of fungi and others related to N_2O production.

5.5. CONCLUSION

The results from this study demonstrated that DCD may be effective at reducing soil N_2O fluxes that occur as a result of nitrification activities through reducing both potential nitrification and nitrate reductase. Increased soil bulk density and WFPS reduced the efficacy of DCD in soils at higher bulk density and therefore, increased WFPS on N_2O fluxes from this grassland soil mesocosmn study indicating at a limitation for DCD use in compacted soils receiving urine inputs.

As the majority of microbial activity was located in the top 0-5 cm layer of soils, this would suggest that surface compaction of soil would play a crucial role in increasing soil N_2O emissions and should therefore. The observation that N_2O fluxes still persisted when DCD inhibited both potential nitrification and nitrate reductase activities indicated that other routes of N_2O prodcution were dominant. The possible route of fungal N_2O production through the high inputs of nitrogen from the urine treatment seems to be a good candidate for these other possbile routes. This also highlights the imporatance of including plant species in soils studied for N_2O fluxes, as they could have large influences on the results. Further study should focus on the distibution of gene abundances of fungi, bacteria and archaea that specifically focus on N_2O producing pathways, the conditions that influence each community and the influences of root exudates on said fluxes.

CHAPTER 6. GENERAL DISCUSSION

6.1. MAIN FINDINGS

6.1.1. The influence of soil bulk density on N_2O fluxes

The work conducted in this thesis examined the impact of commonly occurring grassland management. As identified early on, the effect of both cattle traffic and heavy machinery increases the bulk density of soils which have a negative impact on the physical conditions that play an important role with regard to N_2O emissions such as increased WFPS (Linn and Doran, 1984; Bateman and Baggs, 2005). However, other studies have demonstrated that other soil physical properties may be more applicable in soils of varying bulk densities (van der Weerden et al. (2010). Therefore, a deeper understanding of the contribution of WFPS and soil bulk density was needed. **Chapter 2** set out to investigate the effect of soil physical parameters, under increased bulk densities, on N_2O emissions. Interestingly the oxygen diffusion measurements indicated that the most compacted soils in combination with the highest WFPS demonstrated the largest gas diffusion rates of all the sample treatments. Therefore WFPS alone, although a useful tool for the prediction of soils that are likely to have increased N_2O fluxes as a result of this, may not provide the whole picture. The increased gas diffusivity was attributable to the decreased pore tortuosity in soils at increasing bulk densitites which would seem to be relative to the lower soil moisture contents in the soils at higher bulk densities in order to achieve similar WFPSs. This also indicated that soils at higher bulk densities have the potential to increase soil N_2O emissions even in drier conditions due to the reduced air-filled porosity, and therefore subsequent reductions in air-filled pore sapce. The different contributions from soil nitrogen cycling microorganisms to soil N_2O fluxes are normally associated with nitrifiers, at 60% WFPS and denitrifiers, at 70-80% WFPS (Bateman and Baggs,2005).

Although this association with nitrifiers and denitrifiers may be seen for soils at lower bulk densities, the results from **Chapter 2** may suggest that at increasing soil bulk density the use of these criteria may be less effective, as fluxes of N_2O were seen to be greater in the 1.4 g cm^3 bulk density 60% WFPS treatment than the lower bulk densities at the same WFPS (1.1 g cm^3 and 1.2 g cm^3). There were limitations to the results discussed in **Chapter 2** with respect to the identification of the microbial communities as a source of N_2O emissions as there was no method used to identify them, however, this was discussed in the chapter in context of the current knowledge of these groups.

6.1.2. Efficacy of DCD on soil N_2O fluxes in compacted grassland soils through an improved understanding of soil microbial nitrogen cycling pathways

During the current work, assessing the effect of DCD on two critical stages of soil nitrogen cycling, nitrification and nitrate reduction, its efficacy in reducing N_2O fluxes from compacted grassland soils was further understood. **Chapter 5** aimed to improve on the design from **Chapter 2** by including what is probably one of the most important aspects when studying gaseous fluxes from soil; plant cover. The modifications in **Chapter 5** helped to emulate conditions that would be more native to the conditions in the field. The inclusion of growing rye grass (*Lolium perenne*) in the soil columns ensured the CO_2 fluxes were not diminished after sources of nitrogen were added, and indicated that the microbial community was more stable in the experiment, and less affected by experimental conditions which was seemingly observed in **Chapter 2**. The results from **Chapter 5** also demonstrated the negative impact of soil bulk density, similar to what was oberved in **Chapter 2**; with N_2O fluxes increasing in soils at greater bulk densitiy. Soil N_2O fluxes were significantly higher, as long as nitrogen

wasn't limiting, at higher bulk densities as a result of increased WFPS, as has been observed in other studies (Hammonts et al. 2013). However, the use of DCD did not reduce the impact of increased bulk density on soil N_2O fluxes under urine deposition, demonstrating an inadequacy of DCD not commonly observed in other studies (Di and Cameron. 2002; Di et al. 2009; Podolyan et al. 2014) which could be attributable to the fact that these previous studies have only focused on the efficiacy of DCD on reducing N_2O emission under soils receiving similar bulk density treatments. What was more interesting was the indication that the sources of N_2O under conditions that were observed to inhibit both nitrifying and nitrate reducing soil microorganisms could have been from fungal origin. These findings further indicated the potenttial importance of the soil fungal community in grassland nitrogen cycling and reinforces the importance of including either plants, which can be troublesome in a labortory setting in relation to maintaining certain soil conditions i.e. soil moisture content, or an associated cocktail of root exudates from plants that would be present in the soils of interest i.e. in this case perennial ryegrass, in future experiments studying the fluxes of N_2O in agriculture. The importance of root exudates, or species of carbon, have been increasingly demonstrated to significantly alter the emission rate of N_2O from soil through increased N_2OR activity (Morley et al. 2014). Ideally the inclusion of the host plant would be more preferential, due to the associated developing relationships between the microorganisms and roots during growth would be lost if only root exudates were applied. However, both methodologies include clear advantages over studies that simply include soil. The importance of different soil microbial groups were identified in both **Chapter 3** and **Chapter 4**. In **Chapter 3** the use of the streptomycin and cycloheximide inhibitor method highlighted the importance of soil heterotrophic communities in the oxidation of ammonium and the production of nitrate. As soils

were taken from a grassland site that had received different compaction treatments, an understanding of the effect of compaction could be observed in relation to soil ammonium oxidation. The study, although based on that conducted by Laughlin et al. (2008), included the effect of DCD treatment in order to establish if ammonia oxidation still occurred and if this could be attributed to the heterotrophic nitrifying communities in the soil. Indeed, cycloheximide inhibited ammonium oxidation in the soils collected from the different compaction treatments, and although DCD inhibited ammonium oxidation, nitrate production was still observed to continue by Day 5, which was then inhibited by cycloheximide and not by streptomycin, again further indicating the importance of heterotrophs in nitrate production in grassland soils. Again several limitations were associated with this methodology, which will be discussed later in this chapter. However, the results suggested that heterotrophic nitrogen cycling communities are important with regard to nitrogen cycling in grassland soils.

Chapter 4 sought to look further into the efficacy of DCD in soil receiving different nitrogen sources. The main results showed that DCD inhibited soil nitrification in all fertiliser treatments, therefore demonstrating that DCD efficacy is not limited by nitrogen source. However, the use of the antibiotic cycloheximide, which was observed in an earlier study to inhibit the growth of an ammonia oxidizing archeaon *Nitrosopumilus maritmus* (Vajrala et al. 2014), indicated that soil nitrification potential may be as a result of a 50/50 interaction between both AOB and AOA. However, further study of the literature on the effects of cycloheximide on other organisms in soils indicated that a secondary pathway may also be possible. Due to the common observation that AOB are generally more active and grow at a faster rate than AOA in soils receiving N addition, reinforced by the observation of the lack of

inhibition by DCD on AOA (Zhang et al. 2012); the results obtained in **Chapter 4** from the perspective of our current understanding of the effects of cycloheximide on AOA and more importantly the conditions that effect their growth and activity, as this outweights our current knowledge of the effects of cycloheximide on soil AOA, would more strongly point towards the secondary pathway discussed in **Chapter 4**. Not only has this helped provide a potential understanding of the use of cycloheximide in soil nitrogen cycling studies by offering an alternate route of inhibition, but has also highlighted the potential importance of soil protozoa that may play a more important role in soil nitrification, and therfore denitrifcation, than is currently understood especially in soil under urine deposition, or slurry amended soils.

6.1.3. Conclusions

The result obtained in this thesis has added to the knowledge and helped improve the understanding of regulations of N_2O emissions, including the contribution of the microbial communities to the production of N_2O in grassland soils. Increased soil bulk density is no doubt one of the most important factors in relation to the increased fluxes of soil N_2O from grassland soils, and WFPS alone may not be sufficient to explain the enhanced fluxes of the N_2O from the soil, when refering to its microbial sources i.e. nitrifiers and denitrifiers. Limitations of oxygen through increased moisture content were highlighted as another contributing factor, agreeing with previous studies on soil N_2O fluxes and that WFPS as an indicator of the potential source of N_2O, i.e. nitrifier N_2O and denitrifier N_2O may be less appropriate with increased soil bulk density which warrents further study. Urine deposition to the soil was demonstrated to be a key driver in the amount of N_2O emitted from the soil under higher bulk density, agreeing with other studies (Di and Cameron.2002; Di et al. 2009.). This was

specifically associated with the higher NH_4^+ loading rate that stimulated both the nitrification and denitrification pathways.

Limitations of DCD as a nitrification inhibitor was demonstrated as it was ineffective at reducing N_2O fluxes resulting from urine application to soils. Observations of reduced potential nitrification and nitrate reductase pathways, which are associated with the production of N_2O in soil, indicated an alternative pathway of fungal N_2O production that may have often be overlooked.

The importance of heterotrophic nitrification was highlighted through the subsequent inhibition of ammonium oxidation and nitrate production through the use of cycloheximide. However, a potential implication in the use of cycloheximide was identified, suggesting that other pathways of inhibition may be overlooked in other soil nitrogen cycling studies that employ the use of cycloheximide.

6.2. Limitations of the Methodologies Used in the Thesis

As discussed in previous chapters there were several limitations to the study covered here. First **Chapter 2**, although this chapter did provide useful information on the relationship between soil WFPS and increased bulk density, there were several limitations to the study. The most notable was the lack of microbial differentiation for the sources of N_2O emissions. The simple use of acetylene inhibition would have provided a much needed estimation on the source of N_2O under the conditions presented in the soils, as acetylene would have inhibited production of N_2O from nitrification, or, as a direct result of nitrifier denitrification. This would have been particularly fascinating as it has helped understand whether nitrifer denitrification of heterotrophic denitrification was taking place in the 1.4 g cm^3 bulk density at the highest WFPS (80%) as these samples potentially had the highest oxygen diffusion

rates as measured in a subset of samples. As the air-filled pore space in the 1.4 g cm^3 + 60% WFPS treatment was the third lowest, an understanding of the microbial source in these samples would have provided a fuller picture of the N_2O production. However, the use of acetylene alone might not have been sufficient. The use of ^{15}N labelled NH_4^+ would have helped understand the routes of N_2O production and it would be highly recommended in any future study.

In addition, due to the excessive concentrations of N_2O measured in the study, the use of a more appropriate gas sampling method that had no effect on sample headspace pressure would be essential in order not to introduce artefacts in to the concentrations of N_2O that were clearly observed in **Chapter 2**.

Chapter 3 was designed as a preliminary study, intended to examine the contributions of ammonium cycling between bacteria and fungi in the grassland soils. It has been acknowledged that a dosage response was not conducted for the experiment with regard to ammonium oxidation rates, and a great deal of time was spent trying to optimise the use of the inhibitors for the study of gas fluxes, which was found to simply introduce more problems than solutions, and was subsequently removed from the study. What would have been included in a full study would have been the use of PLFAs to distinguish fungal and bacterial biomass. However, as highlighted in **Chapter 3**, fungi aren't thought to use ammonium oxidation for growth and the use of PLFAs could have either produced an artefact if an increase in fungal biomass was observed in relation to ammonium reduction rates, or, demonstrated no effect. Therefore the use of cycloheximide would have been more appropriate. However, one thing was clear, the identification of a fungal ammonia oxidizing gene is a necessary tool if further studies into fungal ammonium cycling were to be conducted. **Chapter 4** studied the efficacy of DCD on ammonia-oxidizing soil microbial communities

when receiving different nitrogen sources. This was assumed to be important as previous studies had observed that soil ammonia oxidizing communities were not fully inhibited (Podolyan et al. 2014) and it was thought that the efficacy of DCD may have been compromised with different N sources and that an improved understanding of efficacy of DCD in different N treatments would help identify the usefulness of the compound. The recent study by Vajrala et al. (2014), identified the negative impact of cycloheximide on ammonia oxidation in an ammonia oxidizing archaeon *Nitrosopumlius martimus* and raised the question of whether cycloheximide had an impact on soil nitrification potential. As other studies have concluded that cycloheximide inhibited only fungi (Castaldi and Smith . 1998; Laughlin et al. 2008), the impact of cycloheximide on archaea in relation to the study by Vajrala et al. (2014) seemed important. However, the main limitation of the work presented here was the inability to measure abundances of both AOB and AOA genes. The use of this simple technique would have allowed a direct quantitative assessment of any negative impacts of cycloheximide on either groups.

Chapter 5, although a more comprehensive study in comparison to **Chapter 2**, would have been improved through the use of gene abundance analysis of both AOB and AOA as well as denitrifying genes such as *narG, nirK and nosZ*. The inclusion of these assessments would have provided a more clear understanding of the groups of nitrifers and denitrifiers and their distribution in the soil columns, along with their response to the fertilisers and DCD treatments. Acetylene inhibition to determine the total denitrification potential of the soils would also have helped to demonstrate more precisely when the N_2O levels diminished and if this was due to the activation and increased activity of the *nosZ* gene.

6.3. Recommendations for Further Study

Further work leading on from the current studies would link more of the soil physical conditions such as air-filled pore space, oxygen diffusion rates and air permeability, and relate these more closely to the production of N_2O and N_2 gases from grassland soils. Given the increased gas diffusion rates at the higher bulk densities, a ^{15}N labelling study would help to distinguish the sources of N_2O from either nitrifiers or denitrifiers. This would help to increase knowledge between physical characteristics of the soils at increased soil bulk densities which would help estimate the longevity of N_2O fluxes that may occur as a result of these increased soil bulk densities under high nitrogen concentrations. This would add further certainty to estimations of greenhouse gases from agricultural systems.

One of the most interesting results in this thesis was the continued production of N_2O in the presence of DCD. Although DCD is widely used and the subject of a number of studies, the fate of the compound is still poorly understood. Degradation of DCD has been clearly demonstrated to be dependant on soil temperature (Ranjbanshi et al. 1991; Kelliher et al. 2014), which in turn has been linked to microbial degradation (Kelliher et al. 2008). The breakdown and fate of DCD in soils needs to be resolved. A study by Estermaier et al. (1992) demonstrated that DCD was metabolised to equal amounts of cyanourea and ammonia by a bacterial urease from *Bacillus pasteurii*. As *Nitrospira* are also known to contain a urease encoded gene, DCD may potentially be degraded in some part by the same microorganism that it is intended to inhibit.

As DCD was observed to increase CO_2 emissions in the soil **(Chapter 5)** it seems that it is stimulating some portion of the soil microbial communitiy. A doubled labelled ^{15}N ^{14}C experiment with DCD would help to identify both whether the extra CO_2 that is emitted from the soil is a direct result of the degradation of DCD, and also whether

DCD itself is eventually transformed into N_2O. This would clearly make its use less efficient. Labelled DCD would similarly help to understand the best way to use the nitrification inhibitor by linking it to the optimal activities of the microbes that are degrading the compound. This would help identify temperatures and soil conditions that would ensure optimal effect in reducing the losses of N_2O when used.

The effects of cycloheximide on soil potential nitrification rates in soils treated with cattle urine are one of the most important findings from this work. As cycloheximide is used as an inhibitor in soil studies investigating bacterial and fungal nitrogen cycling, the effects of this antibiotoic on soil microorganism needs to be fully resolved. In light of the work conducted by Vajrala et al. (2014), the understanding of the effects of cycloheximide on soil AOA are of high importance, as the potential inhibition of AOA in soils nitrogen cycling studies may lead to an over estimation of the contributions of fungi to both ammonium oxidation and N_2O production. In addition, as this antibiotic has been shown to have the ability to affect growth of soil micro-organisms (Griffiths et al. 1989) and the activity of ammonia oxidizers (Petropoulos et al. 2005; Pogue et al. 2007), the use of cycloheximide could potentially oversestimate the contributions of fungi to the production of N_2O due to the inhibition of soil nitrification as observed in **Chapter 4,** which could potentially be related to the inhibiton of soil protozoa. This only enhances the need for a complete understanding of the other effects of this eukaryotic protein synthesis inhibitor in soil.

As NH_4^+ plays such an important role in regulating that activities of both AOB and AOA in soil, as well as a range of other soil organic compounds, discussed in **Chapter 4**, the use of more sensitive methods for estimating the 'true' chemical composition of the soil would be extremely advantageous. Advances in measuring diffusive fluxes from soil have progressed over the last decade (Inselbacher and

Nasholm. 2012). Inselbacher and Nasholm. (2012) demonstrated that 80% of the soil N supply in boreal forest was compromised of amino acids and that only 10% of the total soil N was attributable to NH_4^+ and NO_3^-. This was accomplished using the method of microdialysis, a method which uses diffusive flux to exhange between high concentrations of compounds, i.e. in the soil, to low concentrations (microdialysis probe perfusing water through the exchange membrane). This method could easily be extended to all soil chemical compounds in order to obtain a more realistic concentration gradient in the soil *in-situ*. The use of microdialysis also highlighted that commonly used extraction methods, such as water extractions, over estimate the concentrations of NH_4^+ and NO_3^-. Given that the activity of soil AOA is highly sensitive to concentrations of ammonium, this uninvasive method would allow a much improved understanding of the compounds that induce the activity of these soil micro-organisms.

Finally, as highlighted earlier in this chapter the importance of soil microbiology studies to include plants that are found in the soils studied if laboratory experiments are being conducted in order to reduce other environmental variables that would otherwise make detailed observations more difficult. When studying the soil microbiome, removing what could be considered one of the most influential factors would seem to create artificial conditions that may favour microbial communities that would naturally be less dominant. It is thought that the observation of continued soil N_2O, even though both potential nitrificaiton and nitrate reductase activity was seemingly fully inhibited, flux may not have occurred in a soil that was deprived of grass roots. However, this would need to be confirmed in a similar study as the suggested continued N_2O flux from fungal activity is only a proposed route.

REFERENCES

Amberger A. 1989. Research on dicyandimide as a nitrification inhibitor and future outlook. *Communications in Soil Science and Plant Analysis*. 20. pg. 1933 – 1955.

Arciero D M and Hooper A B. 1997. Evidence for a crosslink between c-heme and a lysine residue in cytochrome P460 of *Nitrosomonas europaea*. *FEBS Letters*. 410. pg. 457 – 460.

Averill B A and Tiedje J M. 1989. The chemical mechanisms of microbial denitrification. *Federation of European Biochemical Societies Letters*. 138. pg. 8 – 12.

Badalucco L, Pomare F, Grego S and Landi L. 1994. Activity and degredation of streptomycin and cycloheximide in soil. *Biology and Fertilisty of Soils*. 18. pg. 334 – 340.

Badawi N, Johnsen A R, Brandt K K, Sorensen J and Jens Aamand. 2012. Protozoan predation in soil slurries compromises determination of contaminant mineralization potential. *Environmental Pollution*. 170. pg. 32 – 38.

Balaine N, Clough T J, Kelliher F M and van Koten C. 2015. Increasing soil aeration reduces mitigation efficacy of dicyandiamide when targeted at ruminant urine-derived N_2O emissions. *New Zealand Journal of Agriultural Research*. 58. pg. 441 – 453.

Ball B C, Crichton I and Horgan G W. 2008. Dynamics of upward and downward N_2O and CO_2 fluxes in ploughed or no-tilled soils in relation to water-filled pore space, compaction and crop presence. *Soil and Tillage Research*. 101. pg. 20 – 30.

Ball B, Crichton I and Horgan G W. 2008. Dynamics of upward and downward N_2O and CO_2 fluxes in ploughed or no-tilled soils in relation to water-filled pore space, compaction and crop presence. *Soil Tillage and Research*. 101. pg. 20 – 30.

Ball BC, Harris W, and Burford J R. 1981. A laboratory method to measure gas diffusion and flow in soil and other porous materials. *Journal of Soil Science*. 32. pg. 323 – 333.

Ball BC. 2013. Structure of greenhouse gas emissions: a synthesis of 20 years of experimentation. *European Journal of Soil Science*. 64. pg. 357 - 373.

Barenze A S, Minet E P, Cerri C C and Misselbrook T. 2015. The effect of nitrification inhibitors on nitrous oxide emissions from cattle urine depositions to grasslands under summer conditions in the UK. *Chemosphere*. 119. pg. 122 – 129.

Bateman E J and Baggs. 2005. Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space. *Biology and Fertility of Soils*. 41. pg. 279 – 388.

Beauchamp E G. 1997. Nitrous oxide emissions from agricultural soils. *Canadian Journal of Soil Science*. 77. pg. 113 – 123.

Beaumont H J E, Lens S I, Reijnders W N M, Westerhoff H V and Van Spanning R J M. Expression of nitrite reductase in *Nitrosomonas europaea* involves NsrR, a novel nitrite-sensitive transcription repressor. *Molecular Microbiology*. 54. pg. 148 – 158.

Belser L W. 1984. Bicarbonate uptake by nitrifiers: Effects of growth rate, pH, substrate concentration and metabolic inhibitors. *Applied and Environmental Microbiology*. 48. pg. 1100 – 1104.

Bergaust L, Mao Y, Bakken L and Frostegard. 2010. Denitrification response patterns during the transition to anoxic respiration and posttranscriptional effects of suboptimal pH on nitrogen oxide reductase in *Paracoccus denitrificans*. 76. pg. 6387 – 6396.

Bergmann D J, Arciero D M and Hooper A B. 1994. Organization of the hao gene cluster of *Nitrosomonas europaea*: genes for two tetraheme c cytochromes.

Bezkorowajnyj P G, Gordon A M and McBride. 1993. The effect of cattle foot traffic on soil compaction in a silvo-pastoral system. *Agroforestry Systems*. 21. pg. 1 – 10.

Bhandral R, Saggar S, Bolan N S and Hedley. 2007. Transformation of nitrogen and nitrous oxide emission from grassland soils as affected by compaction. *Soil and Tillage Research*. 94. pg. 482 – 492.

Breland T A and Hansen S. 1996. Nitrogen mineralization and microbial biomass as affected by soil compaction. *Soil Biology and Biochemistry*. 28. pg. 655 – 663.

Bremner J M. 1997. Sources of nitrous oxide in soils. *Nutrient Cycling in Agroecosystems*. 49. pg. 1 – 3.

Briggs W R. 2014. Phototropism: some history, some puzzles and a look ahead. *Plant Physiology*. 164. 13 – 23.

Buckingham E. 1904. Contributions to our knowledge of the aeration of soils. *Bureau of Soils Bulletin*. 25. U.S. Department of Argiculture. U.S. Government Printing Office. Washington.

Burchill W, Li D, Langigan G J, Williams M and Humphreys J. 2014. Interannual variation in nitrous oxide emissions from perenninal ryegrass/white clover grassland used for dairy production. *Global Change Biology*. pp 3137 – 3146.

Butterbach-Ball K, Baggs E M, Dannenmann M, Kiese R and Zechmeister-Boltenstern S. 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? *Philosophical Transactions of the Royal Society B*. 368. pg. 1- 13.

Cabera M L, Kissel D E and Bock B R. 1991. Urea hydrolysis in soil: Effects of urea concentration and soil pH. *Soil Biology and Biochemistry*. 23. 1121 – 1124.

Caranto J D, Vilbert A C and Lancaster K M. *Nitrosomonas europaea* cyctochrome P460 is a direct link between nitrification and nitrous oxide emissions. *Proceedings of the National Academy of Sciences of the United States of America (PNAS)*. 113. pg. 14704 – 14709.

Castaldi S and Smith K A. 1998. Effect of cycloheximide on N_2O and NO_3^- production in a forest and an agricultural soil. *Biology and Fertility of Soils*. 27. pg. 27 – 34.

Castellano M J, Schmidt J P, Kaye J P, Walker C, Graham C B, Lin H and Dell C J. 2009. Hydrological and biogeochemical controls on the timing and magnitude of nitrous oxide flux across an agricultural landscape. 16. pg. 2711 – 2720.

Castigentti D and Hollocher T C. 1984. Heterotrophic nitrification among denitrifiers. *Applied and Environmental Microbiology*. 47. pg. 620 – 623.

Chalk P M and Smith C J. 1983. Chemodenitrificaiton. In Freney J R, Simpson J R (eds) Gaseous Loss of Nitrogen from Plant-Soil Systems. *Develoopments in Plant and Soil Sciences*. 9. Springer, Dordrecht, Netherlands. pg. 65 – 89.

Chamindu Deepagoda T K K, Moldrup P, Schjonning P, Wollesen de Jonge L, Kawamoto K and Komatsu T. 2011. Denisty-corrected models for gas diffusivity and air permeability in unsaturated soil.

Chen H, Mothapo N V and Shi W. 2014. The significant contribution of fungi to soil N_2O production across diverse ecosystems. *Applied Soil Ecology*. 73. pg. 70 – 77.

Chen H, Mothapo N V and Shi W. 2015. Soil moisture and pH control relative contributions of fungi and bacteria to N_2O production. *Microbial Ecology*. 69. pg. 180 – 191.

Chen Z, Wu W, Shao X, Li L, Guo Y, Ding G. 2015. Shifts in abundance and diversity of soil ammonia-oxidizing bacteria and archaea associated with land resotration in a semi-arid system.

Christensen S and Tiedje J M. 1998. Denitrification in the field, analysis of spatial and temporal variability. In D. S Jenkinson and K A Smith (Eds), *Nitrogen Efficiency in Agricultural Soils*. pg. 295 – 301. Elsevier, Amsterdam.

Clayton H, McTaggert I P, Parker J, Swan L and Smith K A. 1997. Nitrous oxide emissions from fertilised grassland: A 2-year study of the effects of N fertiliser form and environmental conditions. *Biology and Fertility of Soils*. 25. pg. 252 – 260.

Clough T J, Rolston D E, Stevens R J and Laughlin R J. 2003. N_2O and N_2 gas fluxes, soil gas pressures, and ebullition events following irrigation of $^{15}NO_3^-$ - labelled subsoils. *Soil Research*. 41. pg. 401 – 420.

Crenshaw C L, Lauber R L, Sinsabaugh R L and Stavely L K. 2008. Fungal control of nitrous oxide production in semiarid grassland. *Biogeochemistry*. 87. pg. 17 – 27.

Crutzen P J, Moiser A R, Smith K A and Winiwarter W. 2008. N_2O release from agro-biofuel production negates global warming reduction by replacing fossil fuels. *Atmospheric Chemistry and Physics*. 8. pg. 389 – 395.

Crutzen P J, Mosier A R, Smith K A, Winiwarter W. 2007. N_2O release from agro-biofuel production negates global warming reduction by replacing fossil fuel. *Atmospheric Chemistry and Physics Discussion 7*. pp 11191–11205.

Cumming G, Fidler F and Vaux D L. Error bars in experimental biology. 2007. *The Journal of Cell Biology*. 177. pg. 7 – 11.

Currie J A. 1960. Gaseous diffusion in porous media Part 1. – A non-steady state method. *British Journal of Applied Physics*. 11. pg. 314 – 317.

Daims H, Lebedeva E V, Pjevac P, Han P, Herbold C, Alberston M, Jehmlich N, Palatinszky M, Vierheilig J, Bulaev A, Kirkegaard R H, von Bergen M, Rattei T, Bendinger B, Nielsen P H and Wagner M. 2015. Complete nitrification by *Nitrospira* bacteria. *Nature*. 528. pg. 504 – 509.

Daims H, Lucker S and Wagner M. 2016. A new perspective on microbes formerly known as nitrite-oxidizing bacteria. *Trends in Microbiology*. 24. pg. 699 – 712.

Davidson E A 2009 The contribution of manure and fertilizer nitrogen to atmospheric nitrous oxide since 1860. *Nature Geoscience*. pp 659 – 62.

Davidson E A and Kanter D. 2014. Inventories and scenarios of nitrous oxide emissions. Environmental Research Letters. pp 1 – 12.

Davidson E A and Sietzinger S. 2006. The enigma of progress in denitrification research. *Ecological Applications*. 16. pg. 2057 – 2063.

De Boer W and Kowalchuk G A. 2001. Nitrification in acid soils: micro-organisms and mechanisms. *Soil Biology and Biochemistry*. 33. pg. 853 – 866.

De Boer W and Kowalchuk G A. 2001. Nitrification in acids soils: micro-organisms and mechanisms. *Soil Biology and Biochemistry*. 33. pg. 853 – 866.

de Klein C A M, Letica S A and Macfie P I. 2014. Evaluating the effects of dicyandiamide (DCD) on nitrogen cycling and dry matter production in a 3-year trial on a diary pasture in South Otago, New Zealand. *New Zealand Journal of Agricultural Research*. 4. pg 316 – 331.

DEFRA. 2016. Agriculture in the United Kingdom 2015. (London, UK). [Accessed online: 23-06-2018; 20:00]. https://www.gov.uk/government/statistics/agriculture-in-the-united-kingdom-2016.

DEFRA. 2018. Fertiliser manual (RB209). [Accessed Online 20-06-2018]. https://www.gov.uk/government/publications/fertiliser-manual-rb209--2

Deiglmyar K, Philippot L, Hartwig U A and Kandeler E. 2004. Structure and activity of the nitrate-reducing community in the rhizosphere of *Lolium perenne* and *Trifolium repens* under long-term elevated atmospheric pCO_2. 49. pg. 445 – 454.

Del Grosso S J, Parton W J, Mosier A R, Ojima A E, Kulmala A E and Phongpan S. 2001. General model for N_2O and N_2 gas emissions from soils due to denitrification. *Global Biogeochemical Cycles*. 14. pg. 1045 – 1060.

Denman K L, Brasseur G, Chidthaisong A, Ciais P, Cox P M, Dickinson R E, Hauglustaine D, Heinze C, Holland E, Jacob D, Lohmann U, Ramachandran S, Da Silva Dias P L, Wofsy S C, Zhang X. 2007. Coupling between changes in the climate system and biogeochemistry in: Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt K B, Tignor M, Miller B R (eds). *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press. Cambridge, UK and New York, US. pg. 500–587.

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

Di H J and Cameron K C. 2003. Mitigation of nitrous oxide emissions in spray-irrigated grazed grassland by treating the soil with dicyandiamide, a nitrification inhibitor. *Soil Use and Management*. 19. pg. 284 – 290.

Di H J and Cameron K C. Sources of nitrous oxide from ^{15}N-labelled animal urine and urea fertiliser with and without a nitrification inhibitor dicyandiamide (DCD). *Australian Journal of Soil Research*. 46. pg. 76 – 82.

Di H J, Cameron K C and Sherlock R R. 2007. Comaparison of the effectiveness of a nitrification inhibitor, dicyandiamide, in reducing nitrous oxide emissions in four different soils under different climatic and management conditions. *Soil Use and Management*. 23. pg. 1 – 9.

Di H J, Cameron K C, Podolyan A and Robinson A. 2014. Effect of the nitrification inhibitor dicyandiamide, on ammonia oxidizer and denitrifier growth and nitrous oxide emissions in a grassland soil. *Soil Biology and Biochemistry*. 73. pg. 59 – 68.

Di H J, Cameron K C, Shen J P, Winefield C S, O'Callaghan M, Bowatte S and He J Z. 2009. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature geoscience*. 2. P. 621 – 624.

Di H J, Cameron K C, Shen J P, Winefield C S, O'Callaghan M, Bowatte S and He J Z. 2010. Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. *FEMS Microbiology Ecology*. 72. pg. 386 – 394

Dickinson R E and Cicerone R J. 1986. Future global warming from atmospheric trace gases. *Nature*. 319. pg. 109 – 115.

Eurostat.2018.http://ec.europa.eu/eurostat/statisticsexplained/index.php/Land_cover_statistics. [Accessed online: 23-06-2018; 20:00].

Fernandez N, Sierra-Alvarez, Field J, Amils R and Sanz J L. 2008. Microbial community dynamics in a chemolithotrophic denitrification reactor inoculated with methanogenic granular sludge. *Chemosphere*. 70. pg. 462 – 474.

Firestone M K and Davidson E A. 1989. Microbiological basis of NO and N_2O production and consumption in soil. *Exchange of Trace Gases Between Terrestrial Ecosystems and the Atmosphere*. Andreae M O and Shcimel D S (eds). pg. 7 – 21.

Firestone M K, Firestone R B and Tiedje J M. 1980. Nitrous oxide from soil denitrification: Factors controlling its biological production. Science. 208. pg. 749 – 751.

Fitton N, Datta A, Cloy J M, Rees R M, Topp C F E, Bell M J, Cardenas L M, Williams J, Smith K, Throman R, Watson C J, McGeough K L, Kuhnert M, Hastings A, Anthony A, Chadwick D, Smith P. 2017. Modelling spatial and inter-annual variations of nitrous oxide emissions from UK cropland and grasslands using DailyDayCent. *Agriculture, Ecosystems and the Environment*. pg. 1 – 11.

Flechard C R, Ambus P, Skiba U, Rees R M, Hensen A, van Amstel A, van den Pol-van Dasselaar A, Soussana J F, Jones M, Clifton-Brown J, Raschi A, Horvath L, Neftel A, Jocher M, Ammann A, Leifield J, Fuhrer J, Calanca P, Thalman E, Pilegaard K, Di Macro C, Campbell C, Nemitz E, Hargreaves K J, Levy P E, Ball B C, Jones S K, van de Bulk W C M, Groot T, Blom M, Domingues R, Kasper G, Allard V, Ceschia E, Cellier P, Laville P, Henault C, Bizouard F, Abdalla M, Williams M, Baronti S, Baronti S, Berretti F, Grosz B. 2007. Effects of climate and management intensity on nitrous oxide emissions in grassland systems across Europe. *Agriculture, Ecosystems & Environment*. pp 135–152.

Fortuna A, Honeycutt C W, Vandemark G, Griffin T S, Larkin R P, He Z, Wienhold B J, Sistani K R, Albrecht S L, Woodbury B L, Torbet H A, Powell J M, Hubbard R K, Eigenberg R A, Wright R J, Alldredge J R and Harsh J B. 2012. Links among nitrification, nitrifier communities, and edaphic properties in contrasting soils reciving dairy slurry. *Journal of Environmental Quality*. 41. pg. 262 – 272.

French E, Kozlowski J, Mukherjee M, Bullerjahn G and Bollman A. 2012. Ecophysiological characterisation of ammonia-oxidizing archaea and bacteria from freshwater. *Applied and Environmental Microbiology*. 78. pg. 5773 – 5780.

Fujikawa T and Miyazaki T. 2005. Effects of bulk density and soil type on the gas diffusion coefficient in repacked and undisturbed soils. *Soil Science*. 170. pg. 829 – 901.

Fukushima T, Whang L, Chiang T, Lin Y, Chevalier L R, Chen M and Wu Y. 2012. Nitrifying bacterial commnity structures and their nitrification performance under sufficient and limited inorganic carbon conditions. *Applied Microbiology and Biotechnology*. 97. pg. 6513 – 6523.

Gan X H, Zhang F Q, Gu J D, Guo Y D, Li ZQ, Zhang W Q, Xu X Y, Zhou Y, Wen X Y, Xie G G, Wang Y F. 2016. Differential distribution patterns of ammonia-oxidizing archaea and bacteria in acidic soils of Nanling National Nature Reserve forests in subtropical China. *Antonie Van Leeuwenhoek*. 109. pg. 237 – 251.

Gautier H and Varlet-Grancher C. 1996. Regulation of leaf growth of grass by blue light. *Physiologia Planatrum*. 98. pg. 424 – 430.

Goreau T J, Kaplan W A, Wofsy S C, McElroy M B, Valois F W and Watson S W. 1980. Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen. *Applied and Environmental Microbiology*. 40. pg. 526 – 532.

Gradwell M W. 1960. A laboratory study of the diffusion of oxygen through pasture topsoils. *New Zealand Journal of Science*. 4. pg. 250 – 270.

Griffiths B S. 1989. Enhanced nitrification in the presence of bacteriophagous protozoa. *Soil Biology and Biochemistry*. 21. pg. 1045 – 1051.

Gubry-Rangin C, Nicol G W and Prosser J I. 2010. Arcahea rather than bacteria control nitrification in two agrcultural acidic soils. *FEMS Microbiology Ecology*. 74. pg. 566 – 574.

Gubry-Rangin C, Nicol G W and Prosser J I. 2010. Archaea rather than bacteria control nitrificaiton in two agricultural acidic soils.

Hamonts K, Balaine N, Moltchanova E and Clough T. 2013. Influence of soil bulk density and matric potential on microbial dynamics, inorganic N transformations, N_2O and N_2 fluxes following urea deposition. *Soil Biology and Biochemistry*. 65. pg 1 – 11.

Helen K, Gevers D, Vanparys B, Wittebolle L, Geets J, Boon N and De Vos P. 2006. The incidence of nirS and nirK and their genetic heterogeneity in cultivated denitrifiers. *Environmental Microbiology*. 8. pg. 2012 – 2021.

Helliwell J R, Miller A J, Whalley W R, Mooney S J and Sturrock C J. 2014. Quantifying the impact of microbes on soil structural development and behaviour in wet soils. *Soil Biology and Biochemistry*. 74. pg. 138 – 147.

Hernandez D and Rowe J J. 1987. Oxygen regulation of nitrate uptake in denitrifying *Psuedomonas aeruginosa*. *Applied and Environmental Microbiology*. 53. pg. 745 – 750.

Herndl G J, Reinthaler T, Teira, van Aken H, Veth C, Pernthaler A and Pernthaler J. 2005. Contribution of Archaea to total prokaryotic production in the deep Atlantic ocean. *Applied and Environmental Microbiology*. 71. pg. 2303 – 2309.

Herold M B, Baggs E M and Daniell T J. 2012. Fungal and bacterial denitrification are differently affected by long term pH amendment and cultivation of arable soil. *Soil Biology and Biochemistry*. 54. pg. 25 – 35.

Hirsch P, Overrin L and Alexander M. 1961. Formation of nitrite and nitrate by actinomycetes and fungi. *Journal of Bacteriology*. 82. pg. 442 – 448.

Hooper A B and Terry K R. 1979. Hydroxylamine oxidoreductase of *Nitrosomonas*: Production of nitric oxide from hydroxylamine. *Biochemica et Biophysica Acta (BBA) – Enzymology*. 571. pg. 12 – 20.

Hora T S and Iyengar M R S. 1960. Nitrification by soil fungi. *Archives of Microbiology*. 35. pg. 252 – 257.

Hyman M R and Wood P M. 1985. Suicidal inactivation and labelling of ammonia monooxygenase be acetylene. *Biochemical Journal*. 227. pg. 719 – 725.

Igarashi N, Moriyama H, Fujiwara T, Fukumori Y and Tanaka N. 1997. The 2.8 Å structure of hydroxylamine oxidoreductase from a nitrifying bacterium *Nitrosomonas europaea*.

Ingham E R and Coleman D C. 1984. Effects of streptomycin, cycloheximide, fungizone, captan, carbofuran, cygon and PCNB on soil microorganisms. *Microbial Ecology*. 10. pg. 345 – 358.

Ingham E R and Coleman D C. 1984. Effects of streptomycin, cycloheximide, Fungizone, captan, carbofuran, cygon and PCNB on soil microorganisms. *Microbial Ecology*. 10. pg. 345 – 358.

Inselbacher E and Nasholm T. 2012. The below-ground perspective of forest plants: soil provides mainly organic nitrogen for plants and mycorrihzal fungi. *New Phytologist*. 195. pg. 329 – 334.

IPCC. 2007. Climate change 2007: Synthesis report. Contribution of working groups I, II and III to the fourth assessment report of the intergovernmental panel on climate change. Pachauri R K and Reisinger A (Eds). IPCC, Geneva, Switzerland. pg. 104.

Jager A, Samorski A, Pfeifer F and Lug G. 2002. Individual *gvp* transcript segments in *Haloferax mediterranei* exhibit varying half-lives, which are differentially affected by salt concentration and growth phase. *Nucleic Acids Research*. 30. pg. 5436 – 5443.

Jason J, Cantera L and Stein L Y. 2007. Molecular diversity of nitrite reductase genes (*nirK*) in nitrifying bacteria. *Environmental Microbiology*. 9. pg. 765 – 776.

Jia Z and Conrad R. 2009. Bacteria rather than archaea dominate microbial ammonia oxidation in an agricultural soil. *Environmental Microbiology*. 11. pg. 1658 – 1671.

Jirout J, Simek M and Elhottova D. 2013. Fungal contributions to nitrous oxide emissions from cattle impacted soils. *Chemosphere*. 90. pg. 565 – 572.

Kahlil M A K and Rasmussen R A. 1992. The global source of nitrous oxide. *Journal of Geophysical Research*. 97. pg. 14651 – 14660.

Kahlil M A K, Rasmussen R A and Shearer M J. 2002. Atmospheric nitrous oxide: patterns of global change during recent decades and centuries. *Chemosphere*. 47. pg. 807 – 821.

Kandeler E, Brune T, Enowashu E, Dorr N, Guggenberger G, Lamersdorf N and Philippot L. 2009. Response of total and nitrate-dissimilating bacteria to reduced N deposition in a spruce forest soil profile. *FEMS Microbiology Ecology*. 67. pg. 444 – 454.

Kandeler E. 1996. Ammonium: In Methods in Soil Biology. Schinner F, Ohlinger R, Kandeler E and Margesin R. (Eds.). Springer - Verlag, Berlin, Heidelberg, New York. pg. 406 – 408.

Kandeler E. 1996. Nitrate reductase activity: In Methods in Soil Biology. Schinner F, Ohlinger R, Kandeler E and Margesin R. (Eds.). Springer - Verlag, Berlin, Heidelberg, New York. pg. 176 – 179.

Kandeler E. 1996. Potential nitrification: In Methods in Soil Biology. Schinner F, Ohlinger R, Kandeler E and Margesin R. (Eds.). Springer - Verlag, Berlin, Heidelberg, New York. pg. 146 – 148.

Keil D, Meyer A, Berner D, poll C, Schutzenmeister A, Piepho H, Vlasenko A, Philippot L, Schloter M, Kandeler E and Marhan S. 2011. Influence of land-use intensity on the spatial distribution of N-cycling microorganisms in grassland soils. *FEMS Microbiology Ecology*. 77. pg. 95 – 106.

Kelliher F M, Clough T, Clark H and Sedcole J R. 2008. The temperature dependence of dicyandiamide (DCD) degradation in soils; A data synthesis. *Soil Biology and Biochemistry*. 40. pg. 1878 – 1882.

Kelliher F M, Sedcole J R, Emery I and Condron L M. 2007. Grassland soil microbial repisration responses to urea and litter applications. *Agricultural Research*. 50. pg 321 – 326.

Kelliher F M, van Koten C, Kear M J, Sprosen M S, Ledgard S F, de Klein C A M, Letica S A, Luo J and Rys G. 2014. Effect of temperature on dicyandiamide (DCD) longevity in pastoral soils under field conditions.

Koch H, Lucker S, Alberston M, Kitzinger K, Herbold C, Spieck E, Nielsen P H, Wagner M Daims H. 2015. Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus *Nitrospira*. *Proceedings of the National Academy of Sciences of the United States of America (PNAS)*. 36. pg. 11371 – 11376.

Kowalchuk G A and Stephen J R. Ammonia-oxidizing bacteria: A model for molecular microbial ecology. *Annual Reviews of Microbiology*. 55. pg. 485 – 529.

Kowlachuk G A and Stephen J R. 2001. Ammonia-oxidzing bacteria: a model for molecular microbial ecology. *Annual Review of Microbiology*. 55. pg. 485 – 529.

Kozlowski J A, Price J and Stein L Y. 2014. Revision of the N_2O- producing pathways in the ammonia oxidizing bacterium *Nitrosomonas europaea*. ATCC 19718. *Applied and Environmental Microbiology*. 80. pg. 4930 – 4935.

Kutschera U and Briggs W R. 2012. Root phototropism: from dogma to the mechanism of blue light perception. *Planta*. 235. pg. 443 – 452.

Laudone G M, Matthews G P, Bird N R A, Whalley W R, Cardenas L M and Gregory A S. 2011. A model to predict the effects of soil structure on denitrification and N_2O emission.

Laughlin R J and Stevens R J. 2002. Evidence for fungal dominance of denitrification and codenitrification in a grassland soil. *Soil Science Society of America Journal*. 66. pg. 1540 – 1548.

Laughlin R J, Rutlting T, Muller C, Watson C J and Stevens R J. 2009. Effect of acetate on soil respiration, N_2O emissions and gross N transformations related to fungi and bacteria in a grassland soil. *Applied Soil Ecology*. 42. pg. 25 – 30.

Laughlin R, Stevens R J, Muller C and Watson C J. 2008. Evidence that fungi can oxidize NH_4^+ to NO_3^- in a grassland soil. *European Journal of Soil Science*. 59. pg. 285 – 291.

LeBauer D S and Treseder K K. 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecological Society of America*. 89. pg. 371 – 379.

Li Y, Xu M, Sun O J and Cui W. 2004. Effect of root litter exclusion on soil CO_2 efflux and microbial biomas in wet tropical ecosystems. *Soil Biology and Biochemisty*. 36. pg. 2111 – 2114.

Linn D M and Doran J W. 1984. Effect of water-filled pore space on carbon dioxide and nitrous oxide emissions in tilled and non-tilled soils. *Soil Science Society of America Journal*. 48. pg. 1267 – 1272.

Liscum E, Askinosie S K, Leuchtman D L, Morrow J, Willenburg K T and Coats D R. 2014. Phototropism: growing towards an understanding of plant movement. *The Plant Cell*. 26. pg. 38 – 55.

Liu B, Frostegard A and Bakken L. 2014. Impaired reduction of N_2O to N_2 in acid soils is due to a posttranscriptional interference with the expression of *nosZ*.

Loick N, Dixon E, Abalos D, Vallejo A, Matthews P, McGeough K, Watson C, Baggs E M, Cardenas L M. 2017. 'Hotspots' of N and C impact nitric oxide, nitrous oxide and nitrogen gas emissions from a UK grassland soil. *Geoderma*. 305. pg. 336 – 345.

Luo J, de Klein C, Shepard M and Ledgard S. 2010. Effect of nitrification inhibitor on nitrous oxide emissions in pasture soils. Conference Paper: *19th World Congress of Soil Science: Soil Solutions for a Changin World*. pg 1 – 6. August 2010, Brisbane, Australia.

Luo J, Ledgard S F, Lindsey S B. 2007. Nitrous oxide emissions from application of urea on New Zealand pasture. *New Zealand Journal of Agricultural Research*.50. pg. 1–11.

Ma W K. Farrell R E and Siciliano S D. 2008. Soil formate regulates the fungal nitrous oxide emission pathway. *Applied and Environmental Microbiology*. 74. pg. 6690 – 6696.

Maalcke W J, Dietl A, Marritt S J, Butt J N, Jetten M S, Keltjens J T and Kartal B. *Journal of Biological Chemistry*. 289. pg. 1228 – 1242.

Maeda K, Spor A, Edel-Hermann V, Heraud C, Breuil M, Bizouard F, Toyoda S, Steinberg C and Philippot L. 2015. N_2O production, a widespread trait in fungi. *Scientific Reports*. 5. 1 – 7.

Marhan S, Philippot L, Bru D, Rudolph S, Franzaring J, Hogy P, Fangmeier A and Kandeler E. 2011. Abundance and variability of nitrate reducers in an arable soil are more affected by temporal variation and soil depth than by elevated atmospheric [CO_2]. *FEMS Microbiology Ecology*. 76. pg. 209 – 219.

Marshall K C and Alexander M. 1962. Nitrification by *Aspergillus flavus*. *Journal of Bacteriology*. 83. pg. 572 – 578.

Mazzetto A M, Barneze A S, Feigl B J, Van Groenigen J W, Oenema O, de Klein C A M and Cerri C C. 2015. Use of the nitrification inhibitor dicyandiamide (DCD) does not mitigate N_2O emissions from bovine urine patches under Oxisol in Northwest Brazil.

Merino P, Stavillo J M, Besga G, Pinto M and Gonzalez-Murua C. 2001. Nitrification and denitrification derived N_2O production from a grassland soil under application of DCD and Actilith. *Nutrient Cycling in Agroecosystems*. 60. pg. 9 – 14.

Miller M, Palojarvi A, Rangger A, Reesiev M and Kjoller A. 1998. The use of Fluorogenic substrates to measure fungal presence and activity in soil. *Applied and Environmental Microbiology*. 64. pg. 613 – 617.

Misselbrook T H, Cardenas L M, Camp V, Throman R E, Williams J R, Rollett A J and Chambers B J. 2014. An assessment of nitrification inhibitors to reduce nitrous oxide emissions from UK agriculture. *Environmental research Letters*. 9. pg. 1 – 11.

Moiser A, Kroeze C, Nevison C, Oenema O, Seitzinger S, van Cleemput O. 1998. Clsing the global N_2O budget: nitrous oxide emissions through the agricultural nitrogen cycle. *Nutrient Cycling in Agroecosystems*. 52. pg. 224 – 248.

Monaghan R M and Barraclough D. 1993. Nitrous oxide and dinitrogen emissions from urine effected soil under controlled conditions. *Plant Soil*. 151. pg. 127 – 138.

Morley N J, Richardson D J and Baggs E M. 2014. Substrate induced denitrificaiton over or under estimates shifts in soil N_2/N_2O ratios.

Nicol G W, Leininger S, Chelper C and Prosser J I. 2008 The Influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archae and bacteria. *Environmental Microbiology*. 10. pg. 2966 – 2978.

Norman J S, Lin L and Barret J E. 2015. Paired carbon and nitrogen metabolism by ammonia-oxidizing bacteria and archaea in temperate forest soils. *Ecosphere*. 6. pg. 1 – 11.

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

Obaton M, Amarger N and Alexander M. 1968. Heterotrophic nitrification by *Psuedomonas aeruginosa*. *Archives of Microbiology*. 63. pg. 122 – 132.

Offre P, Prosser J I and Nicol G W. 2009. Growth of ammonia-oxidizing archaea in soil microcosms is inhibited by acetylene. *FEMS Microbiology Ecology*. 70. pg. 99 – 108.

Oren A. 2009. Chemolithotrophy. In: Encylopedia of Life Sciences (ELS). John Wiley & Sons, Ltd: Chichester, UK. DOI 10.1002/9780470015902.a0021153. pg. 1 -7.

Ouverney C C and Fuhrman J A. 2000. Marine planktonic archaea take up amino acids. *Applied and Environmental Microbiology*. 66. pg. 4829 – 4833.

Panday D and Nkongolo N V. 2016. Comparison of models for predicting space indicies and their realtionships with CO_2 and N_2O fluxes in a corn-soybean field. *Canadian Journal of Soil Science*. 96. pg. 328 – 335.

Park B, Park S, Yoon D, Schouten S, Sinninghe J S and Rhee S. 2010. Cultivation of autotrophic ammonia-oxidizing archaea from marine sediments in coculture with

sulfur-oxidizing bacteria. *Applied and Environmental Microbiology*. 76. pg. 7575 – 7587.

Paul E A, Beauchamp E G and Trevors J T. 1989. Acetate, propionate, butyrate, glucose and surcose as carbon sources for denitrifying bacteria in soil. *Canadian Journal of Soil Sciences*. 35. pg. 754 – 759.

Pedersen H, Dunkin K A, Firestone M K. 1999. The relative importance of autrotrophic and heterotrophic nitrificaiton in a conifer forest soils as measured by ^{15}N tracer and pool dilution techniques. *Biogeochmistry*. 44. pg. 135 – 150.

Pendergast-Miller M T, Baggs E M and Johnson D. 2011. Nitrous oxide production by the ectomycorrhizal fungus *Paxillus involutus* and *Tylospora fibillosa*. *FEMS Microbiology Letters*. 316. pg. 31 -35.

Pennington P I and Ellis R C. 1993. Autotrophic and heterotrophic nitrification in acidic grassland forest and native grassland soils. *Soil Biology and Biochemistry*. 25. pg. 1399 – 1408.

Petersen S O, Henriksen K and Blackburn T H. 1991. Coupled nitrification-dentirification associated with liuid maure in a gel-stabilized model system. *Biology and Fertility of Soils*. 12. pg. 19 – 27.

Petropoulos P and Gilbridge K A. 2005. Nitrification in activated sludge batch reactors is linked to the grazing of the bacterial population. *Canadian Journal of Microbiology*. 51. pg. 791 – 799.

Pett-Ridge J, Petersen D G, Nuccio E and Firestone M K. 2013. Influence of oxic/anoxic fluctuations on ammonia oxidizers and nitrification potential in a wet tropical soil. *FEMS Microbiology Ecology*. 85. pg. 179 – 194.

Philippot L and Hoberg O. 1999. Dissimilatory nitrate reductases in bacteria. *Biochimica et Biophysica Acta (BBA)*. 1577. pg 1 – 23.

Philippot L, Mirleau S, Mazurier S, Siblot S, Hartmann S, Lemanceau P and Germon J C. 2001. Characterization and transcriptional analysis of *Psuedomonas fluorecens* denitrifying clusters containing the *nar*, *nir*, *nor* and *nos* genes. *Biochimica et Biophysica Acta (BBA) – Gene Structure and expression*. 1517. pg. 436 – 440.

Phillipot L, Andert J, Jones C M, Bru D and Hallin S. 2010. Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N_2O emissions from soil. *Global Change Biology*. 17. pg. 1497 – 1504.

Pilegaard K. 2013. Processes regulating nitric oxide emissions from soils. *Philosophical Transactions of the Royal Society B*. 368. pg. 1 – 8.

Ping G, Li-li Z, Zhi-Jie W, Zhen-Hua and Li-Jun C. 2013. Responses of ammonia-oxidizing bacteria and archaea in two agricultural soils to nitrification inhibitors DCD and DMPP: A pot experiment. *Pedosphere*. 6. pg. 729 – 739.

Ping G, Li-Li Z, Zhi-Jie W, Zhen-Hua C, Li-Jun C. 2013. Responses of ammonia-oxidizing bacteria and archaea in two agricultural soils to nitrificatiom inhibitors DCD and DMPP: a pot experiment. *Pedosphere*. 23. pg. 729 – 739.

Podolyan A, Di HJ, Cameron K C, Clough T, de Klein C and Saggar S. 2014. Ammonia oxidising populations and relationships with N_2O emissions in three New Zealand soils. *New Zealand Journal of Agricultural Research*. 57. pg. 228 – 243.

Pogue A J, Gilbride K A. 2007. Impact of protozoan grazing on nitrification and the ammonia- and nitrite-oxidizing bacterial communities in activated sludge. *Canadian Journal of Microbiology*. 53. pg. 559 – 571.

Potter L, Angove H, Richardson D and Cole J. 2001. Nitrate reduction in the periplasm of gram-negative bacteria. *Advances in Microbial Physiology*. 45. pg. 51 – 86.

Prosser J I and Nicol G W. 2012. Archaeal and Bacterial ammonia-oxidizers in soil: the quest for niche specialisation and differentiation. 20. pg. 523 – 531.

Prosser J I and Nicol G W. 2012. Archael and bacterial ammonia-oxidizers in soil: the quest for niche specialisation and differentiation. *Trends in Microbiology*. 20. pg. 523 – 531.

Pupin B, da Silva Freddu O and Nahas E. 2009. Microbial alterations of the soil influenced by induced compaction. *Revista Brasileira de Cienda do Solo*. 33. pg. 1207 – 1213.

Rees R M, Augustin J, Alberti G, Ball B C, Boeckx P, Cantarel, Castaldi S, Chirinda N, Chojnicki B, Giebels M, Gordon H, Grosz B, Horvath L, Jusczak R, Kasimir Klemedtsson A, Klemedtsson L, Medinets S, Machon A, Mapanda F, Nyamangara J, Olesen J E, Reay D S, Sanchez L, Sanz Cobena A, Smith K A, Sowerby A, Sommer M, Soussana J F, Stenberg M, Topp C F E, van Cleemput O, Vallejo A, Watson C A and Wuta M. 2013. Nitrous oxide emissions from European agriculture – an analysis of variability and drivers of emissions from field experiments. *Biogeosciences*. 10. pg. 2671 – 2682.

Rex D, Clough T J, Richards K G, de Klein C, Morales S E, Samad M S and Grant J. 2018. Fungal and bacterial contributions to codenitrification emissions of N_2O and N_2 following urea deposition to soil. *Nutrient Cycling in Agroecosystems*. 110. pg. 135 – 149.

Richard G, Cousin I, Silion J F, Bruand A and Guerif J. 2001. Effect of compaction on the porosity of a silty soil: influence on unstaturated hydraulic properties. *European Journal of Soil Science*. 52. pg. 49 – 58.

Ruser R, Felssa H, Russow R, Schmidt G, Buegger and Munch J C. 2006. Emissions of N_2O, N_2 and CO_2 from soils fertilised with nitrate: effect of compaction, soil moisture and rewetting. *Soil Biology and Biochemistry*. 38. pg. 263 – 274.

Ruser R, Flessa H, Russow R, Schmidt G, Buegger F and Munch J C. 2006. Emission of N_2O, N_2 and CO_2 from soils fertilized with nitrate: effect of compaction, soil moisture and rewetting. *Soil Biology and Biochemistry*. 38. 263 – 274.

Saggar S, Giltrap D L, Li C, Tate K R. 2007. Modelling nitrous oxide emissions from grazed grasslands in New Zealand. *Agriculture, Ecosystems and the Environment*. pp 205 – 216.

Saggar S. Luo J. Giltrap, D L Maddena. M. 2009. Nitrous oxide emissions from temperate grasslands: processes, measurements, modeling and mitigation. In: Sheldon A I, Barnhart E.P. (Eds.). Nitrous oxide Emissions Research Progress. Nova Science Publishers Inc, New York. US, pg. 1–66 (Chapter 1).

Sayavedra-Soto L A, Hommes N G, Russell S A and Arp D J. 1996. Induction of ammonia monooxygenase and hydroxylamine oxidoreductase mRNAs by ammonium in *Nitrosomonas europaea*. 3. pg. 541 – 548.

Schimel J P, Firestone M K and Killham S. 1984. Identificaiton of heterotrophic nitrification in a Sierran forest soil. *Applied and Environmental Microbiology*. 48. pg. 802 – 806.

Schmidt E L. 1954. Nitrate formation by a soil fungus. *Science*. 119. pg. 187 – 189.

Sere C H, Steinfeld H and Groenwold J. 1995. World live-stock production systems: current status, issues and trends. Food and Agriculture Organization of the United Nations, Rome, Italy. FAO animal production and health paper No. 107. pg. 1 – 48.

Sere C, Stenifeld H, Groenewold J. 1995. World Live-stock Production Systems: Current status, Issues and Trends. Food and Agriculture Organisation of the United Nations, Rome, Italy.

Sexstone A J, Parkin T B and Tiedje J M. 1985. Temporal responses of soil denitrification rates to rainfall and irrigation. *Soil Science Society of America*. 49. pg. 99 – 103.

Shaw L J, Nicol G W, Smith Z, Fear J, Prosser J I, Baggs E M. 2006. *Nitrospira* spp. Can produce nitrous oxide via a nitrifier denitrification pathway. *Environmental Microbiology*. 8. pg. 214 – 222.

Shen T, Stieglmeier M, Dai J, Urich T and Schelper C. 2013. Responses of the terrestrial ammonia-oxidizing archaeon *Ca. Nitrososphaera viennenisis* and the ammonia-oxidizing bacterium *Nitrospira multiformis* to nitrification inhibitors.

Sherlock R R and Goh K M. Initial emissions of nitrous oxide from sheep urine applied to pasture soil. *Soil Biology and Biochemistry*. 15. pg. 615 – 617.

Shestek C J and Busse M D. 2005. Compaction alters physical but not biological indices of soil health. *Soil Sience Society of America Journal*. 69. pg. 236 – 246.

Shinn M B. 1941. Coloremetric method for determination of nitrate. *Industrial and Engineering Chemistry Analytical Edition*. 13. pg. 33 – 35.

Shjonning P, Lamande M, Berisso F E. 2013. Gas diffusion, non-darcy air permeability and computed tomography images of a clay subsoil affected by compaction. *Soil Science Society of America Journal*. 77. pg. 1977 – 1990.

Shoun H, Kim D, Uchiyama H and Sugiyama J. 1992. Denitrification by fungi. *FEMS Microbiology Letters*. 94. pg. 277 – 282.

Siddiqui R A, Warnecke-Eberz U, Hengsberger A, Schneider B, Kostka S and Friedrich B. 1993. Structure and function of a periplasmic nitrate reductase in *Alcaligenes eutrophus* H16. 175. pg. 5867 – 5876.

Signor D, Cerri C E P. 2013. Nitrous oxide emissions in agricultural soils: a review. *Pesquisa Agropecuaria Tropical*. 43. pg. 322 – 338.

Singh J, Saggar S, Giltrap D L and Bolan N S. 2008. Decomposition of dicyandiamide in three contrasting soils and its effect on nitrous oxide emission, soil repiratory activity, and microbial biomass - an incubation study. *Australian Journal of Soil Research*. 46. pg. 517 – 525.

Sitaula B K, Hansen S, Sitaula J I B, Bakken L R. Methane oxidation potentials and fluxes in agricultural soils: Effects of fertilisation and soil compaction. 48. pg. 323 – 339.

Skiba U and Ball B. 2002. The effect of soil texture and soil drainage on emissions of nitric oxide and nitrous oxide. 2002. *Soil Use and Management*. 18. pg 56 – 60.

Skiba U, Jones S K, Dragosits U, Drewer J, Fowler D, Rees R M, Pappa V A, Cardenas L, Chadwick D, Yamulki, Manning A J. 2012. UK emissions of the greenhouse gas nitrous oxide. *Philisophical transactions of the Royal Society B*. pp 1175 – 1185.

Skipp R A and Christensen. 1989. Host specificity of root-invading fungi from New Zealand pasture soils. *Australasian Plant Pathology*. 18. pg. 101 – 103.

Smith K A McTaggart J P and Tsuruta H. 1997. Emissions of N2O and NO associated with nitrogen fertilization in intensive agriculture, and the potential for mitigation. *Soil Use and Management*. 13. pg. 296 - 304.

Smith M, Firestone M K and Tiedje J M. 1978. The acetylene inhibition method for short-term measurement of soil denitrification and its evaluation using nitrogen-13.

Stark J M and Hart S C. 1997. High turnover rates of nitrification and nitrate turnover in undisturbed coniferous forests. *Nature*. pg. 385. pg. 61 – 64.

Starkenburgh S R, Arp D J and Bottomley P J. 2008. Expression of a putative nitrite reductase and the reversible inhibition of the nitrite-dependant respiration by nitric oxide in *Nitrobacter winogradsky* Nb-255. *Environmental Microbiology*. 10. pg. 3036 – 3042.

Stein L Y and Arp D J. 1998. Loss of ammonia monooxygenase activity in *Nitrosomonas europaea* upon exposure to nitrite. *Applied and Environmental Microbiology*. 64. pg. 4098 – 4102.

Stepniewski W, Glinksi J and Ball B C. 1981. Chapter 8 – Effects of compaction on soil aeration properties. *Developments in Agricultural Engineering*. 11. pg. 167 – 189.

Sterngren A E, Hallin S and Bengston. 2015. Archaeal ammonia oxidizers dominate in numbers, but bacteria drive gross nitrification in N amended soil. *Frontiers in Microbiology*. 6. pg. 1 – 8.

Strauss E A and Lamberti G A. 2000. Regulation of nitrificaiton in aquatic sediments by organic carbon. *Limnology and Oceanography*. 45. pg. 1854 – 1859.

Strauss E A and Lamberti G A. 2002. Effect of dissolved organic carbon quality in microbial decomposition and nitrificaiton rates in stream sediments. *Freshwater Biology*. 47. pg. 65 – 74.

Stroo H F, Klein T M and Alexander M. 1986. Heterotrophic nitrification in an acid forest soil and by an acid tolerant fungus. 52. pg. 1107 – 1111.

Taylor A E, Vajrala N, Giguere A T, Gitelman A I, Arp D J, Myrold D D, Sayavedra-Soto L and Bottomley P J. 2013. Use of aliphatic *n*-alkynes to discriminate soil nitrification activities of ammonia oxidizing *Thaumarchaea* and bacteria. *Applied and Environmental Microbiology*. 79. pg. 6544 – 6551.

Taylor A E, Zeglin L H, Dooley S, Myrold D D and Bottomley P J. Evidence for the different contribution of archaea and bacteria to the ammonia-oxidizing potential of diverse Oregon soils. *Applied and Environmental Microbiology*. 76. pg. 7691 – 7698.

Taylor S A. 1949. Oxygen diffusion in porous media as a measure of soil aeration. *Soil Science Society of America. Proceedings*. 14. pg. 55 – 61.

Teira E, van Aken H, Veth C and Herndl G J. 2006. Archaeal uptake of enantiormeric amino acids in the meso-bathypelagic waters of the North Atlantic. *Limnology and Ocenaography*. 51. pg. 60 – 69.

Tora J A, Baeza J A and Carrera J. 2010. Combined effect of inorganic carbon limitation and inhibition by free ammonia and free nitrous acid on ammonia oxidizing bacteria. *Bioresource Technology*. 101. pg. 6051 – 6058.

Tourna M, Stieglmeier M, Spang A, Konneke M, Shintlmeister, Urich T, Engel M, Schloter M, Wagner M, Richter A and Shelper C. 2011. Nitrosphaera viennensis, an ammonia oxidizing archaeon from soil. *Proceedings from the National Academy of Sciences of the United States of America (PNAS)*. 108. pg. 8420 – 8425.

Tseng J T, Bryan L E and Van den Elzen H M. 1972. Mechanism and spectrum of streptomycin resistance in a natural population of *Psuedomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*. 2. pg. 136 – 141.

Tsilingiridis G and Papakostas K. 2014. Invertigating the relationship between air and ground temperature vairations in shallow depths in Northern Greece. *Energy*. 73. pg. 1007 – 1016.

Vachaud G, Gaudet J P and Kuraz V. 1974. Air and water flow during ponded infilitration in a vertical bounded column of soil. *Journal of Hydrology*. 22. pg. 89 – 108.

Vajrala N, Bottomly P J, Stahl D A, Arp D J, Sayavedra-Soto L A. 2014. Cycloheximide prevents the *de novo* polypeptide synthesis required to recover from acetylene inhibition in *Nitrosopumilus maritimus*. *FEMS Microbiology Ecology*. 88. pg. 495 – 502

Vajrala N, Martens-Habbena W, Sayavedra-Soto L, Schauer A, Botttomley P J, Stahl D A and Arp D J. 2013. Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea. *Proceedings of the National Academy of Sciences of the United States of America (PNAS)*. 110. pg. 1006 – 1011.

Vallejo A, Garcia-Torres L, Diez J A, Acre A and Lopez-Fernandez S. 2005. Comparison of N losses (NO_3^-, N_2O, NO) from surface applied, injected or amended (DCD) pig slurry of an irrigated soil in a mediterranean climate. *Plant and Soil*. 272. pg. 313 – 325.

Van der Weerden T J, Kelliher F M and de Klein C A M. 2012. Influences of pore size distribution and soil water content on nitrous oxide emissions. *Soil Research*. 50. pg. 125 – 135.

Van Gool A P and Schmidt E L. 1973. Nitrification in relation to growth in *Aspergillus flavus*. *Soil Biology and Biochemistry*. 5. pg. 259 – 265.

Van Veen J A and Kuikman P J. 1990. Soil structural aspects of decomposition of organic matter by micro-organisms. *Biogeochemistry*. 11. pg. 213 – 233.

Verhamme D T, Prosser J I and Nicol G W. Ammonia concentration determines differential growth of ammonia-oxidizing arcahea and bacteria in soil microcosms. *The ISME Journal*. 5. pg. 1067 – 1071.

Wakelin S A, Clough T J, Gerard E M and O'Callaghan M. 2013. Impact of short-interval repeat application of dicyandiamide of soil N transformation in urine patches. *Agriculture, Ecosystems & Environment*. 167. pg. 60 – 70.

Ward B B. 2013. Nitrification. In: Encyclopedia of Ecology 2008. Reference Module in Earth Systems and Environmental Sciences. Elsevier. https://doi.org/10.1016/B978-0-12-409548-9.00697-7.

Wilks J C and Slonczewski J L. 2007. pH of the cytoplasm and periplasm of *Escherichia coli*: rapid measurement by green fluorescent protein fluorimetry. *Journal of Bacteriology*. 189. pg. 5601 – 5607.

Wrage N, Velthof G L, Laanbroek H J and Oenema O. 2004. Nitrous oxide emissions in grassland soils: assessing the contribution of nitrifier denitrification. *Soil Biology and Biochemistry*. 36. pg. 229 – 236.

Wrage N, Velthof G L, van Beusichem M L and Oenem O. 2001. Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry*. 33. pg. 1723 – 1732.

Xue C, Zhang X, Zhu C, Zhao J, Zhu P, Peng C, Ling N and Shen Q. 2016. Quantitative and compositional responses of ammonia-oxidizing archaea and bacteria to long-term field fertilisation. *Scientific Reports*. 6. pg. 1 – 11.

Xue X, Nleber J L and Gupta S C. 1992. Compaction effect on the gas diffusion coefficient in soils. *Soil Science Society of America*. 56. pg. 1743 – 1750.

Zahlnina K. Dorr de Quadros P, Camargo F A O and Triplett. 2012. Drivers of ammonia-oxidizing communities in soil. *Frontiers in Microbiology*. 3. pg. 1 – 9.

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

Zhang L, Hu H, Shen J and He J. 2012. Ammonia-oxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. *The ISME Journal*. 6. pg. 1032 – 1045.

Zhang L, Offre P, He J, Verhamme D T, Nicol G W and Prosser J I. 2012. Autotrophic ammonia oxidation by soil thaumarchaea. *Proceedings of the National Academy of Sciences of the United States of America (PNAS)*. 107. pg. 17240 – 17245.

Zhou Z M, Takaya N, Nakamura M, Takeo K and Shoun H. 2001. Oxygen requirement for denitrification by the fungus *Fusarium oxysporum*. *Archives of Microbiology*. 175. pg. 19 – 25.

Zhu K, Brunn S and Jensen L S. 2016. Nitrogen transformations in and N_2O emissions from soil amended with manure solids and nitrification inhibitor. *European Journal of Soil Science*. 67. pg. 792 – 803.

Zumft W G. 1997. Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews*. 61. pg. 533 – 616.

7. APPENDIX

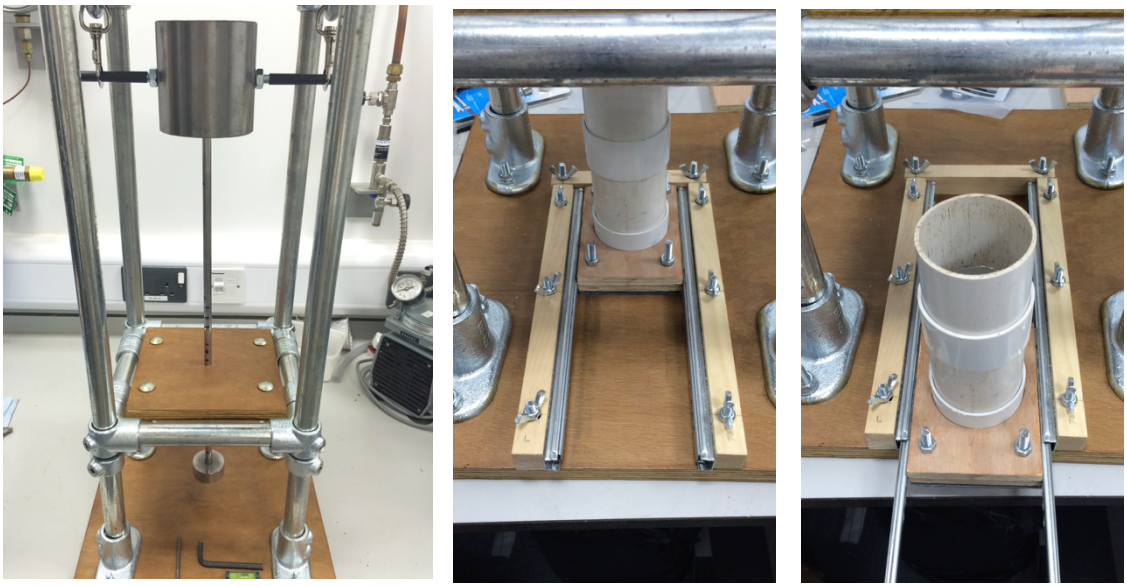


Fig. 7.1 Constructed soil columns preperation device.

Table. 7.1. Total nitrogen and carbon, dissolved organic carbon, inorganic carbon and ammonium (NH_4^+) concentrations for cattle urine and slurry

Nitrogen & Carbon Content	Fertiliser	
	Urine	Slurry
TN g L^{-1}	15.0 ± 0.210	3.1 ± 0.01
TC g L^{-1}	11.8 ± 0.001	4.4 ± 0.001
DOC g L^{-1}	9.4 ± 0.01	3.9 ± 0.01
IC g L^{-1}	2.4 ± 0.85	0.5 ± 0.5
NH_4^+ mg L^{-1}	105.2 ± 2.2	85.7 ± 4.3



Fig. 7.2. Acetone removal system for acetylene gas. Acetyelen gas was passed through (1) concentrated sulphric acid (H_2SO_4) to remove acetone from the acetylene gas. Acetone free gas was then passed through deíonsed water (2) to further clean aceylene gas.

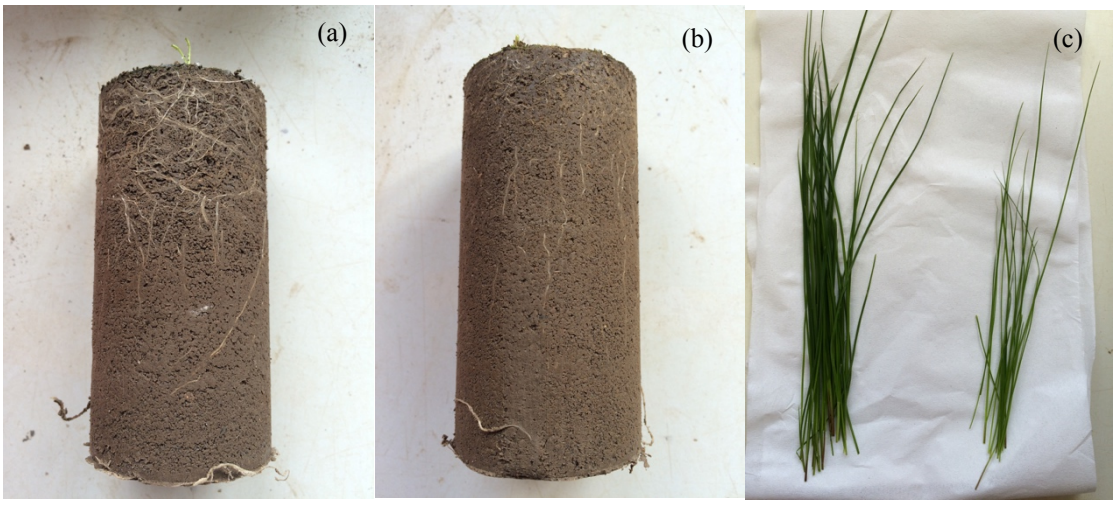


Fig. 7.3. Reduced root and shoot growth of *Lolieum perenne* in soil at greater bulk density. Difference in root growth betweens soils at 1.1 g cm^3 (a) and 1.3 g cm^3 (b) bulk densities. Difference of grass shoot development at higher soil bulk densities 1.1 g cm^3 (a) and 1.3 g cm^3 (c).

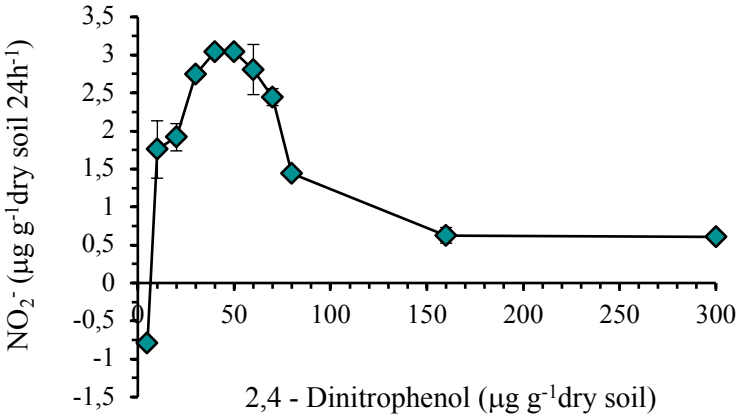


Fig. 7.4. Dosage response cure for 2,4 -Dinitrophenol for inhibition of nitrite reductase (*nirK*) activity for nitrate reductase enzyme assay (Kandeler. 1996). Mean values of nitrite production over the 24 hour incubation are displayed for each 2,4 – Dinitrophenol concentration (µg g^{-1} dry soil). Error bars represent the standard error of the mean. n = (3).