Effect of different forms of farm dairy effluent, with and without animal urine, on nitrification, denitrification and N_2O emissions

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Master of Agricultural Science.

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Nitrous oxide (N_2O) is one of the most important greenhouse gases. Agricultural soils are the largest source of N_2O emissions. In New Zealand, the application of farm dairy effluent (FDE) on pasture soils is the third largest source of N_2O emissions from grazed grassland. Recently, new FDE treatment technologies have been developed to separate out solids from liquids to produce treated clear water and treated effluent, aimed at recycling water, increasing the storage pond capacity and minimising contamination of waterways. However, it is not known how the treated clear water and treated effluent would affect the N_2O emission factors compared with the standard untreated effluent, and whether the treated effluent would interact with animal urine to result in different N_2O emission factors for the animal urine-N.

Thus, the objectives of this research were: a) to determine N_2O emissions from treated effluents (including treated effluent and treated clear water) compared with standard FDE applied to soil; b) to determine N_2O emissions from treated FDE and standard FDE co-applied with animal urine; c) to determine ammonia oxidising bacteria (AOB), ammonia oxidising archaea (AOA), denitrying microbial functional genes, and mineral N dynamics following the application of treated FDE and, standard FDE with or without urine; and d) to determine the relationships between N_2O emissions and the FDE properties.

Two incubation experiments were conducted to measure N_2O emissions, mineral N dynamics, soil pH and the abundance of AOB, AOA, and denitrifying functional genes (*nir*S, *nir*K and *nos*Z). A Templeton silt loam soil was collected from Lincoln University Research Dairy Farm and packed into gas sampling jars and soil sampling pottles. FDE and cow urine were collected from Lincoln University Demonstration Dairy Farm and FDE was treated to produce treated clear water and treated effluent by the latest effluent treatment technology. The treated effluents and the original FDE, with or without animal

urine, were applied to the soils, and were incubated at 12°C for 244 days for gas sampling jars and 210 days for soil sampling pottles.

Results showed that there were no significant differences among the treated clear water, treated effluent or standard FDE in N_2O emissions, mineral N dynamics, soil pH and the abundance of AOB, AOA, and denitrifying functional genes (*nir*S, *nir*K and *nos*Z). Similarly, when animal urine was co-applied with the three different effluents, there were also no significant differences among the effluent plus urine treatments in these parameters. Therefore, it is concluded that applying the treated effluent or treated clear water produced from the new treatment technology will not increase N_2O emissions nor change the mineral N dynamics, soil pH and the abundance of AOB, AOA, and the denitrifying functional genes (*nir*S, *nir*K and *nos*Z), even on the urine patches of grazed pasture soils, compared with applying the standard FDE. Future research could assess potential long-term effects on these parameters and potential effects on other microbial communities in the soil.

Keywords: Nitrous oxide, farm dairy effluent, effluent treatment technology, treated clear water, treated effluent, urine, ammonium, nitrate, soil pH, ammonia oxidising bacteria, ammonia oxidising archaea, denitrifiers.

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Chapter 1

Introduction

1.1 Introduction

In New Zealand, dairy farming has been expanding rapidly. In 1982, the population of dairy cattle was roughly 3 million and the numbers have increased to nearly 5 million in 2015/16 (Dairy NZ, 2016). About 60% of dairy cows are located in the North Island and 40% in the South Island (Fig. 1.1).

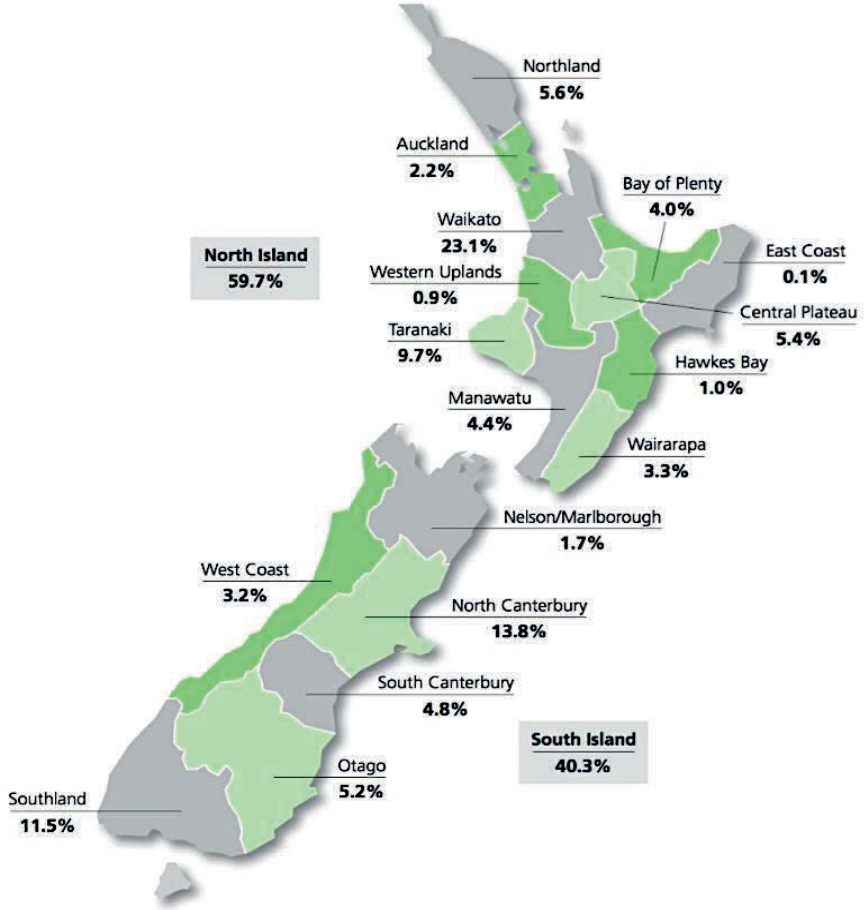

Fig. 1.1 Regional distribution of dairy cows in 2015/16 (Dairy NZ, 2016).

Globally, it has been identified that the livestock sector is one of the major contributors to the contamination of water and global warming (Di and Cameron, 2016). Greenhouse gases (GHG's) provide radiative forcing in the atmosphere, which can cause the climate to warm (IPCC, 2007). Nitrous oxide (N_2O) is one of the most important anthropogenic greenhouse gases, which has a global warming potential 298 times that of carbon dioxide (CO_2) and N_2O is the largest contributor to stratospheric ozone depletion (IPCC, 2007). N_2O is produced in the soil during the processes of nitrification and

denitrification. Agriculture is the largest source of N_2O emissions, comprising 66% of total N_2O emissions (Davidson and Kanter, 2014; van der Weerden et al., 2016a). One of the significant factors is the increasing application rate of synthetic N fertilisers, which has increased the N_2O concentration in the atmosphere in recent decades (Davidson, 2009; van der Weerden et al., 2016b). Globally, fertiliser use is expected to double between 2006 and 2050 (Sutton and Bleeker, 2013; van der Weerden et al., 2016a). In New Zealand, pasture grazing generally occurs all year round owing to the mild winter climate (Luo et al., 2013). The intensification in livestock farming has led to a growing volume of manure and animal excreta (including urine and farm dairy effluent) being produced, which also contribute to the rising atmospheric N_2O concentration.

In New Zealand, the N_2O produced from agriculture is high and represented 94.8% of total N_2O emissions in 2015 (Ministry for the Environment, 2017). N_2O emissions from urine and dung excreted by the grazing animal accounted for 63% of the agricultural N_2O and synthetic N fertiliser accounted for 16.1% of the agricultural N_2O emissions in New Zealand in 2015 (Ministry for the Environment, 2017). Dairy cattle excreta is deposited in and around the milking shed and washed and collected into a pond or sump (Chung et al., 2013). This is known as farm dairy effluent (FDE), which is the most common form of manure collected and applied to pastoral soils in New Zealand (van der Weerden et al., 2016a). In New Zealand, the application of FDE on pasture is the third largest source of N_2O emissions.

Recently, some new FDE treatment technologies have been developed to separate solids out from the liquids and produce treated clear water for re-use and treated effluent (the more concentrated effluent with higher amounts of solids) (Cameron and Di *Pers. Comm.*). However, it is not known how the new treated effluent and treated clear water produced from this treatment technology would affect N_2O emissions compared with the original standard non-treated FDE, and whether the treated FDE would interact with animal urine to result in different N_2O emission factors. This research is aimed at bridging these knowledge gaps.

1.2 Aims and objectives

The aim of this study is to improve knowledge and fundamental understanding of the effect of different forms of FDE (including standard FDE, treated clear water and treated effluent), with and without animal urine, on N_2O emissions, mineral nitrogen dynamics, soil pH and the abundance of ammonia oxidising bacteria (AOB), ammonia oxidising archaea (AOA), and denitrifying functional genes (*nirS*, *nirK* and *nosZ*).

The objectives of this project were:

a) To determine N_2O emissions from treated effluents (including treated effluent and treated clear water) compared with standard FDE applied to soil;

b) To determine N_2O emissions from treated FDE and standard FDE co-applied with animal urine;

c) To determine ammonia oxidising bacteria (AOB), ammonia oxidising archaea (AOA), denitrying microbial functional genes, and mineral nitrification rate dynamics following the application of treated FDE and standard FDE, with or without urine;

d) To determine the relationships between N_2O emissions and the FDE properties.

These objectives will be achieved by conducting laboratory incubation studies.

1.3 Hypotheses

This research programme tested the following hypotheses:

a) That the treated effluent and treated clear water would have different N_2O emission factors compared with the untreated standard FDE when applied to soil;

b) That there would be different interactive effects between the treated effluents (treated clear water and treated effluent) and standard FDE and animal urine when they are co-applied to soil.

1.4 Structure of the thesis

Chapter Two of this thesis is the review of previously published literature relevant to the factors affecting N_2O emissions from nitrification and denitrification. Chapter Three describes the experimental design and methods of sampling and analysis in this research. The research results and discussion are presented in Chapter Four. Chapter Five summarizes the conclusions of this research and provides some suggestions for future research.

Chapter 2

Literature review

2.1 Introduction

Nitrous oxide (N_2O) is an important greenhouse gas (GHG), which is almost 300 times greater than carbon dioxide (CO_2) in terms of long-term global warming potential (IPCC, 2007).It is also the largest contributor to the depletion of stratospheric ozone (Ravishankara et al., 2009). Mostly, the N_2O is produced from biological processes, nitrification and denitrification, as a part of the nitrogen (N) cycle in soil (Thomson et al., 2012).

The dominant land use in New Zealand is pastoral agriculture (Di and Cameron, 2017). In grazed grassland soils, the N associated with animal excreta (both urine and dung) is an important part of the N cycle (Saggar et al., 2013). On average, 290 g N cow^{-1} day^{-1} can be returned as excreta by dairy cattle in the New Zealand grazing system (Saggar et al., 2004b). In New Zealand, N_2O emissions from urine and dung excreted by the grazing animal accounted for 63% of the agricultural N_2O and synthetic N fertiliser accounted for 16.1% of the agricultural N_2O emissions in 2015 (Ministry for the Environment, 2017). Farm dairy effluent (FDE) is the most common form of manure that is collected and applied to the pastoral soils, and is the third largest source of N_2O emissions in New Zealand (van der Weerden et al., 2016a).

In New Zealand, a series of mitigation options are being considered to decrease N_2O emissions from grazed farming systems including restricted grazing, using winter feed pads and low N animal feed (Di and Cameron, 2002a, 2002b; Kramer et al., 2006; Monaghan et al., 2007; Di and Cameron, 2012). In addition, the nitrification inhibitor, dicyandiamide (DCD), has been shown to decrease both NO_3^- leaching and N_2O emissions in grazed pasture soils (Di and Cameron, 2007, 2012). However, the use of DCD has been suspended in New Zealand awaiting the establishment of a food standard by the FAO's Codex Committee.

Nitrous oxide emissions are part of the N cycle in the soil, and are affected by a number of soil, environmental and management factors. This review will first provide a brief summary of the N cycle in the soil, followed by a review of the factors that affect N_2O emissions, and microbial communities responsible for the production of N_2O.

2.2 The nitrogen cycle

There are 18×10^{15} tonnes of N in the earth's crust and 3.8×10^{15} tonnes as nitrogen gas (N_2) in the atmosphere (McLaren and Cameron, 1996). In the soil, there are four major forms of N: (a) N in organic

matter, humus, plant material and animal material; (b) N in soil organisms; (c) ammonium ions, which are held by organic matter and clay minerals and, (d) mineral-N forms in soil solution, such as nitrate (NO_3^-), ammonium (NH_4^+), and nitrite (NO_2^-) in low concentration. The N cycle in the plant/soil system includes three processes: gains, transformations and losses (Fig. 2.1). These processes affect N availability for plants and N transformations in the wider environment.

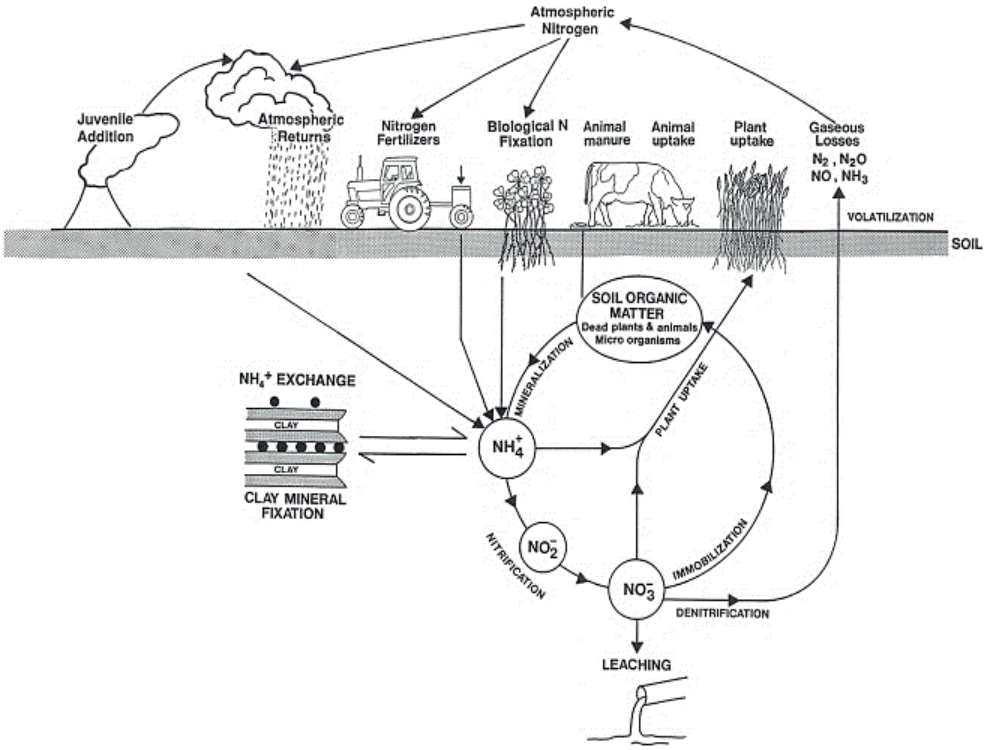

Fig. 2.1 The soil/plant nitrogen cycle (Cameron et al., 2013).

2.2.1 Gains

There are four main pathways for soil to gain N: (a) atmospheric returns from atmosphere N and juvenile addition; (b) N fertilisers; (c) biological N fixation which is carried out by specific bacteria, such as *Rhizobia* and free-living N_2 fixing soil bacteria and (d) animal manure (Fig. 2.1).

2.2.2 Transformation

In the soil, N can be transformed into different forms through many chemical and biochemical reactions. Soil organic matter, including animal manure, microorganisms and dead plants and animals, is transformed into ammonium ions (NH_4^+) by mineralisation. Through nitrification, NH_4^+ is transformed into nitrite (NO_2^-) and nitrate (NO_3^-) by specific nitrifying bacteria. Ammonium and nitrate can also be transformed into organic forms through the process of immobilisation by soil microorganisms.

2.2.3 Losses

Nitrogen can be lost from soil through leaching or through gaseous emissions in the forms of N_2, N_2O and NO by denitrification, or in the form of ammonia by volatilization. Nitrogen is also lost from the N cycle through removal in plant and animal products (Fig. 2.1).

2.3 Nitrification and denitrification

In the soil/plant N cycle system, nitrification and denitrification are two important processes that produce N_2O. Through nitrification, NH_4^+ is converted to NO_2^- and then to NO_3^- by nitrifying microbes under aerobic conditions (Fig. 2.2) (Di et al., 2014). N_2O is a by-product that can be produced during nitrification. In the process of NH_4^+ oxidation, there are two stages, the conversion of NH_4^+ to NH_2OH (Equation 2.1) by *amoA* ammonia monooxygenase and from NH_2OH to NO_2^- by hydroxylamine oxidoreductase (Equation 2.2) (Bothe et al., 2000).

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (2.1)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (2.2)$$

The transformed NO_2^- is rapidly oxidised to NO_3^-. N_2O is produced chemically because of the decomposition of NH_2OH during the oxidation of NH_4^+ (Braker and Conrad, 2011).

For over a century, ammonia oxidising bacteria (AOB) was thought to mostly perform in the first and rate-limiting step of nitrification, the oxidation of NH_3 to NH_2OH (Kowalchuk and Stephen, 2001; Di and Cameron, 2017). However, it was found recently that the populations of ammonia oxidising archaea (AOA) in a range of soils were more abundant than AOB (He et al., 2007; Prosser and Nicol, 2008; Di and Cameron, 2017). This suggested that AOA could potentially have a greater role than AOB in ammonia oxidation. However, further research has found that the activity and population abundance of AOB and AOA in ammonia oxidation can vary depending on soil and environmental conditions (Di and Cameron, 2017). This will be discussed in **Section 2.4**.

Denitrification is the reduction of NO_3^- or NO_2^- to NO, N_2O and N_2 gases under anaerobic conditions (Fig. 2.2). Under anaerobic conditions, heterotrophic denitrifiers use NO_3^- or NO_2^- to replace O_2 as the electron acceptor for the oxidation (van Spanning et al., 2007) and release N_2O and N_2 and gain energy. Nitrate can be reduced to NO_2^- by nitrate reductase (encoded by the *narG* gene). The nitrite reductase (encoded by *nirS* and *nirK* genes) reduces NO_2^- to NO. N_2O and N_2 are produced because of the reduction of NO by nitric oxide reductase and nitrous oxide reductase (*nosZ*), respectively. N_2O is released as an intermediate product.

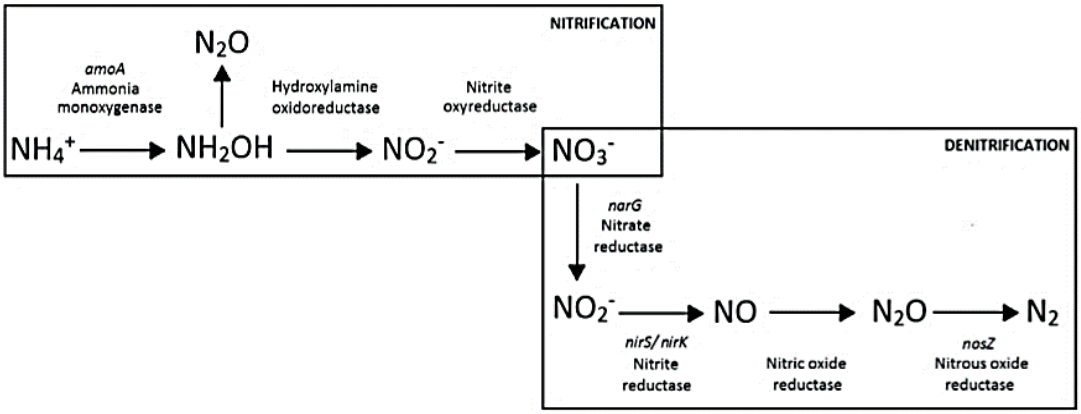

Fig. 2.2 Nitrification and denitrification processes with associated enzymes and functional genes (adapted from Di et al. (2014)).

2.4 Factors affecting nitrification, denitrification and N_2O emissions

2.4.1 Soil moisture content

Soil moisture content plays a significant role in nitrification, denitrification and N_2O emissions since it affects the soil aeration status (Cameron et al., 2013). For example, Osborne et al. (2016) reported that nitrification increased with increasing moisture content but only whilst the soil remained aerobic. Under aerobic conditions, nitrification occurs and N_2O can be produced under certain soil conditions (Fig. 2.3) (Di et al., 2014). Maag and Vinther (1996) reported that when soil moisture content increased, the N_2O-N produced from nitrification increased. Less than 60% of water filled pore space (WFPS) is found as the optimal soil moisture content for nitrification, since the O_2 or substrates are not restricted (Bateman and Baggs, 2005). WFPS between 35% and 60% leads to N_2O emissions which is produced predominantly by autotrophic nitrification (Bateman and Baggs, 2005). A similar result was reported by Ussiri and Lal (2013) (Fig. 2.4).

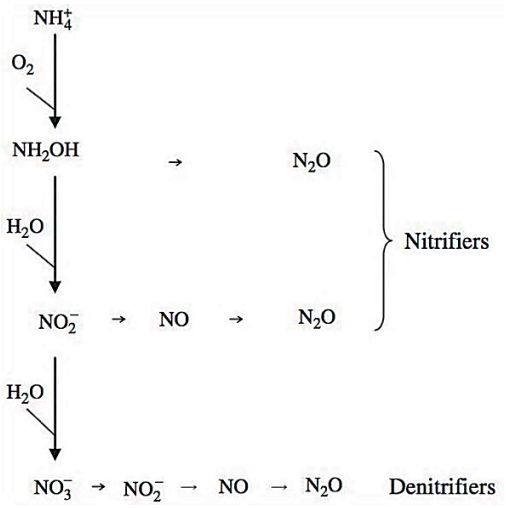

Fig. 2.3 The processes of nitrification (Cameron et al., 2013).

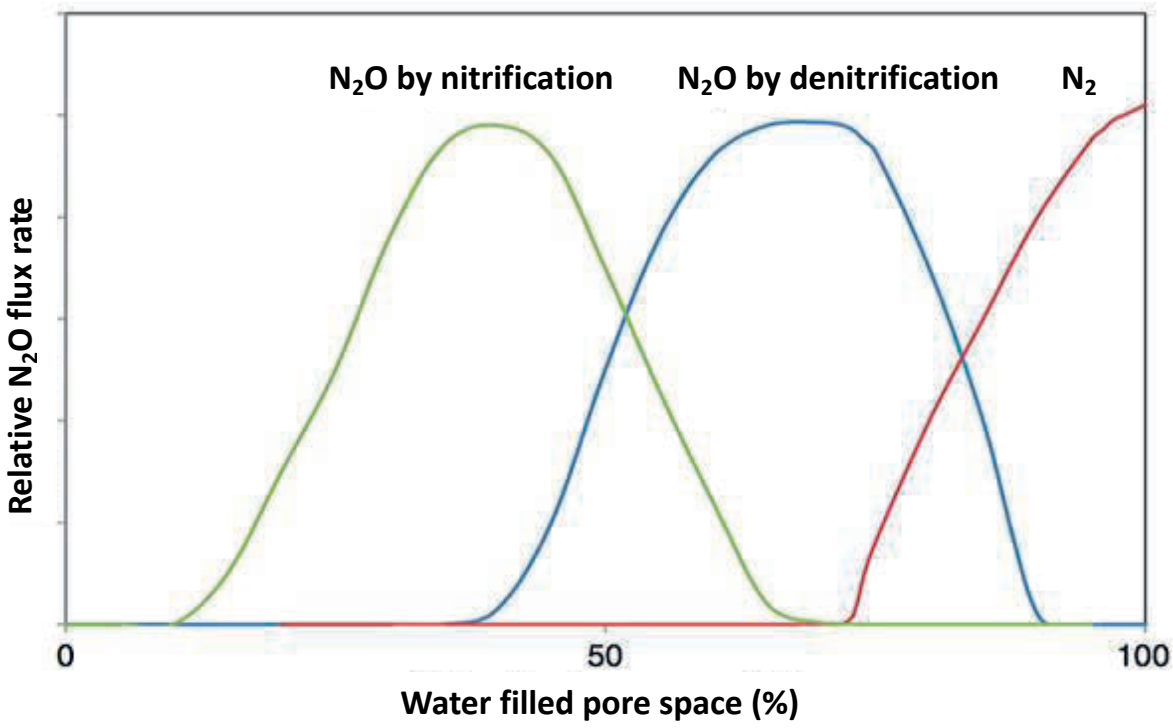

Fig. 2.4 A generalized schematic indicating the relationship between water-filled pore space (WFPS) of soils and relative fluxes of N_2O and N_2. N_2O can be produced by both nitrification and denitrification and N_2 is produced by denitrification (Ussiri and Lal, 2013).

Under anaerobic conditions, denitrification occurs producing N_2O and N_2 gases(Di et al., 2014). The rate of denitrification increases significant when soil moisture content is greater than field capacity (FC) (Mosier et al., 1986; de Klein and van Logtestijn, 1996; Saggar et al., 2009; Cameron et al., 2013). Thus, the losses of N due to denitrification are greatest in late-autumn, winter and early-spring and under the conditions of heavy rainfall or irrigation (Phillips et al., 2007; Cameron et al., 2013).

Since nitrification and denitrification can be affected by soil moisture content, the emissions of N_2O are also impacted. The relationship between WFPS and N_2O/N_2 flux is shown in Fig. 2.4. The emissions of N_2O produced by nitrification or denitrification change depending on the WFPS. Di et al. (2014) found that the N_2O emissions from urine-treated soil were dominantly driven by soil moisture content. The total N_2O emissions at 100% and 130% soil FC were 9 and 400 times that at 60% FC, respectively (Di et al., 2014). The soil becomes increasingly anaerobic when soil moisture content increases, which leads to higher denitrification rates and N_2O emissions. It was also reported by Banerjee et al. (2016) that the N_2O emissions in wetter soils were higher than those in drier soils. Higher N_2O emissions can occur when WFPS is around 60% because of the combination of autotrophic nitrification and denitrifier nitrification since the limitation of O_2 may occur in a short term (Bateman and Baggs, 2005). However, a decrease of N_2O emission was shown when WFPS was higher than 90% because the conversion of N_2O to N_2 occurs under complete anaerobic conditions (Smith et al., 1998). In addition, the emission

of N_2O is high when soil moisture alternates between wetting and drying since both nitrification and denitrification occur and the N_2O produced cannot be reduced to N_2 because there is insufficient time (Sherlock et al., 1992).

The growth of ammonia oxidisers (AOB and AOA) and denitrifiers can also be affected by soil moisture content. It was reported by Di et al. (2014) that the abundance of the AOB *amoA* gene in the urine treatment increased with the increase of soil moisture content. The AOA *amoA* gene also increased with soil moisture content but it was higher in the control than in the urine treatment. At low soil moisture content (60% FC), the growth of both AOB and AOA was restricted (Di and Cameron, 2017).

According to Di et al. (2014) the abundance of measured denitrifier genes all increased with the increase of soil moisture content, except *narG*. Although most denitrifiers are facultative aerobes, they can use various forms of nitrogen oxides as electron acceptors in place of O_2 when under anaerobic conditions (Wrage et al., 2001). In addition, the growth of microbial communities was limited when the soil was too dry (< 60% FC) (Di et al., 2014).

2.4.2 Temperature

Under higher temperatures, nitrification and denitrification rates are both higher, compared with rates under low temperatures. Avrahami et al. (2003) found that the N_2O release rate increased steadily between 4 and 37°C (Fig. 2.5) after a short-term temperature adaptation when ammonium was not a limitation. About 35-50 % of N_2O production was released through nitrification between 4 and 25°C, and nitrification was most active at the intermediate temperatures, i.e. 15-25°C. However, it was reported by Hu et al. (2014b) that the rates of nitrification at high temperatures, such as at 25°C, 35°C and 40°C, were significantly greater than the rates under lower temperatures (Fig. 2.6). The process of nitrification is inhibited at the lower temperature because of the reduction in microbial activity. In addition, the rate of denitrification can also be enhanced with the increase in temperature (Dobbie and Smith, 2001; Cameron et al., 2013). It was reported by de Klein and van Logtestijn (1996) that when temperature increased from 10°C to 20°C, a 10-fold increase in denitrification rate was recorded.

Cooler temperatures result in lower microbial activity, higher N_2O solubility and a slower gas diffusion, which leads to lower N_2O emissions (Sherlock et al., 1992). However, because of the incomplete denitrification and the low proportion of N_2O converted to N_2, thus N_2O emissions were reported to be higher when soil temperature was below 15°C (Keeney et al., 1979). It was reported by Keeney et al. (1979) that because of incomplete denitrification, even though the denitrification rate was low under 15°C, the amount of N_2O released was equivalent to that under 25°C. For N_2O emitted from nitrification, the emission rate at 5°C was 3 times higher than that at 20°C (Maag and Vinther, 1996).

This is because NO_3^- accumulates at low temperatures, which results in an increase of the nitrifiers' contribution to N_2O emissions.

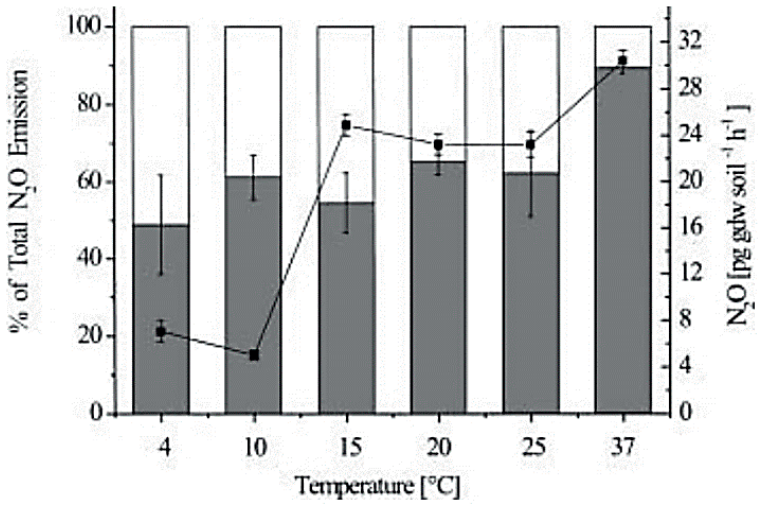

Fig. 2.5 Pattern of N_2O production and percentage contribution of nitrification and denitrification to total N_2O emission after 5 days of incubation at different temperatures. Stacked bars indicate percentage contribution of nitrification and denitrification to total N_2O emission (white, nitrification; grey, denitrification), and squares indicate rates of total N_2O emission. Mean ± SE; note that x-axis is not to scale (Avrahami et al., 2003).

2.4.3 Soil texture

N_2O emissions are reported to be lower in free-draining soils than in poorly drained soils (Luo et al., 2010). It was reported by Jamali et al. (2016) that greater N_2O emissions occurred in fine-textured soil than in free-draining soils, in the order: clay loam>loam>sand. The authors explained that the hydraulic conductivity, porosity, water-holding capacity and aeration status were different in the different soil types, and these affected soil moisture content. Similar results were also reported by Lan et al. (2013) and Wlodarczyk et al. (2011).

However, contradictory results have been reported by some other researchers. It was found by Rafique et al. (2011) that higher N_2O emissions were produced from free-draining podzols, compared with poorly drained gley soils. This was because the porosity in the podzols was higher, which led to an enhancement of nitrification rate. Moreover, soil is frequently waterlogged in gley soils, which causes a very high WFPS and almost completely anaerobic conditions (Rafique et al., 2011). This reduces the nitrification rate in the soil and the denitrification pathway may be more complete, leading to less production of N_2O gas.

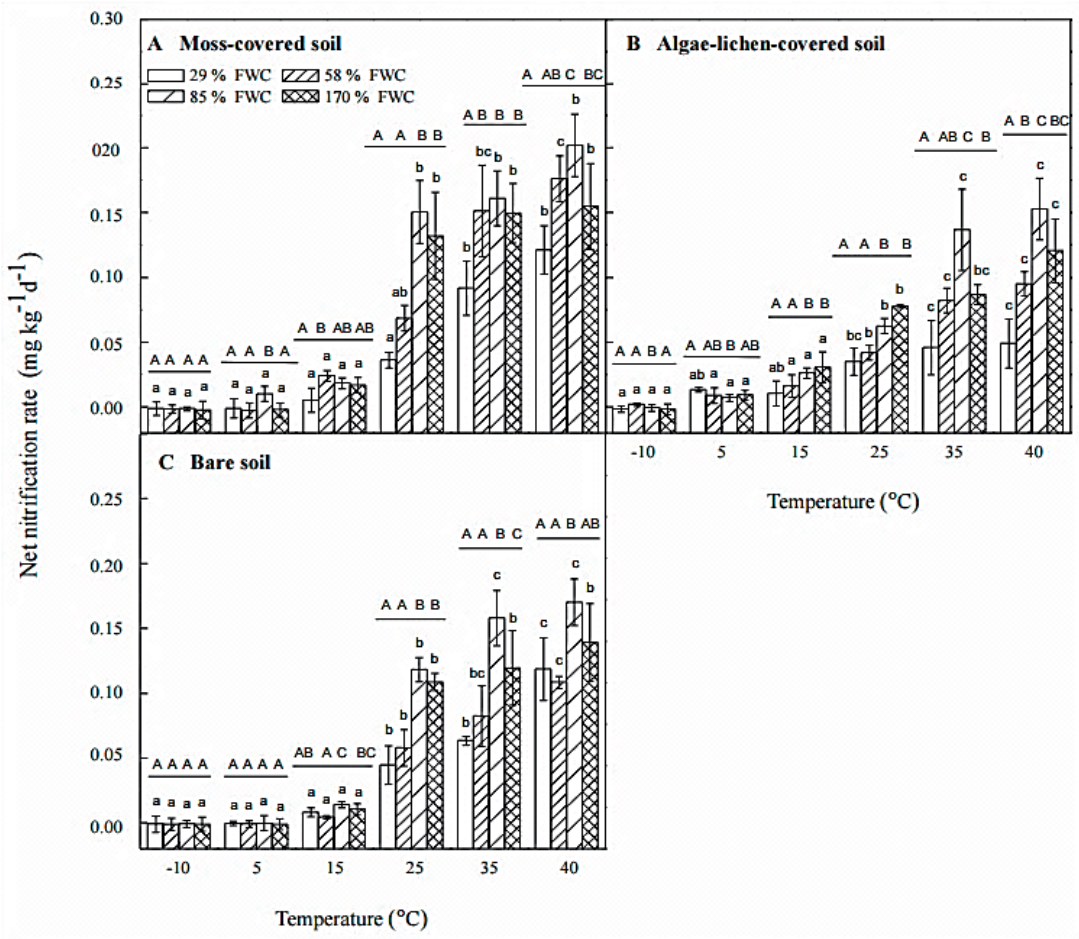

Fig. 2.6 Effect of temperature and moisture on the net nitrification rate in three soil microhabitats. Different capital letters indicate significant differences in net nitrification rate of the same temperature among different soil moistures; different small letters denote significant differences among different temperatures in the same soil moisture (P< 0.05). Error bars represent the standard errors of the mean values for three replicates (Hu et al., 2014b).

2.4.4 Soil pH

Both nitrification and denitrification rate can be affected by soil pH. The process of nitrification is sensitive to soil pH (Curtin et al., 1998; Jiang et al., 2015). Darrah et al. (1986) reported that the relative nitrification rate reached the highest point when soil pH was around 6.5 (Fig. 2.7). The optimal range of soil pH for nitrifying bacteria is from 4.5 to 7.5 (McLaren and Cameron, 1996). In very acid soils, the toxicity of Al and/or the deficiencies of Ca and Mg may inhibit nitrification (McLaren and Cameron, 1996). In addition, nitrification rate, especially NH_3 oxidation, is significantly lower when soil pH is less than 5.5 since NH_3 is protonated to NH_4^+ at a low pH. When the pH decreases to 5.0 or lower, the activity of nitrifiers becomes negligible (Foth, 1997). In alkaline soils, the toxicity of NH_3 may become a limitation of nitrifiers activity (McLaren and Cameron, 1996).

Both AOA and AOB are players of ammonia oxidation (Che et al., 2015). The growth and activity of AOB and AOA can be affected by soil pH (Luo et al., 2007; Di and Cameron, 2017). In acidic soil ecosystems, archaea may play an important role in ammonia oxidation (He et al., 2007; Yao et al., 2011; Zhang et al., 2012; Che et al., 2015; Jiang et al., 2015) since they can adapt to extreme environment conditions (Di et al., 2010). If high N concentrations do not inhibit AOA growth then AOA may become significant in NH_3 oxidation under strongly acidic conditions (He et al., 2012; Zhang et al., 2012; Hu et al., 2014a; Di and Cameron, 2017). Robinson et al. (2014) reported that when the urine substrate was applied to soil, AOA growth was favoured in the acid soil treatment once the urine was hydrolysed (after day 28), and AOB growth was favoured in the alkaline treatment.

The rate of denitrification is also affected by soil pH (McLaren and Cameron, 1996). The rate of denitrification increases with the increase of soil pH (ŠImek and Cooper, 2002; Hansen et al., 2014). Compared with neutral or alkaline soils, the denitrification rate is slower in acid soils (Nägele and Conrad, 1990; ŠImek and Cooper, 2002; Fageria and Baligar, 2008), especially when the pH is less than 5 (McLaren and Cameron, 1996). However, denitrification can still occur when soil pH is as low as 3.5 (Parkin et al., 1985). The optimum soil pH for denitrifying organisms is between 7 and 8 (Sherlock et al., 1992).

The product ratio of N_2O:N_2 from denitrification is impacted by the level of soil acidity (Saggar et al., 2013). Čuhel et al. (2010) reported that with a decrease of pH, the N_2O/ (N_2O+ N_2) ratio increased because of the changes in the total denitrification activity (Fig. 2.9B). In acid soils, N_2O appears as the dominant product frequently (Christensen, 1985) and the N_2O: N_2 ratio increases at lower soil pH (Nägele and Conrad, 1990; Liu et al., 2010; Saggar et al., 2013; Samad et al., 2016). Robinson et al. (2014) reported that the total N_2O emissions were significantly higher in acid-treated soils than in the untreated native and alkaline-treated soils when urine (700 kg N ha^{-1}) was applied. When soil was at a high pH level, complete denitrification was favoured, which produced more N_2 than N_2O (Fig. 2.8a) (Čuhel et al., 2010).

2.4.5 Organic carbon

Organic carbon (C) can affect the denitrification process (Giles et al., 2017). Denitrification can be stimulated by increasing the amount of readily available organic C, such as from animal excreta deposition and organic waste applications to soil (de Klein et al., 2001; Di and Cameron, 2003; Cameron et al., 2013). Since most of the denitrifiers are heterotrophs, the addition of organic C not only stimulates the respiration and growth of microbes in the soil but also provides the organic C needed by the denitrifiers (Cameron et al., 2013). Therefore, heterotrophic denitrification is often limited by labile C in agricultural soils (Saggar et al., 2013). In most soils, the rate of denitrification decreases with soil depth since both the microbial population and the organic matter content are higher at the soil

surface (Rolston, 1981). In addition, any process that can impact the C mineralisation rate in soils, such as incorporation of crop residue, root exudates, organic or inorganic fertiliser application, temperature, liming and drying-wetting, can have a major effect on the rate of denitrification. The application of farm effluents has been shown to increase N_2O emissions by enhancing the soil C availability (Bhandral et al., 2007).

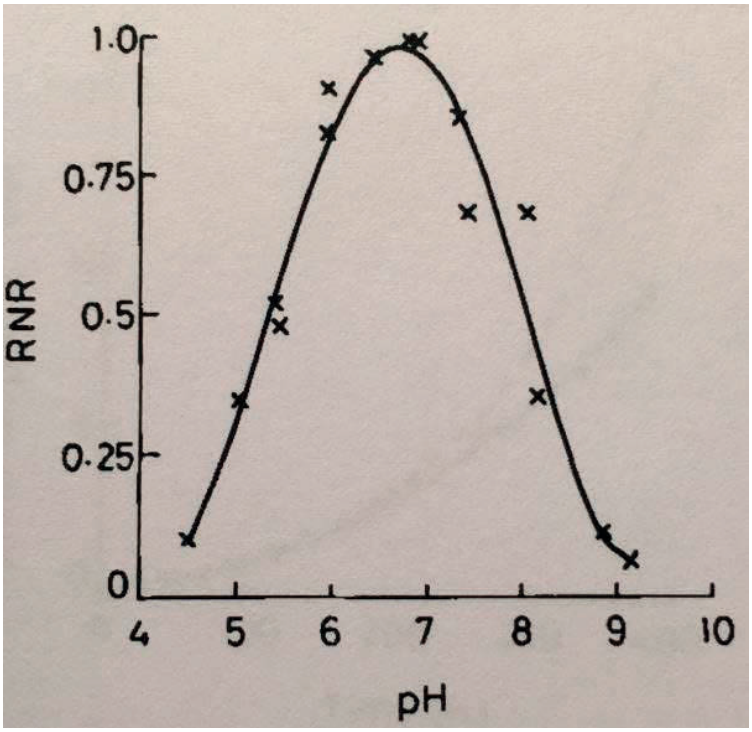

Fig. 2.7 Relative nitrification rate (RNR) expressed as a fraction of the maximum rate, as a function of pH in the short-term nitrification assay (Darrah et al., 1986).

N_2O production was positively correlated with total C, and highly correlated with water soluble organic C (Burford and Bremner, 1975) (Fig. 2.9). This is because water soluble C can be used by the denitrifying bacteria, resulting in higher N_2O emissions. Compared with ammonium-based fertilisers, the application of urea fertiliser leads to increased denitrification rates because of the direct supply of dissolved organic C from urea and an increase of soil C solubilisation from an increase in soil pH caused by urea hydrolysis (Barton et al., 1999). However, N_2O emissions have also been reported to be reduced by applying urea fertiliser with a C source (green manure and wheat straw), compared with applying urea fertiliser alone (Aulakh et al., 2001). This is probably because of the dissimilatory nitrate reduction to ammonium (Matheson et al., 2002) or microbial immobilisation of some of the added N (Tiedje, 1988). In addition, it is generally considered that the ratio of N_2O: N_2 decreases with the enhancement of C availability (Saggar et al., 2013).

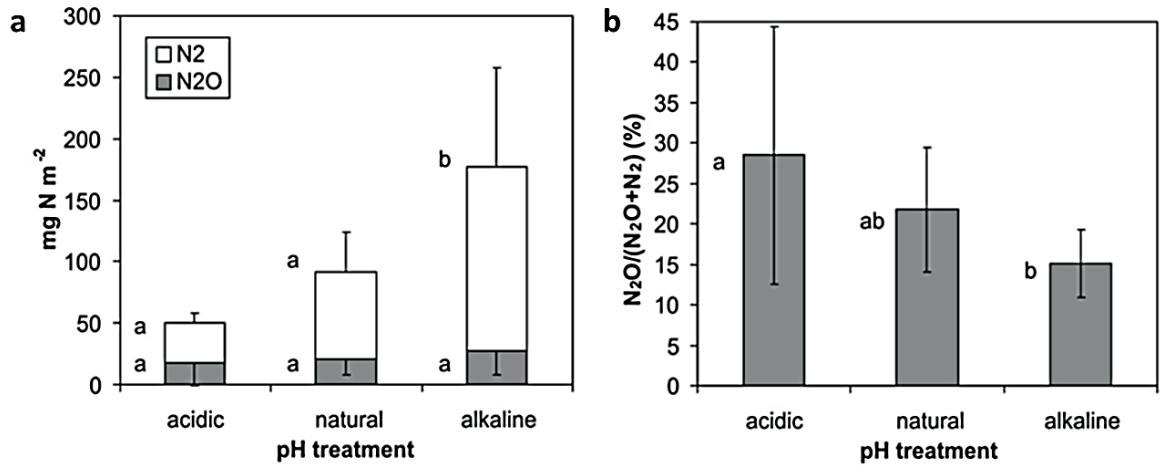

Fig. 2.8 In situ cumulative losses of N (separately as N_2O and N_2) (a) relative N_2O production expressed as the $N_2O/(N_2O+ N_2)$ molar ratio (b) over the 74 h after the addition of 15N-labeled KNO_3 to acidic, natural pH, and alkaline soils. Mean values and standard deviations are shown (n=12). The different letters next to the bars indicate significant differences between the specific pH treatments (*P*<0.05)(Čuhel et al., 2010).

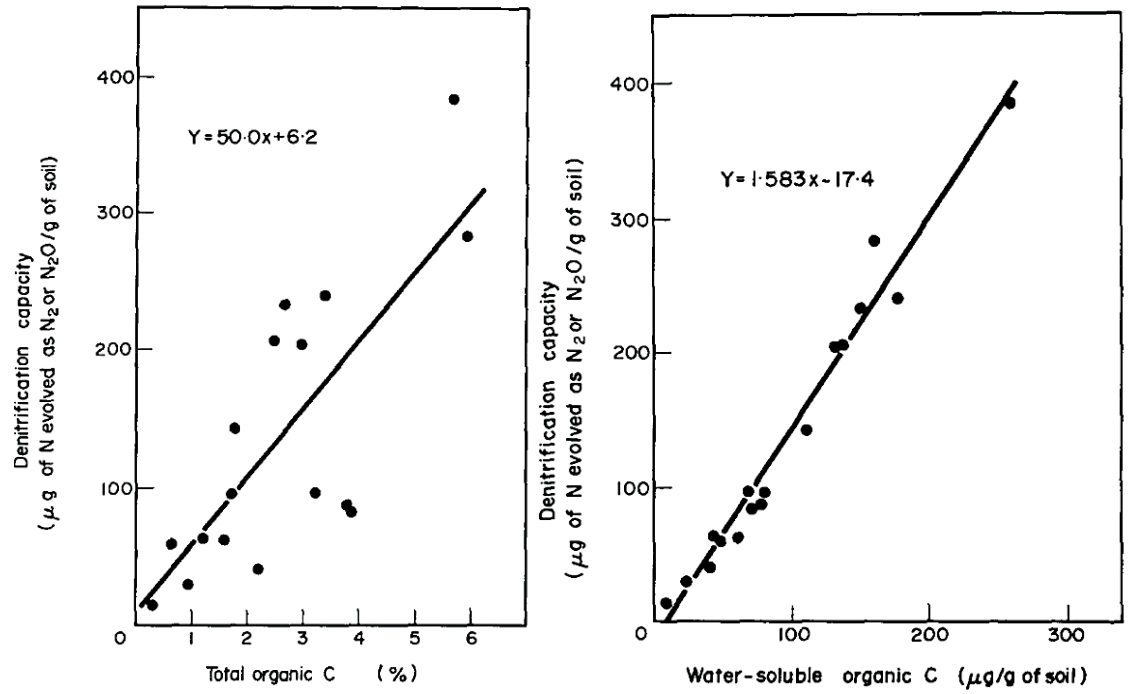

Fig. 2.9 Relationship between denitrification loss and total organic carbon in 17 soils (left) and relationship between denitrification loss and water soluble organic carbon in 17 soils (Burford and Bremner, 1975).

2.4.6 Synthetic N fertiliser applications

The application of N fertilisers in New Zealand increased from 59,265 tonnes in 1990 to 428,682 tonnes in 2015, and the proportion of urea application has increased to over 80% of all synthetic fertilisers at

the same time (Ministry for the Environment, 2017). The rate of nitrification and denitrification are influenced by the increased availability of N (including NH_4^+ and NO_3^-) (Cameron et al., 2013). Cardenas et al. (2010) reported that the emissions of N_2O were increased with the increase in the application rate of N fertilisers (Fig. 2.10).

The type of N fertiliser also affects N_2O emission levels. In some field studies, the N_2O from oxidised N fertilisers (nitrate based) was higher than reduced N fertilisers (Smith et al., 2012; Hinton et al., 2015). Eckard et al. (2006) found that nitrate fertiliser released more N_2O than urea fertiliser and N_2O emissions were positively correlated to N fertiliser application rate (Fig. 2.11). However, some other studies reported conflicting results, where reduced N forms had higher N_2O emissions (Lebender et al., 2014; Soares et al., 2016). Kroeze (1994) found that anhydrous NH_3 fertiliser produced higher N_2O emissions, compared with NH_4^+ and urea, while NO_3^- fertiliser led to the lowest N_2O emission levels. Since many factors can affect nitrification and denitrification, the comparative advantages of oxidised or reduced N fertilisers regarding N_2O emission potential strongly depend on weather and site specific conditions (Snyder et al., 2009; Tierling and Kuhlmann, 2018). In addition, it was found from several field studies that more N_2O can be emitted from urea than ammonium N forms (Tenuta and Beauchamp, 2003). Tierling and Kuhlmann (2018) reported 2.7-3.8 fold higher cumulative N_2O emissions from urea than from ammonium sulphate, and N_2O emission rates from urea were accompanied by elevated soil nitrite levels while those of ammonium sulphate were not. This indicated that the alkalizing hydrolysis of urea increased the risk of nitrite accumulation and thus higher N_2O emissions as well.

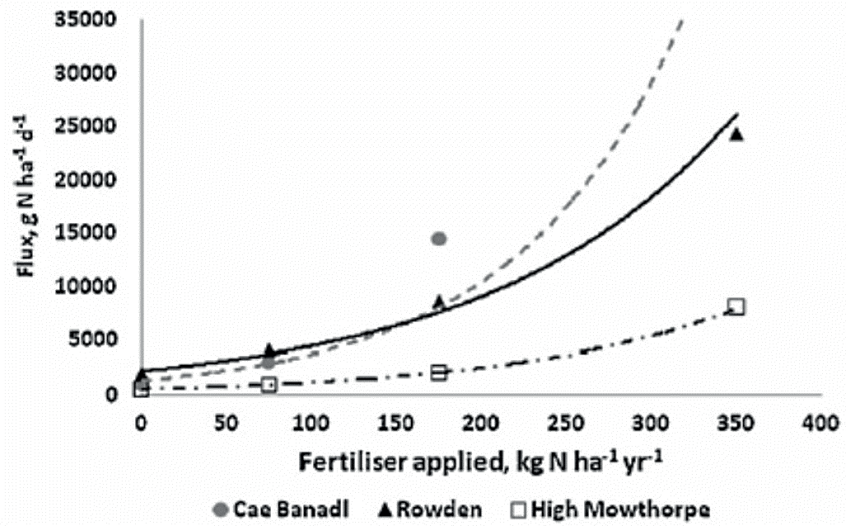

Fig. 2.10 N_2O flux related to fertiliser-N applied in three study sites (Cardenas et al., 2010).

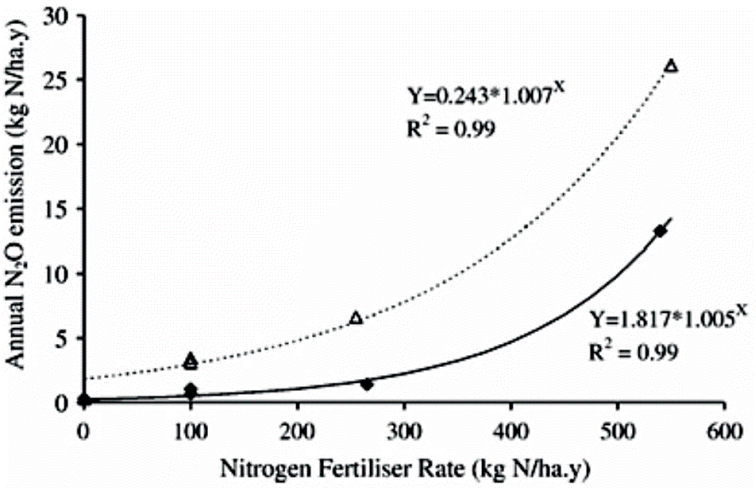

Fig. 2.11 Predicted annual N_2O emissions with increasing N fertiliser application rates with two different fertilisers; urea (closed diamond) and nitrate (open triangle) (Eckard et al., 2006).

2.5 N_2O emissions from farm dairy effluent (FDE)

In New Zealand, dairy cows deposit the majority of their excreta (dung and urine) onto pastures in a typical outdoor grazing system. Commonly, only the excreta that is deposited in and around the milking shed is managed actively (Chung et al., 2013). These excreta are washed from the milking parlour and yard into a pond or sump. Farm dairy effluent (FDE) is a combination of dairy cow excreta and wash-down water from the dairy cow milking shed and the associated yards. This FDE is the most common form of manure collection applied to the pastoral soils in New Zealand (van der Weerden et al., 2016a). The concentration of dry matter (DM) classifies the produced effluent into FDE, slurries or solids (Li et al., 2015b). FDE contains less than 5% DM whilst the DM content of slurry is between 5 and 15%. Solid manure contains more than 15% DM (Longhurst et al., 2012).

The application of FDE on New Zealand pastures is the third largest source of N_2O emissions (van der Weerden et al., 2016a). The 'Emission factor' (EF) is used to describe the proportion of excreta N that is emitted as N_2O-N (Cameron et al., 2013). For animal manures, the range of emission factors is from 0% to 5% (de Klein and van Logtestijn, 1996; Cameron et al., 2013). Currently, the New Zealand emission factor for FDE is 0.25% (Ministry for the Environment, 2017). Some reported EF values of FDE are given in Table 2.1. Many factors can affect the EF of FDE, such as season and prior excreta deposition.

There are two reasons why N_2O emissions are increased by applying FDE. One is because of the addition of N and available C, and another is because of the enhancement of soil moisture and the increase of anaerobic conditions in the soil (Bhandral et al., 2007). Higher N_2O emissions can result from the high total N concentrations in FDE (Li et al., 2015b). Some reports show that there is a

significant relationship between FDE total N concentration and N_2O EF (Fig. 2.12). Li et al. (2015a) reported that some other components such as C content in FDE can also affect the EF of FDE.

Since there is a range of C concentrations in FDE, the different amounts of organic C applied will affect soil denitrifiers, and subsequently impact denitrification rate and N_2O emissions (Cameron et al., 2013). The growth and respiration of microorganisms will be stimulated by adding effluent C to soils. Pelster et al. (2012) suggested that FDE contained greater C content than pig manure and that this was the reason why a higher N_2O EF was recorded for FDE. Compared with inorganic N fertiliser, FDE leads to higher N_2O EF because the denitrification activity is increased by the increased C availability and/or by the decline of soil aeration and the increase of respiration (Barton and Schipper, 2001). A significant exponential relationship between N_2O EF and C concentration in effluent was found by Li et al. (2015b) through analysing results reported in the literature (Fig. 2.13). They explained that the increase of N_2O EF may be not only because of the enhancement of C content but also because of some other factors. The highest N_2O emissions are not necessarily caused by applying fresh FDE, which contains the highest C concentration (Laubach et al., 2015). It was suggested by Bhandral et al. (2007) that both availability of C and N within FDE, and C:N ratio can influence the denitrification rate and the N_2O:N_2 emission ratio.

Table 2.1 N_2O emission factors resulting from land application of FDE.

Soil type	Country	N source	N input (kg N ha^{-1})	N_2O emission (kg N ha^{-1})	Emission factor (%)	Reference
Silt loam	New Zealand	FDE(Spring)	23.9	1.357	2.00	(Bhandral et al., 2010)
		FDE(Summer)	25.2	1.922	4.90	
Sandy loam	New Zealand	Treated FDE (Autumn)	21.8	0.382	2.00	(Bhandral et al., 2007)
		Treated FDE (Winter)	13	0.102	0.80	
		Untreated FDE (Autumn)	61	0.585	2.20	
		Untreated FDE (Winter)	49.3	0.153	0.30	
		Treated piggery FDE (Autumn)	27.5	0.585	2.20	
		Treated piggery FDE (Winter)	23.1	0.13	0.60	
Silt loam	New Zealand	Fresh FDE	100	2.34	0.62	(Li et al., 2016)
Silt loam	New Zealand	Fresh FDE(Spring)	98	1.62	1.65	(Li et al., 2015a)
		Fresh FDE(Summer)	101	0.01	0.01	
		Fresh FDE(Autumn)	101	0.57	0.56	
		Stored FDE(Spring)	60	0.48	0.80	
		Stored FDE(Summer)	53	0.13	0.25	
		Stored FDE(Autumn)	100	0.27	0.27	
Sandy loam	New Zealand	FDE	23.9	0.493	2.00	(Saggar et al., 2004)
		FDE	25.2	1.433	5.70	
		FDE	18	0.449	2.50	
Silt loam	New Zealand	Piggery effluent	368	6.99	1.90	(Khan, 1999)
Sandy loam	New Zealand	FDE	400	1.2	0.30	(Khan, 1999)
Silt loam	New Zealand	FDE	50	0.015	0.03	(Luo et al., 2008)
		FDE	50	0.004	0.01	
		Fresh FDE	100	0.14	0.14	(Li et al., 2014)
		Stored FDE	100	0.03	0.03	

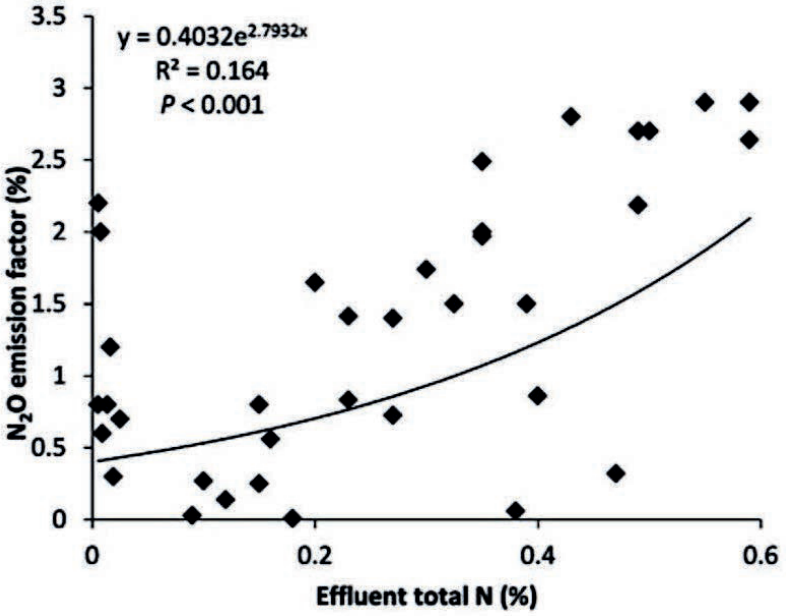

Fig. 2.12 Relationship between nitrous oxide emission factors and effluent total N% (adapted from Li et al. (2015b)).

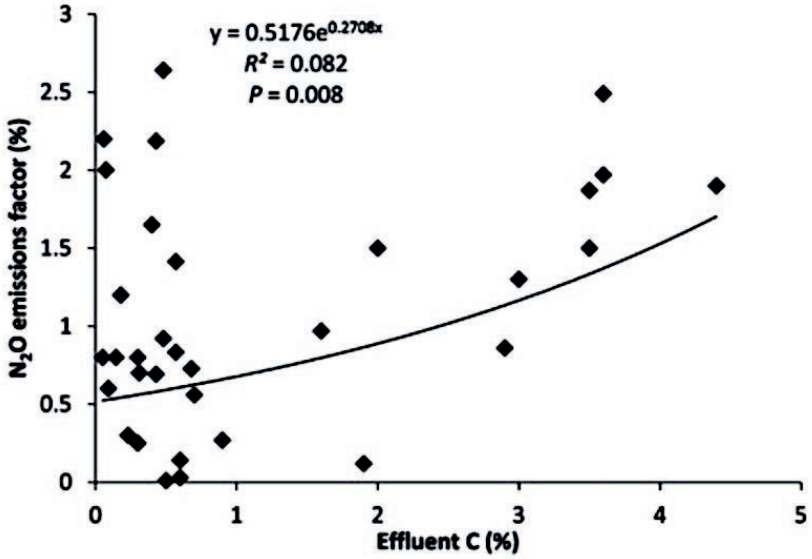

Fig. 2.13 Relationship between nitrous oxide emission factors from effluent application (% of applied N lost as N_2O) and effluent C concentration (adapted from Li et al. (2015b)).

The change of soil moisture and the increase in anaerobic conditions following FDE application will also exert impacts on N_2O emissions. It was found by several researchers that the emissions of N_2O reach the highest point immediately after adding FDE (Barton and Schipper, 2001; Sistani et al., 2010; Li et al., 2014). Li et al. (2015b) suggested that a possible reason was that the trapped air in the soil was driven out by the liquid from FDE, and that this trapped air could contain high N_2O concentrations. Schils et al. (2013) reported that N_2O emissions increased immediately after the injection of liquid

manure and then the emission shifted to N_2. Many researchers have studied the seasonal change of N_2O emissions under FDE irrigation and reported an interaction of soil moisture and temperature. It was found by Bhandral et al. (2007) that the highest N_2O emission occurred in autumn in both treated and untreated FDE treatments, reaching 2.0% and 0.7%, compared with 0.8% and 0.3% in winter, respectively (Table 2.1). During autumn and winter in New Zealand, anaerobic conditions can be created when soil is saturated, and the water-filled pore space (WFPS) will be increased, following rainfall. These conditions can lead to higher N_2O emissions.

Some other properties of FDE, for example the DM content, also exert influences on the value of N_2O EF. Li et al. (2015b) reported that higher N_2O EF generally could be from a higher DM content (Fig. 2.14). Some interacting factors, such as higher C and N concentrations, with increasing DM content, will also affect N_2O emissions. However, van der Weerden et al. (2016a) suggested that the EF values of slurries and FDE applied to pastoral soils were similar, although a higher N content existed in slurries, compared with FDE. Bourdin et al. (2014) also reported that the total solid content had no significant influence on EF when the proportion was in the range from 3.3% to 6.3%, with 0.67% as the EF value. In addition, solid manures generally have lower N_2O emissions than slurries because of the lower N contents in solid manures.

The application of FDE to pasture is beneficial for soil fertility by recycling the nutrients. However, there is a need to manage this practice to minimise N_2O emissions from the FDE applied (Luo et al., 2008).

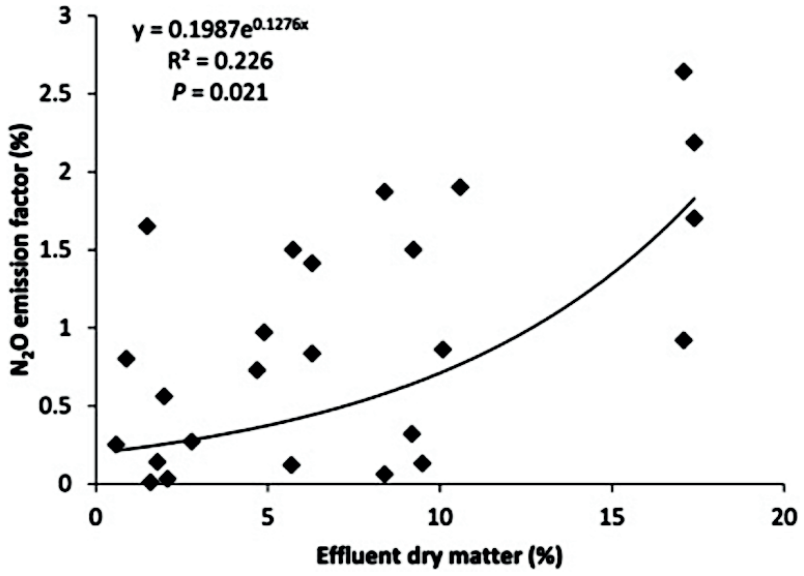

Fig. 2.14 Relationship between nitrous oxide emission factors from effluent application (% of applied N lost as N_2O-N) and effluent dry matter content (adapted from Li et al. (2015b)).

2.6 Methods to reduce N_2O emissions

A number of methods have been proposed to reduce N_2O emissions from pasture applied FDE and increase the N use efficiency (Saggar et al., 2013).

Reducing the numbers of animal livestock is the simplest approach to decreasing N_2O emissions (Li et al., 2015b). However, reducing the number of animals is unlikely to be acceptable from a financial perspective. Increasing milk production efficiency from dairy cows and growing meat-producing animals more quickly will potentially reduce N_2O emissions since N excretion would be decreased (Satter et al., 2002; de Klein and Eckard, 2008). Reduced N content in effluent can decrease the applied effluent N so that the potential risk can be minimised. Keeping a better N balance in feeding rations by avoiding excessive N in diet will reduce effluent N concentration (Li et al., 2015b). Decreasing the crude protein (CP) content of animal diets can also reduce N excretion (Hristov et al., 2011).

Nitrification inhibitors can reduce N losses by reducing the nitrification rate in soil (Di and Cameron, 2002b; Cameron et al., 2013). It was reported by Li et al. (2014) that using dicyandiamide (DCD) at 10 kg ha^{-1} just before applying FDE resulted in a 51% - 90% decrease in N_2O emissions, compared with applying untreated FDE. It was reported by Li et al. (2015a) that 10 kg ha^{-1} of DCD decreased the N_2O emission factor by 40-80% and 24-84% from fresh FDE and stored FDE, and reduced the N_2O emission factor by 69-76% and 60-70% from fresh manure and stored manure, respectively. In addition, they also reported that DCD was more effective during the spring and autumn to reduce N_2O emissions than in summer. However, the use of DCD in New Zealand has been suspended awaiting the establishment of a standard for food by the FAO's Codex Committee.

Choosing the optimal timing of FDE application can improve the N use efficiency of FDE by plants. Key soil and climatic factors should be considered, such as soil drainage and rainfall, before applying FDE (Li et al., 2015b). It was found by Luo et al. (2008) that up to 96% of N_2O emissions were reduced when effluent was strategically applied to pastures under the condition of dry soil-moisture status. Both N_2O emissions and N leaching could be potentially reduced by decreasing the use of FDE in wet winter and spring (Houlbrooke et al., 2004). Saggar et al. (2013) suggested that the application of the right amount of effluent close to the time that the nutrients are needed by the crop is the best strategy of N losses reduction.

A high application rate of FDE increases the availability of N and C in soil so that denitrification is promoted, generally leading to higher N_2O emissions (Saggar et al., 2004a). The particular production system depends the level of optimum N concentration in applied FDE (Li et al., 2015b). Computer models such as, Overseer® are useful tools to build a nutrient budget to guide the application of FDE on farm.

In addition, a pre-treatment that decreases the pH level to less than 7 before FDE is applied to pasture can reduce the emissions of NH_3 (Petersen and Sommer, 2011). Addition of nitric or sulphuric acid to the effluent can reduce NH_3 losses by up to 75% (Ndegwa et al., 2011). In the following application, N_2O emissions can be potentially reduced by the acidification of FDE and the effluents with reduced C. It was suggested that the emissions of NH_3 and N_2O were significantly lower after applying digested and acidified FDE than untreated FDE (Li et al., 2015b).

Recently, advanced FDE treatment technologies have been developed to separate the solids in the FDE from the liquid in order to recycle the water in FDE to wash the farm yards and reduce the size of effluent pond required to store the FDE. These treatment technologies produce treated effluent (the effluent with greater amounts of solids) and treated clear water (which can be re-used for washing the yard) with properties which are different from the original un-treated FDE in terms of solid contents and organic C content (Cameron and Di, *pers. comm*) (see more details in Materials and methods in Chapter 3). These different properties may impact on the nitrifying and denitrifying microbes in the soil, the nitrification rate dynamics and subsequently N_2O emissions. However, there is a lack of detailed knowledge and understanding about the effect of the application of treated effluent and treated clear water to the soil on microbial processes and N_2O emissions. There is therefore a need to conduct scientific studies to determine the impacts of these treated effluents on soil microbial activities and N_2O emissions compared with the standard FDE in order to understand potential impacts and develop appropriate management strategies to reduce N_2O emissions.

2.7 Conclusions

N_2O is produced during the processes of nitrification and denitrification. Nitrification, denitrification and the N_2O emission rate can be affected by many factors, such as soil moisture, temperature, soil texture, soil pH, soil organic C and N fertiliser application. FDE is an important source of N_2O emissions in New Zealand. FDE with different properties may produce different N_2O emission factors. The relationships between the treated FDE (treated clear water and treated effluent, using the latest FDE treatment technology) and N_2O emissions and soil microbes are unclear. In addition, it is not clear if the treated FDE would have a different interactive effect when co-applied with animal urine compared with the standard FDE. Therefore, there is a need to determine the effect of the treated FDE compared with the standard FDE on N_2O emissions, nitrifying and denitrifying microbes, and possible interactive effects on these parameters when the treated FDE is co-applied with animal urine onto soil.

Chapter 3

Materials and methods

3.1 Introduction

Two parallel laboratory incubation studies were conducted to determine the impact of applying three different types of effluents on soil nitrification rate dynamics and nitrous oxide (N_2O) emissions. One experiment was conducted to determine the effect of the effluents, with or without animal urine, on N_2O emissions. At the same time, a companion experiment was conducted to determine the effect of the different effluents, with or without animal urine, on mineral nitrification rate dynamics and the abundance of ammonia oxidising bacteria (AOB) and archaea (AOA), and denitrifying functional genes (*nirS*, *nirK*, *nosZ* (I)and *nosZ* (II)).

3.2 Experiment preparation and setup

3.2.1 Soil

A Templeton silt loam, classified as Immature Pallic soil (Hewitt, 1993); Udic Haplustepsts (Soil Survey Staff, 2014), was used in this study. The top 10 cm of soil was collected from the Lincoln University Research Dairy Farm, about 20 km south of Christchurch (43°38'S, 172°27'E), thoroughly mixed, with the roots and stones removed, and sieved through a 5 mm sieve (Fig. 3.1). The properties of the soil are shown in Table 3.1. The annual rainfall in the region is about 650 mm, and the annual average temperature is about 12.1°C. The soil moisture content was measured, by drying 6 replicate samples for 24 hours at 105°C in the oven, and was 16.97 % on average. The soil was acclimatised in the incubator at 12°C for one week before treatments were applied.

3.2.2 Farm dairy effluent

Farm dairy effluent was collected from the Lincoln University Demonstration Dairy Farm. A farm effluent treatment technology has been developed at Lincoln University to separate the solids out from the effluent in order to purify and recycle the water to wash the yard. The treatment technology involves adding a ferric iron (Fe^{3+}) compound to coagulate the colloidal solids in the FDE in a treatment tank (Cameron and Di, *pers. comm*). Once the solids are settled at the bottom of the treatment tank, which takes about 30-60 minutes after treatment application, the treated clear water (about the top two thirds of the tank) has a turbidity less than 50 NTU and can be used to recycle the water for cleaning the farm yard (Fig. 3.2). The treated effluent (the more concentrated effluent that has settled at the lower part of tank) is emptied out of the tank and put in the storage pond before being irrigated to pasture when conditions are suitable. The properties of the original FDE, the treated clear water,

and the treated effluent were analysed by Hills Laboratory, for total solids, pH, total nitrogen, total N, NH_4^+, NO_3^-, total P, dissolved reactive P (DRP), total carbon (C), soluble C and carbonaceous biochemical oxygen demand (cBOD) (Table 3.2). The concentration of total N in the standard FDE, treated clear water and treated effluent was 495 mg N L^{-1}, 311 mg N L^{-1} and 570 mg N L^{-1}, respectively, and was adjusted by adding 2.15 g, 2.55 g and 1.99 g urea to 1 L volumes of the different effluents, respectively, to produce the same N content of 1500 mg N L^{-1}. This limited the volume of the effluent applied to the pottles and jars so that the soil would not become too wet. The volume of effluent needed for each replicate was calculated based on the total N concentration and the weight of the soil.

Cow urine was also collected from the Lincoln University Demonstration Dairy Farm (Fig. 3.3) and was analysed for N concentration. The concentration of total N in the urine was 5.65 g N L^{-1} and was standardized by adding 5.77 g urea to 2 L of urine to give a standard concentration of 7 g N L^{-1}.

Fig. 3.1 The collection of the Templeton silt loam soil from the Lincoln University Research Dairy Farm.

3.2.3 Treatments

Eight treatments, each with four replicates, were established for the soil sampling (pottles) or N_2O sampling (jars) experiments (Table 3.3). The treatments were: (i) standard FDE, (ii) treated clear water, (iii) treated effluent and (iv) water (Control); all with and without cow urine. The effluents were applied

at the equivalent of 100 kg N ha^{-1} and the urine was applied at the equivalent of 700 kg N ha^{-1} (equivalent to 91 mg N kg^{-1} dry soil and 636 mg N kg^{-1}, respectively).

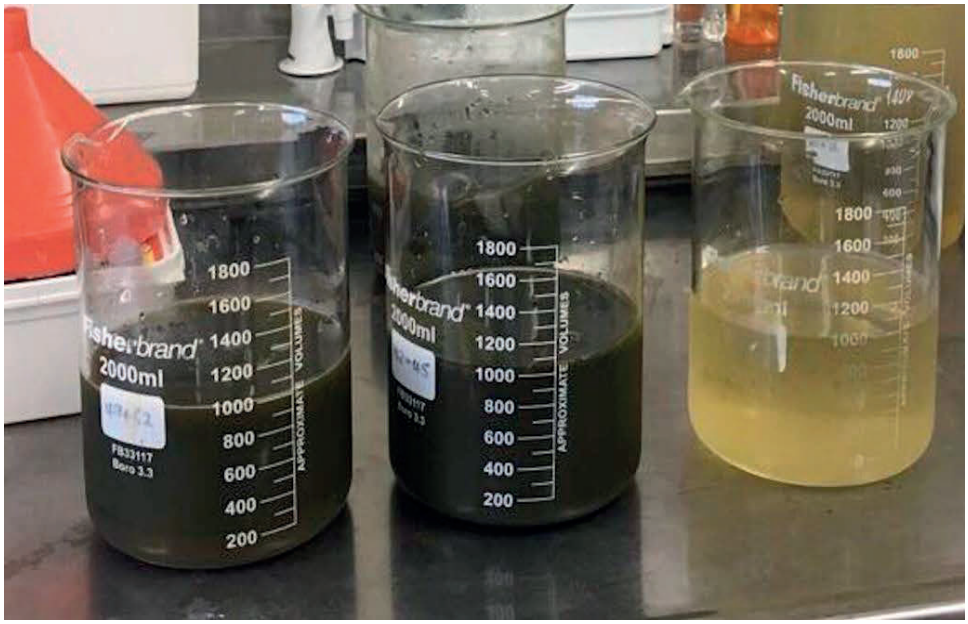

Fig. 3.2 Different types of effluent. From left to right is standard FDE, treated effluent and treated clear water, respectively.

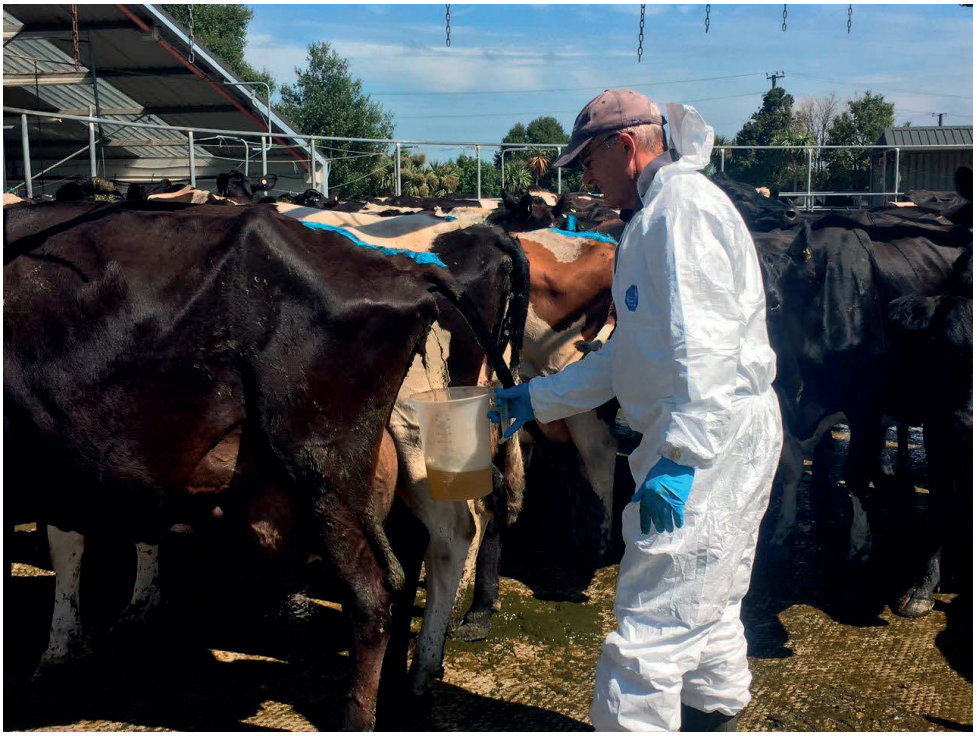

Fig. 3.3 The collection of cow urine from the Lincoln University Demonstration Dairy Farm.

Table 3.1 The properties of the soil used in this study.

Property	Value
Organic matter (g kg^{-1})	5.0
Total Nitrogen (g kg^{-1})	2.7
Total Carbon (g kg^{-1})	29.0
pH (H_2O)	6.1
Olsen P (mg kg^{-1})	45.7
CEC ($cmol_c\,kg^{-1}$)	13.3
Exchangeable Calcium ($cmol_c\,kg^{-1}$)	7.5
Exchangeable Magnesium ($cmol_c\,kg^{-1}$)	1.2
Exchangeable Potassium ($cmol_c\,kg^{-1}$)	1.0
Exchangeable Sodium ($cmol_c\,kg^{-1}$)	0.3
Base saturation (%)	74.0
Sulphate Sulphur (mg kg^{-1})	6.7

Table 3.2 Original properties of the three different types of effluent used in this study.

Chemical property	Standard FDE	Treated clear water	Treated effluent
Turbidity (NTU)	2277.0	10.7	4882.7
Total solid (g m^{-3})	4233.3	1706.7	11266.7
pH	7.2	5.9	5.9
Total nitrogen (g m^{-3})	495.0	311.0	570.0
Ammonium (g m^{-3})	115.3	119.0	121.0
Nitrate + nitrite (g m^{-3})	0.1	4.0	2.8
Total phosphorus (g m^{-3})	42.0	0.8	77.7
Dissolved reactive phosphorus (g m^{-3})	23.3	0.0	0.0
Total carbon (g m^{-3})	1270.0	655.0	2933.3
Soluble carbon (g m^{-3})	713.3	596.7	670.0
cBOD (g m^{-3})	936.7	520.0	1390.0
Fe (g m^{-3})	9.3	10.7	794.0

Table 3.3 Description of the treatments.

Treatment number	FDE type (100 kg N ha^{-1})	Urine (700 kg N ha^{-1})	Replicates
1	Water (0)	0	4
2	Standard FDE	0	4
3	Treated clear water	0	4
4	Treated effluent	0	4
5	Water (0)	700	4
6	Standard FDE	700	4
7	Treated clear water	700	4
8	Treated effluent	700	4

The pottles for soil sampling and the jars for N_2O gas sampling were placed inside the incubator following a randomized block design. The incubator was set to a temperature of 12°C (simulating the autumn/winter soil temperature in New Zealand).

The experimental design for the trials was a randomized block design with four replicates of each treatment (Table 3.3). Treatments were randomly numbered and allocated to pottles and jars and were randomly placed within each block inside the incubator. The soil moisture content was adjusted to and maintained at field capacity during the incubation (30% gravimetric water content), which equalled 56.4% water filled pore space (WFPS).

3.2.4 Gas sampling jars

There were 32 glass jars (1 litre) used in the N_2O emissions sampling trial (Fig. 3.4). Each jar was packed with 600 g soil (dry equivalent) to a bulk density of 1 g cm^{-3}. The effluent and cow urine treatments were applied evenly over the soil surface (Table 3.4). The total weight of each jar (without lid) was recorded. The maintenance of soil moisture content (30%) was done twice a week, after taking gas samples and in between gas sampling, by the addition of de-ionized water until the jar reached the recorded weight. There were two 1 cm diameter holes in the lid of the sampling jar to allow aeration.

Fig. 3.4 Incubation jars inside the incubator for N_2O sampling.

3.2.5 Soil sampling pottles

A total of 32 pottles were established for soil sampling to determine nitrification rate dynamics, ammonia oxidisers and denitrifiers (Fig. 3.5). Each pottle contained 500 g of soil (dry equivalent). After the effluent and urine treatments were applied, the soil was thoroughly mixed (Table 3.5). Subsamples of soil were taken after 1, 7, 14, 28, 63, 91, 119, 154 and 210 days of incubation to determine the NH_4^+, NO_3^-, pH, AOB, AOA, *nirS*, *nirK*, *nosZ* and soil moisture content. There were two 1 cm diameter holes in the lid of soil sampling pottle to allow aeration. The weight of each pottle (without lid) was recorded after each subsampling in order to maintain soil moisture content by adding de-ionized water. The maintenance of soil moisture content was done twice a week as described for the gas sampling jars.

Table 3.4 The volumes of effluents and/or cow urine applied to the incubation jars.

Treatment	Water (mL)	Standard FDE (mL)	Treated clear water (mL)	Treated effluent (mL)	Urine (mL)	Replicates
1	91	0	0	0	0	4
2	55	36	0	0	0	4
3	55	0	36	0	0	4
4	55	0	0	36	0	4
5	36	0	0	0	55	4
6	0	36	0	0	55	4
7	0	0	36	0	55	4
8	0	0	0	36	55	4

Table 3.5 The volumes of effluents and/or cow urine applied to the soil sampling pottles.

Treatment	Water (mL)	Standard FDE (mL)	Treated clear water (mL)	Treated effluent (mL)	Urine (mL)	Replicates
1	75	0	0	0	0	4
2	45	30	0	0	0	4
3	45	0	30	0	0	4
4	45	0	0	30	0	4
5	45	0	0	0	45	4
6	0	30	0	0	45	4
7	0	0	30	0	45	4
8	0	0	0	30	45	4

3.3 N_2O gas sampling and analysis

The method used to measure N_2O gas emissions in this study was similar to that of Hutchinson and Mosier (1981). During N_2O sampling, the gas jars were taken out of the incubator and the lids were removed and replaced with gas sampling lids, which contained a rubber septum, tap and needle (Fig. 3.6). During each sampling event, a 10 mL gas sample was taken, 30 minutes apart (one at time 0, and one 30 minutes later), and was injected into a pre-evacuated 6 mL glass vial using a hypodermic needle

and 60 mL syringe. Before taking the gas sample, the air in the jar was mixed 3 times by recirculating 10 mL of air three times using the syringe.

After gas sampling, the soil moisture content was maintained by weighing each jar and adding de-ionized water to achieve the initial weight. The incubation lids were then replaced before returning the jars to the incubator. N_2O gas samples were taken twice a week for the first 70 days and then taken weekly for the remainder of the study. The glass vial rubber septum was replaced after each sampling.

Fig. 3.5 The soil sampling pottles inside the incubator.

Fig. 3.6 An example of N_2O gas sampling. Top left: the gas sampling lids used. Bottom left: A syringe was used to mix the gas and then inject 10 mL into an evacuated vial. Middle: the preparation just before gas sampling. Right: gas sampling lids in place.

The concentration of N_2O was measured using a gas chromatograph (SRO8610 linked to a Filson 222XL autosampler) using an Electron Capture Detector (ECD) (SRI Instruments, USA). N_2O gas emissions were calculated by comparing the N_2O concentration in the two samples (A and B) extracted from the headspace of the incubation jars. A preliminary study showed a linear increase of N_2O during the 30 min sampling interval.

The hourly N_2O emissions for each sampling day were calculated using the following equation:

$$N_2O\ flux = \frac{(c_1 - c_0) \times HV \times P \times C_L \times M_{N_2O} \times C_{mg}}{(t_1 - t_0) \times SA \times R \times T_K}$$

Where:

N_2O flux = Hourly N_2O emissions (mg N_2O-N m^{-2} hr^{-1})

c_0 = N_2O concentration at t_0 (µL L^{-1})

c_1 = N_2O concentration at t_1 (µL L^{-1})

HV = Headspace volume of the chamber (L)*

P = Atmospheric pressure (1 atm)

C_L = Conversion factor µL to L (0.000001 L μL^{-1})

M_{N_2O} = Molecular weight of N in N_2O (28.01 g mol^{-1})

C_{mg} = Conversion factor µg to mg (1000 mg μg^{-1})

t_0 = Time 1st sample taken (0 hours)

t_1 = Time 2nd sample taken (0.5 hours)

SA = Surface area of gas jar (0.0064 m^2)

R = Universal gas constant (0.0821 L atm mol^{-1} K^{-1})

T_K = Temperature (K) **

* Chamber headspace volume (HV) = SA (0.0064 m^2) × headspace height (m) × 1000(L m^{-3}).

** Temperature (°C) at the soil surface (12°C).

The hourly N_2O emissions were used to calculate daily emissions by assuming it represented the average hourly flux of the day (de Klein et al., 2003). The cumulative N_2O emissions were calculated by integrating the calculated daily N_2O fluxes and linearly interpolating between measurements for each jar.

The emission factor (EF) of N_2O was calculated for each treatment in each block using the following equation:

$$EF(\%) = \frac{N_2O\text{-}N\ total\ (treatment) - N_2O\text{-}N\ total\ (control)}{Total\ N\ (applied)} \times 100$$

Where:

EF (%) = Emission factor (N_2O-N emitted as % effluent and/or urine applied)

N_2O-N total (treatment) = Total N_2O emissions (kg N ha^{-1}) from effluent or urine treatments

N_2O-N total (control) = Total N_2O emissions (kg N ha^{-1}) from the control

Total N (applied) = Total N application rate (kg N ha^{-1})

3.4 Soil sampling and analysis

In total, 9 batches of soil subsamples were taken after 1, 7, 14, 28, 63, 91, 119, 154 and 210 days of incubation to determine the concentration of mineral N (including NH_4^+ and NO_3^-), soil moisture content, soil pH and the abundance of AOB, AOA, and denitrifying functional genes (including *nirS*, *nirK* and *nosZ*) (Fig. 3.7). For each sampling date, the 32 pottles were removed from the incubator. Soil moisture content was maintained before taking the subsamples, by weighing each pottle and adding de-ionized water to the recorded weight.

Fig. 3.7 Taking soil subsamples.

3.4.1 Soil moisture content

The soil moisture content was maintained during the experiment. However, an accurate moisture content at the time of sampling was confirmed by taking a subsample. Approximately 20 g of soil from each pottle was dried in an oven for 24 hours at 105°C and then reweighed. The calculation of soil moisture content used the following formula:

$$Soil\ moisture\ content\ (\%) = \frac{wet\ soil\ (g) - dry\ soil\ (g)}{dry\ soil\ (g)} \times 100$$

3.4.2 Soil mineral nitrogen

Five grams of soil were taken for each pottle and placed into a labelled 50 mL PP Labserv disposable centrifuge tube. Then 25 mL of 2 M KCl solution was added into each tube to extract NH_4^+ and NO_3^-. The tubes were shaken for one hour at the speed of 120 oscillations per minute on a Ratek Platform Mixer (Fig. 3.8). The samples were then centrifuged at 4000 rpm for 10 minutes on a Thermo Multifuge 3S-R Centrifuge and then filtered through 110 mm Advantec 5 C filter paper into 30 mL PP Labserv white cap vials (Fig. 3.8). Two blanks were prepared. The extracts were frozen at -20°C until analysed by Flow Injection Analyser (FIA) (FOSS FIAstar 5000 Flow Injection Analyser with SoFIA software version 2.00).

Ammonium-N was determined using a gas diffusion membrane on the FIA. Sodium hydroxide was added to increase the pH of the sample stream. All the ammonium ions present were converted into ammonia gas. The ammonia gas diffused into an indicator stream through the membrane. The indicator stream changed colour from red to blue with measurement made at 590 nm. The extent of the colour change was proportional to the concentration of ammonium ions (NH_4^+-N) present in the sample.

Nitrate-N was analysed by initial reduction of nitrate-N to nitrite-N using a packed cadmium reduction coil in the FIA, followed by the reaction of nitrite-N with sulphanilamide/NED to form an azo dye compound. The intensity of this compound was determined spectrophotometrically at 540 nm.

3.4.3 Soil pH

Fifteen grams of soil were weighed from each pottle and placed into a labelled 70 mL specimen bottle. Then, 25 mL de-ionized water was added, and the bottle shaken well. Subsamples were settled overnight (at least 12 hours) before measuring pH by a pH meter (Mettler Toledo SevenCompact) (Fig. 3.9).

Fig. 3.8 KCl extraction. Left, soil subsamples shaken with 25 mL KCl; right, filtering.

Fig. 3.9 Soil pH measurement.

3.4.4 Functional gene abundance quantification

The abundance of AOB, AOA (*amoA*) and denitrifier functional genes (*nirS*, *nirK*, *nosZ* (Clades I and II) were analysed as follows. The subsamples from the pottles were collected at the soil sampling dates above and were frozen at -20°C until analysis using the method described by Di et al. (2014).

DNA extraction

DNA was extracted from 0.25 g soil subsamples using NucleoSpin® Soil Kit (Macherey-Nagel,Düren, Germany) according to the manufacturer's instructions. From each pottle, 0.25 g soil was collected as

subsample and transferred to a NucleoSpin® bead tube. Then, 700 µL buffer SL2 and 150 µL enhancer SX was added into the tube and then processed in the MP FastPrep-24 for 1 min at a speed of 4.0 m s^{-1} to homogenize the samples. The samples were centrifuged for 2 min at a speed of 11000 g. The supernatant was transferred to a new tube and then 150 µL Buffer SL3 was added. The samples were vortexed for 5 sec to mix before incubated for 5 min at 4°C, and then centrifuged at 11000 g for 1 min. 700 µL of supernatant was loaded up from the previous step and transferred to a NucleoSpin® inhibitor removal column (red ring) in a collection tube and then centrifuged at 11000 g for 1 min. This step was repeated once. In each column, 250 µL Buffer SB was added and then vortexed for 5 sec. A Nucleospin® soil column (green ring) was placed on a collection tube. Then, a 550 µL sample was loaded onto the green-ring column, centrifuged for 1 min at 11000 g and then the flowthrough was discarded and returned the column back into the collection tube. This step was repeated with the remaining sample. There was 500 µL buffer SB added to each Nucleospin® soil column and centrifuged for 30 sec at 11000 g with the flowthrough discarded. This step was repeated using 550 µL buffer SW1. Then 700 µL buffer SW2 was added to each column, vortexed for 2 sec and centrifuged for 30 sec at 11000 g with the flowthrough discarded. This step was repeated once. The columns and collection tubes were then centrifuged for 2 min at 11000g to make the columns dry. To elute DNA, the green-ring columns were transferred to the new collection tubes and 100 µL of buffer SE were added to each column. The samples were incubated at room temperature for 1 min with the lids open before centrifuged with the lids closed for 30 sec at 11000 g. Purified DNA was eluted with 100 µL of Elution buffer and was stored at -20°C until analysis by real-time qPCR.

Real-time qPCR analysis

The abundance of AOB *amoA*, AOA *amoA*, *nirS*, *nirK*, *nosZ* (I) and *nosZ* (II) was measured using real-time qPCR on a Rotor-Gene™ 6000 (Corbett Life Science). CAS-1200 Robotic liquid handling system was used to set up all PCR reactions (Corbett Life Science, Australia). All the soil DNA extraction samples were diluted 1:10 with deionized water and used as a template in PCR reactions. The primer pairs used are shown in Table 3.6. A 1.5 µL aliquot of 1:10 diluted template soil genomic DNA was mixed with 8 µL of SYBR Premix Ex Taq (TaKaRa, Nori Biotech, Auckland, New Zealand), and 0.4 µL-1.6 of each primer (final concentrations of each primer pair combination used was shown in Table 3.6) was added into 16 µL reaction mixture. The running of PCR was in accordance with the temperature profiles shown in Table 3.6. After the amplification, a melting curve analysis was done to confirm the specificity of PCR product by measuring the fluorescence continuously during the increase of temperature from 72°C to 99°C. Then the data was analysed by Rotor Gene 6000 series software 1.7.

Table 3.6 The primer pairs and PCR conditions used in real-time qPCR analysis.

Target group	Primer name	Sequence (5'-3')	Length of amplicon (bp)	Primer final concentration (nM)	Thermal profile	Amplification efficiency (R^2 >0.99)(%)	References
Bacterial *amoA*	amoA1F amoA2R	5'-GGGGTTTCTACTGGTGGTGGT-3' 5'-CCCCTCKGSAAAGCCTTCTTC-3'	491	250	95°C for 2 min - × 1 cycle; 95°C for 20 s, 57°C for 30 s, 72°C for 30 s, 85°C for 10 s - × 40 cycles;	96-98	(Rotthauwe et al., 1997)
Archaeal *amoA*	Arch-amoAF Arch-amoAR	5'-STAATGGTCTGGCTTAGACG-3' 5'-GCGGCCATCCATCTGTATGT-3'	635	250	95°C for 2 min - × 1 cycle; 95°C for 20 s, 55°C for 20 s, 72°C for 30 s, 80°C for 10 s - × 40 cycles;	92-94	(Francis et al., 2005)
nir S	cd3af R3cd	5'-GTSAACGTSAAGGARACSGG-3' 5'-GASTTCGGRTGSGTCTTGA-3'	410	750	95°C for 2 min - × 1 cycle; 95°C for 45 s, 55°C for 45 s, 72°C for 45 s, 85°C for 20 s - × 40 cycles;	93-95	(Michotey et al., 2000) (Throbäck et al., 2004)
nir K	FlaCu R3Cu	5'-ATCATGGTSCTGCCGCG-3' 5'-GCCTCGATCAGRTTGTGGTT-3'	474	780	95°C for 2 min - × 1 cycle; 95°C for 20 s, 55°C for 30 s, 72°C for 30 s, 85°C for 10 s - × 40 cycles;	98-100	(Hallin and Lindgren, 1999)
nos Z (I)	nosZ-F nosZ1622R	5'-CGYTGTTCMTCGACAGCCAG-3' 5'-CGSACCTTSTTGCCSTYGCG-3'	424	750	95°C for 2 min - × 1 cycle; 95°C for 20 s, 55°C for 30 s, 72°C for 30 s, 85°C for 15 s - × 40 cycles;	94-99	(Kloos et al., 2001) (Throbäck et al., 2004)
nos Z (II)	nosZ-II-F nosZ-II-R	5'-CTIGGICCIYTKCAYAC-3' 5'-GCIGARCARAAITCBGTRC-3'	698	1000	95°C for 2 min - × 1 cycle; 95°C for 30 s, 50°C for 30 s, 72°C for 45 s, 80°C for 10 s - × 40 cycles;	76-81	(Jones et al., 2013)

Standard curves for real-time qPCR were performed using the following process. The primers aforementioned in Table 3.6 were used to amplify AOB *amoA*, AOA *amoA*, *nirS*, *nirK*, *nosZ* I and *nosZ* II from the extracted DNA. To purify the products of PCR, a clean-up kit (Axygen) was used and then cloned into the pGEM-T Easy Vector (Promega, Madison, WI). The resulting clones were then transformed into *Escherichia coli* JM109 competent cells (Promega) according to manufacturer's instruction. After the transformation, *E. coli* cells were grown on LB plates overnight at 37°C. Then ten to fifteen bacterial colonies from the LB plate were individually inoculated into a 3 mL LB broth medium and incubated in an orbital incubator-shaker at 37°C and 250 rpm overnight. The plasmids were then extracted from overnight cultures using PureLink™ Quick Plasmid Miniprep Kit (Life Technologies, Auckland, New Zealand). To generate the PCR amplicons containing each gene of interest, the plasmids were then used as a template in the reactions of PCR with T7 and SP6 primers. Further details can be found in Di et al. (2014).

Chapter 4

The effect of different forms of effluent, with and without animal urine, on nitrification, denitrification and N_2O emissions

4.1 Introduction

N_2O is one of the most significant non-CO_2 greenhouse gases because it has a global warming potential around 298 times greater than CO_2 (IPCC, 2007) and is also becoming the largest contributor to stratospheric ozone depletion (Ravishankara et al., 2009). In New Zealand, 93.7% of N_2O emissions are from agricultural soils (Ministry for the Environment, 2017). The widespread use of nitrogenous fertilisers and increasing inputs of manure are partly responsible for the increase of N_2O emissions (Reay et al., 2012). In New Zealand, N_2O emissions from urine and dung excreted by the grazing animal accounted for 63% of the agricultural N_2O and synthetic N fertiliser accounted for 16.1% of the agricultural N_2O emissions in 2015 (Ministry for the Environment, 2017). In grazed grassland, around 70%-90% of the ingested N is returned to the animal-grazed pastures and approximately 80% of that is in the form of urine (Di and Cameron, 2017). Urine makes a greater contribution to gaseous N losses than N fertiliser (Saggar et al., 2013).

Farm dairy effluent (FDE) is the most common animal manure collected and applied to grazed grassland in New Zealand. FDE is a mixture of animal excreta and wash-down water in the milking shed (van der Weerden et al., 2016a). FDE represents around one-quarter of lactating dairy cattle excreta on New Zealand dairy farms (Luo et al., 2013). The application of FDE onto pasture land can recycle the nutrients which improves soil fertility and increases farming system sustainability (Luo et al., 2008). However, it is also a source of N_2O emissions from soils. The high total N concentration, the addition of available C and the increase of soil moisture content and anaerobic conditions are the reasons for the enhancement of N_2O emissions after FDE application (Bhandral et al., 2007). In New Zealand, the application of FDE on pastures is the third largest source of N_2O emissions from agriculture (van der Weerden et al., 2016a).

It has been reported by van der Weerden et al. (2016a) that the mean emission factor (EF) of FDE is usually around 0.5% to 1%. Recently, new FDE treatment technologies have been developed where the solids are separated out from the liquid to produce treated effluent (with increased solid content compared with the standard FDE) and treated clear water (with significantly reduced solid content) (Cameron and Di, *pers. comm.*). However, there is currently no information available on potential impacts of these different types of effluents on N_2O emissions, nitrification, denitrification, and

nitrifiers and denitrifiers in the soil. This Chapter (4) reports the results from a laboratory incubation study to assess the impacts of these different effluents on these parameters.

4.2 Materials and methods

4.2.1 Experiment methods

Details of the materials and methods have been described in Chapter Three. Only a brief summary is presented here.

Two parallel laboratory incubation studies were conducted to determine the impact of applying three different types of effluents on N_2O emissions, soil nitrification rate dynamics, soil pH and abundance of ammonia oxidising bacteria (AOB) and archaea (AOA), and denitrifying functional genes (*nirS*, *nirK*, *nosZ* I and *nosZ* II).

4.2.2 Experimental design

Both experiments, involving (1) jars for gas sampling and (2) pottles for soil sampling, were laid out in randomized complete block designs with eight treatments and four blocks, simultaneously in the same incubator. The treatment structure was a 4 (effluent types) by 2 (urine, or not) factorial.

4.2.3 Statistical analysis

All variables were statistically analysed using analysis of variance for a randomized complete block design. For N_2O-N emissions (and the emission factor) plus NH_4-N and NO_3-N variables, the data values were an order of magnitude different between “no urine” and “urine” treatments, so these two sets of treatments were analysed separately (to avoid violating the essential ANOVA assumption of homogeneity of variance). For all other variables, data from all eight treatments were analysed as a 4 x 2 factorial with 4 blocks. In the cases of AOA and AOB, data values were logarithmically transformed prior to analysis to ensure the homogeneity of variance assumption was met.

4.3 Results

4.3.1 Soil moisture content

During the incubation period, the soil moisture content was adjusted to and maintained at around 30% of gravimetric water content (Fig. 4.1).

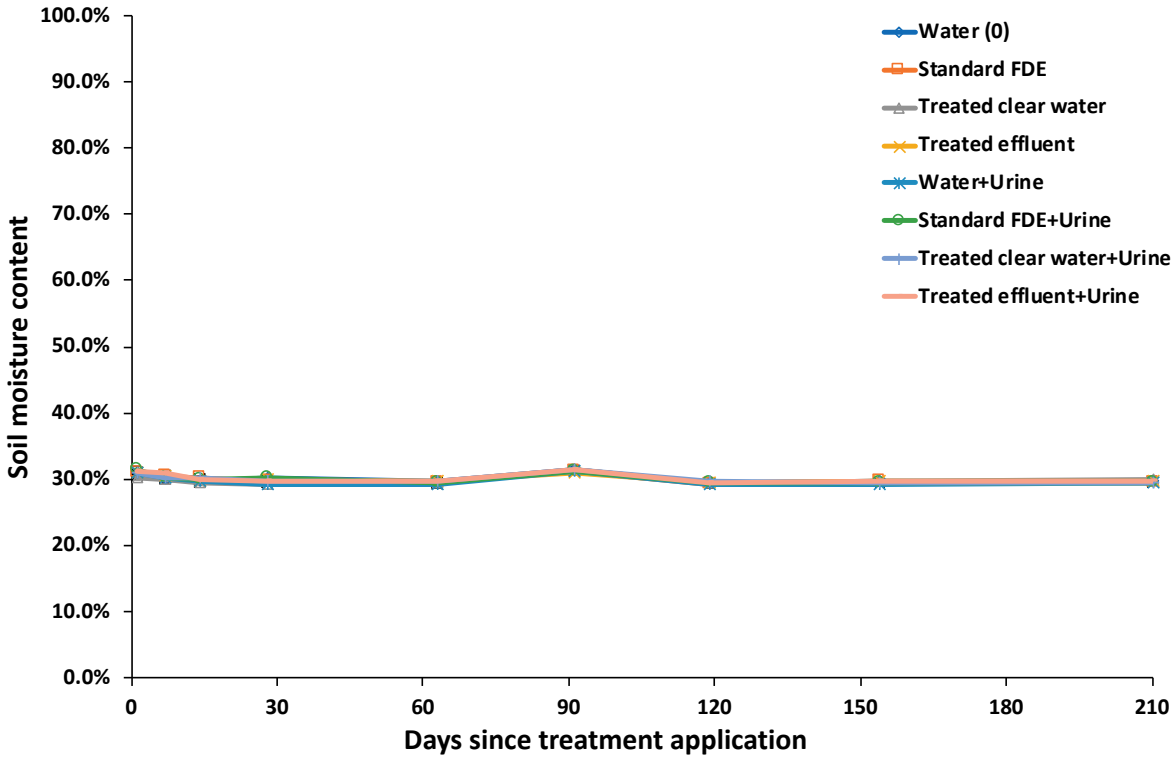

Fig. 4.1 Soil moisture content over the experiment period. The error bars represent the standard error of the mean (n=4).

4.3.2 N_2O emissions

Daily emissions

The application of the different effluents alone resulted in significantly higher daily N_2O emissions compared to the control straight after application (P<0.05, Fig. 4.2a). However, there was no significant difference in daily N_2O emissions between the different effluent treatments. Daily N_2O emissions decreased rapidly with time, reaching background levels after about 30 days.

The application of the different effluents plus animal urine also significantly increased daily N_2O emissions compared to that in the control straight after application. However, daily N_2O emissions decreased slightly before rising again, reaching peak values of between 68 and 109 g N_2O-N ha^{-1} day^{-1} about 75 and 100 days after treatment application (Fig. 4.2b). The peak daily N_2O emissions recorded between 75 to 100 days were significantly higher in the effluent plus urine treatments compared to that in the urine alone treatment (P<0.05). However, there was no significant difference among the standard FDE plus urine, treated clear water plus urine and treated effluent plus urine treatments. Daily N_2O emissions gradually declined with time reaching background values in about 250 days after treatment application.

Total emissions

Total N_2O emissions from the standard FDE and treated effluent were significantly higher than that from the control (P<0.05), but there was no significant difference in total N_2O emissions between the treated clear water and control (Fig. 4.3a). The difference in total N_2O emissions was not significant between the three different effluent treatments (P>0.05).

The application of the three different effluents plus animal urine resulted in significantly higher total N_2O emissions (at between 8.77 and 7.67 kg N_2O-N ha^{-1}) than that in the water plus urine treatment (at 5.79 kg N_2O-N ha^{-1}) (P<0.05). However, there was no significant difference between the standard FDE plus urine, treated clear water plus urine and treated effluent plus urine treatments (P>0.05, Fig. 4.3b).

4.3.3 Soil ammonium

The application of the standard FDE, treated clear water and treated effluent all resulted in significantly higher NH_4^+-N concentrations in the soil, reaching between 77.9 and 63.1 mg NH_4^+-N kg^{-1} soil, than that in the control straight after application (at 1.3 mg kg^{-1} NH_4^+-N soil) (P<0.05, Fig. 4.4a). The NH_4^+-N concentration in the treated clear water treatment was slightly higher than those in the standard FDE and treated effluent during the first 7 days (P<0.05). The NH_4^+-N concentration declined rapidly with time reaching background values about 30 days after treatment application.

The application of animal urine resulted in a significant increase in NH_4^+-N concentration in the soil well above those in the effluent only treatments (Fig. 4.4a and b). The NH_4^+-N concentrations in the effluents plus urine treatments were higher than those in the urine alone treatment throughout most of the incubation period. The NH_4^+-N concentration decreased rapidly with time (Fig. 4.4b). There was no significant difference in the NH_4^+-N concentration among the standard FDE plus urine, treated clear water plus urine and treated effluent plus urine treatments.

4.3.4 Soil nitrate

The application of the different effluents resulted in significantly higher NO_3^--N concentrations in the soil than those in the water (control) treatment (P<0.05, Fig. 4.5a). Similar NO_3^--N concentrations were recorded in the three different effluent treatments.

The application of urine resulted in significantly higher NO_3^--N concentrations than those in the effluent only treatments (cf. Fig. 4.5 a and b). However, there was no significant difference in NO_3^--N concentrations between the different effluent plus urine treatments (P>0.05, Fig. 4.5 b).

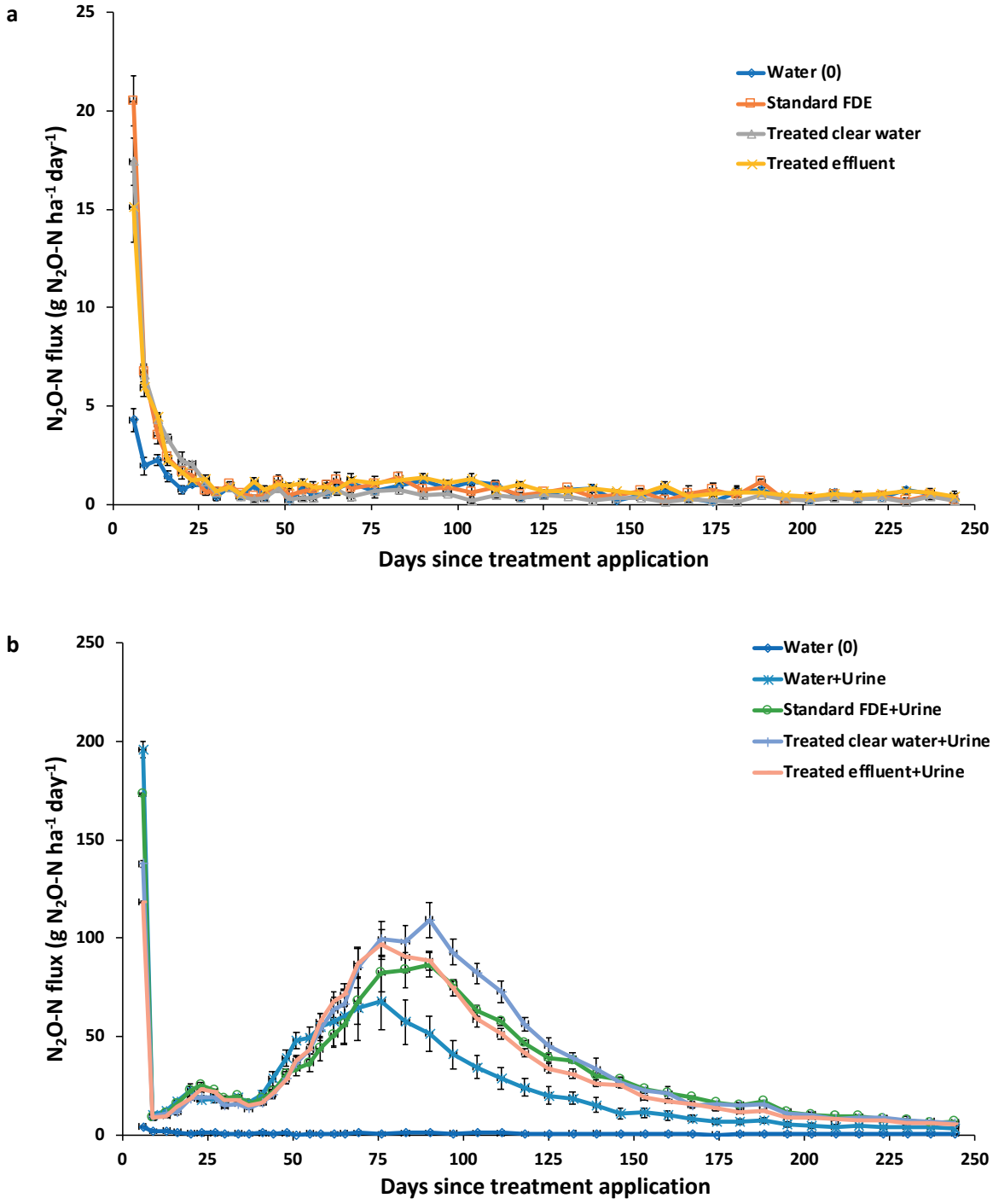

Fig. 4.2 Daily N_2O-N emissions. (a): Effluent treatments; (b): Effluents plus urine treatments. The error bars represent the standard error of the mean (n=4).

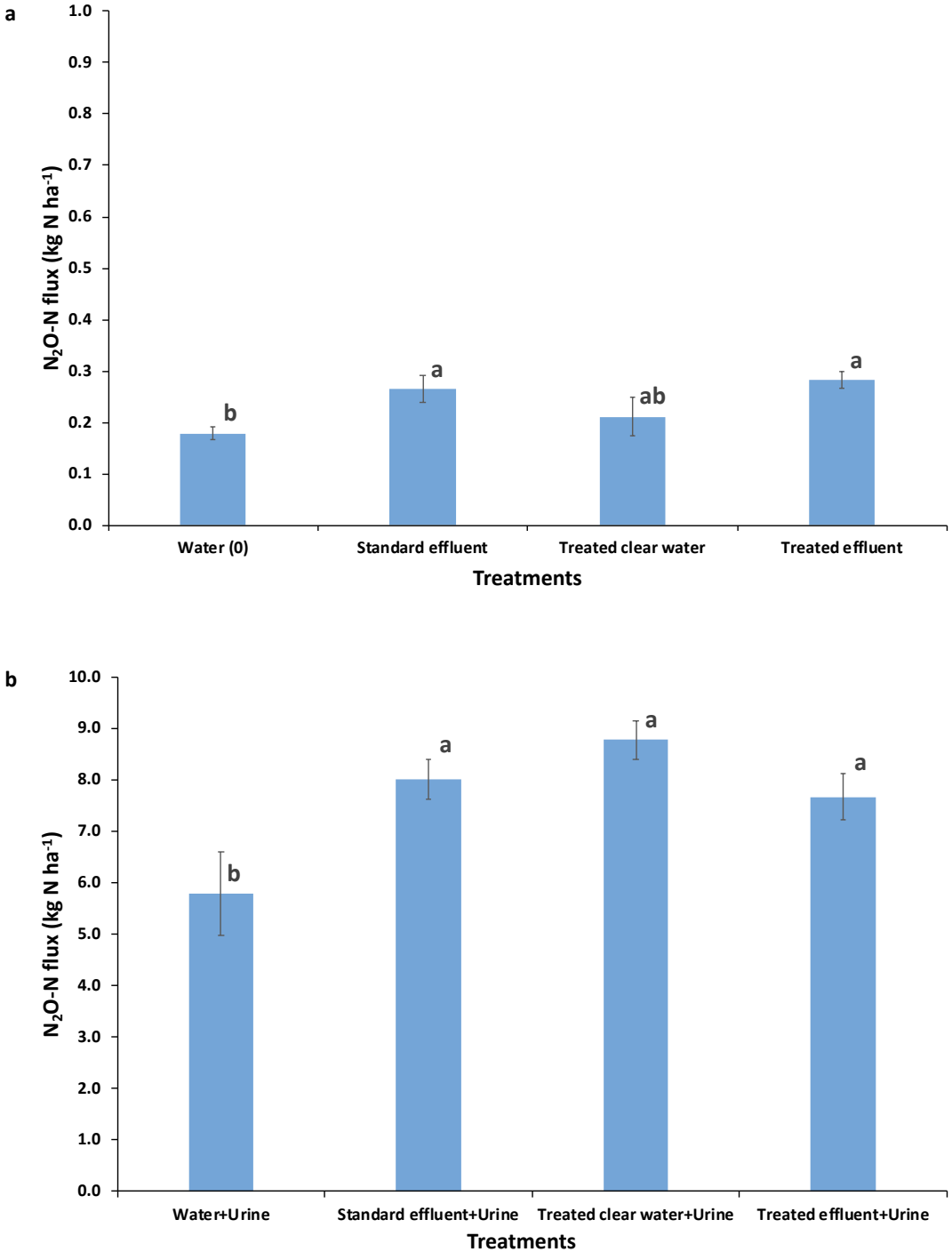

Fig. 4.3 Total N_2O-N emissions over the experiment period. (a): Effluent treatments; (b): Effluents plus urine treatments. The error bars represent the standard error of the mean (n=4). The treatments with different lower-case letters are significantly different at *P<0.05*. Note the difference in the y-axis scale between the Figure a and b.

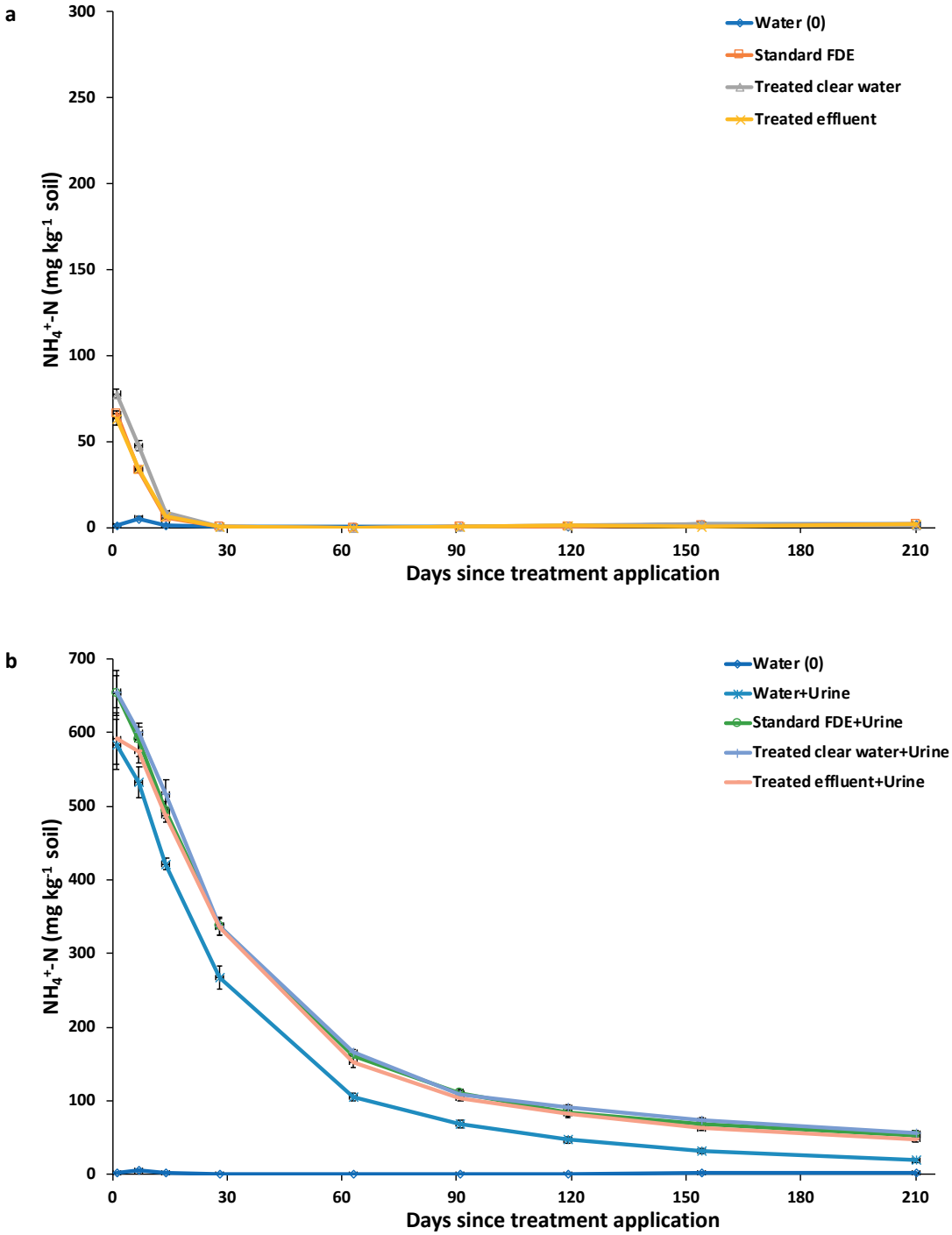

Fig. 4.4 Soil ammonium-N concentration. (a): Effluent treatments; (b): Effluents plus urine treatments. The error bars represent the standard error of the mean (n=4). Note the difference in the y-axis scale between a and b.

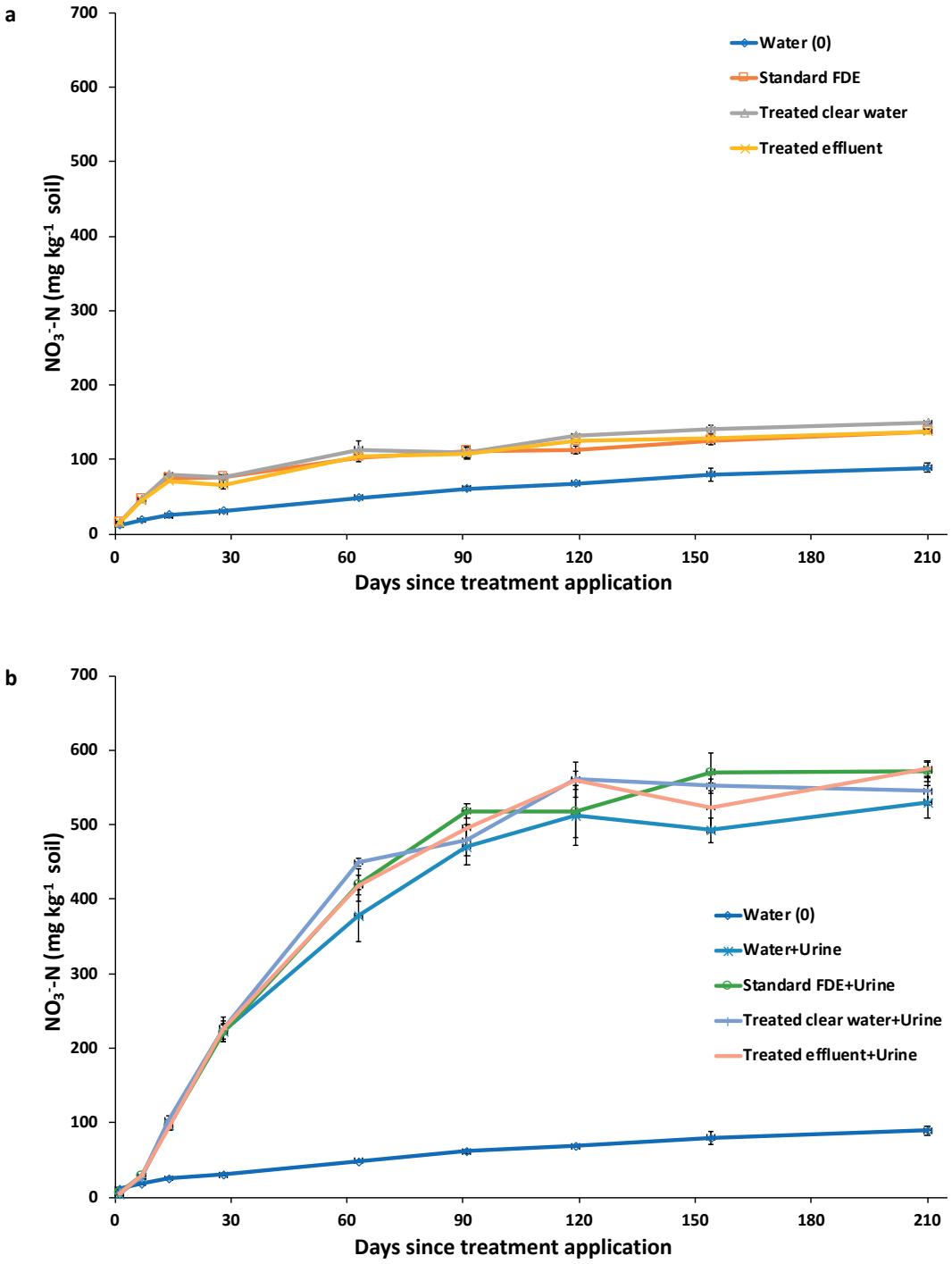

Fig. 4.5 The nitrate-N concentration. (a): Effluent treatments; (b): Effluents plus urine treatments. The error bars represent the standard error of the mean (n=4).

4.3.5 Soil pH

The application of the three effluents decreased the soil pH from 6.2 at the start of the incubation to between 5.0 and 5.5 after 14 days of incubation (Fig. 4.6a). There was no significant difference in soil pH between the effluent treatments (P>0.05).

However, the application of animal urine in addition to the effluents increased the soil pH to 7.6 at the start, which then declined to between 4.7 and 4.2 after 60 days of incubation (Fig. 4.6b). There was no significant difference in soil pH between the different effluent plus urine treatments (P>0.05).

4.3.6 Functional gene abundance

AOB

The application of the dairy effluents increased the AOB *amoA* gene copy numbers to between 6.75×10^7 and 7.65×10^7 copies g^{-1} soil after 14 days of incubation (Fig. 4.7a). These AOB *amoA* gene copy numbers were significantly higher than that in the control (P<0.05). The AOB abundance then gradually declined over time, particularly after 120 days of incubation. There was no significant difference in AOB abundance among the three effluent treatments (P>0.05).

The application of animal urine plus the different effluents increased the AOB *amoA* gene copy numbers to between 1.69×10^8 and 1.93×10^8 copies g^{-1} soil after 28 days of incubation (Fig. 4.7b). These peak AOB *amoA* gene copy numbers were significantly higher than those in the effluent treatments without urine (P<0.05). The AOB abundance then declined rapidly dropping to similar levels to those in the control after 210 days of incubation. There was no significant difference in the AOB abundance among the different effluent plus urine treatments.

AOA

The AOA *amoA* gene copy numbers remained relatively stable between 0 and 150 days of incubation. The AOA abundance then increased after 210 days of incubation (Fig. 4.8a). There was no significant difference among the effluent treatments throughout the entire incubation period (P>0.05).

The AOA *amoA* gene copy numbers in the effluent plus urine treatments were similar to those in the effluent only treatments between 0 and 90 days (Fig. 4.8b). The AOA abundance then decreased after 90 days of incubation to levels significantly below those in the control and all the effluent only treatments (P<0.05, Fig. 4.8b). There was no significant difference in AOA *amoA* gene copy numbers among the different effluent plus urine treatments (P>0.05).

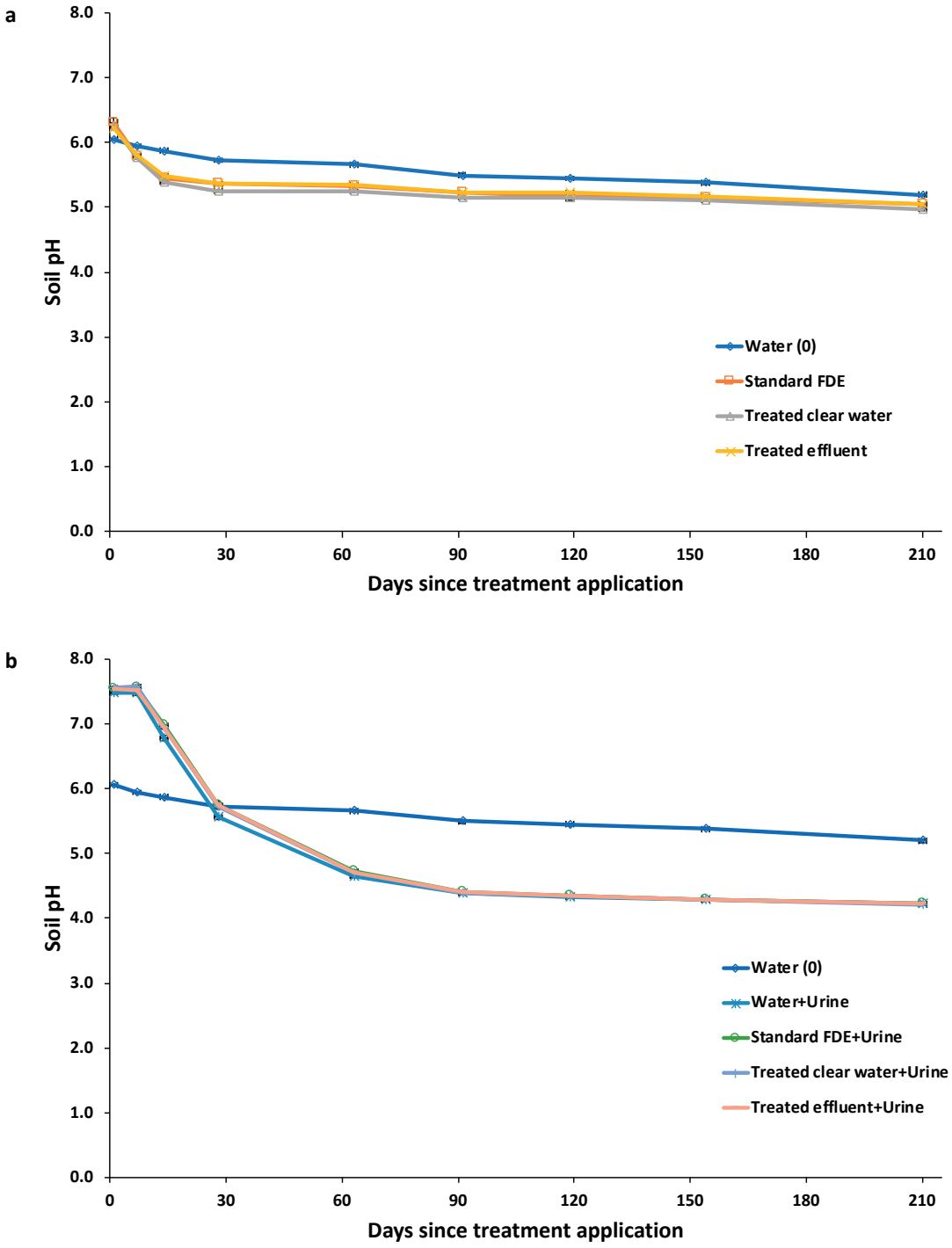

Fig. 4.6 The soil pH. (a): Effluent treatments; (b): Effluents plus urine treatments. The error bars represent the standard error of the mean (n=4).

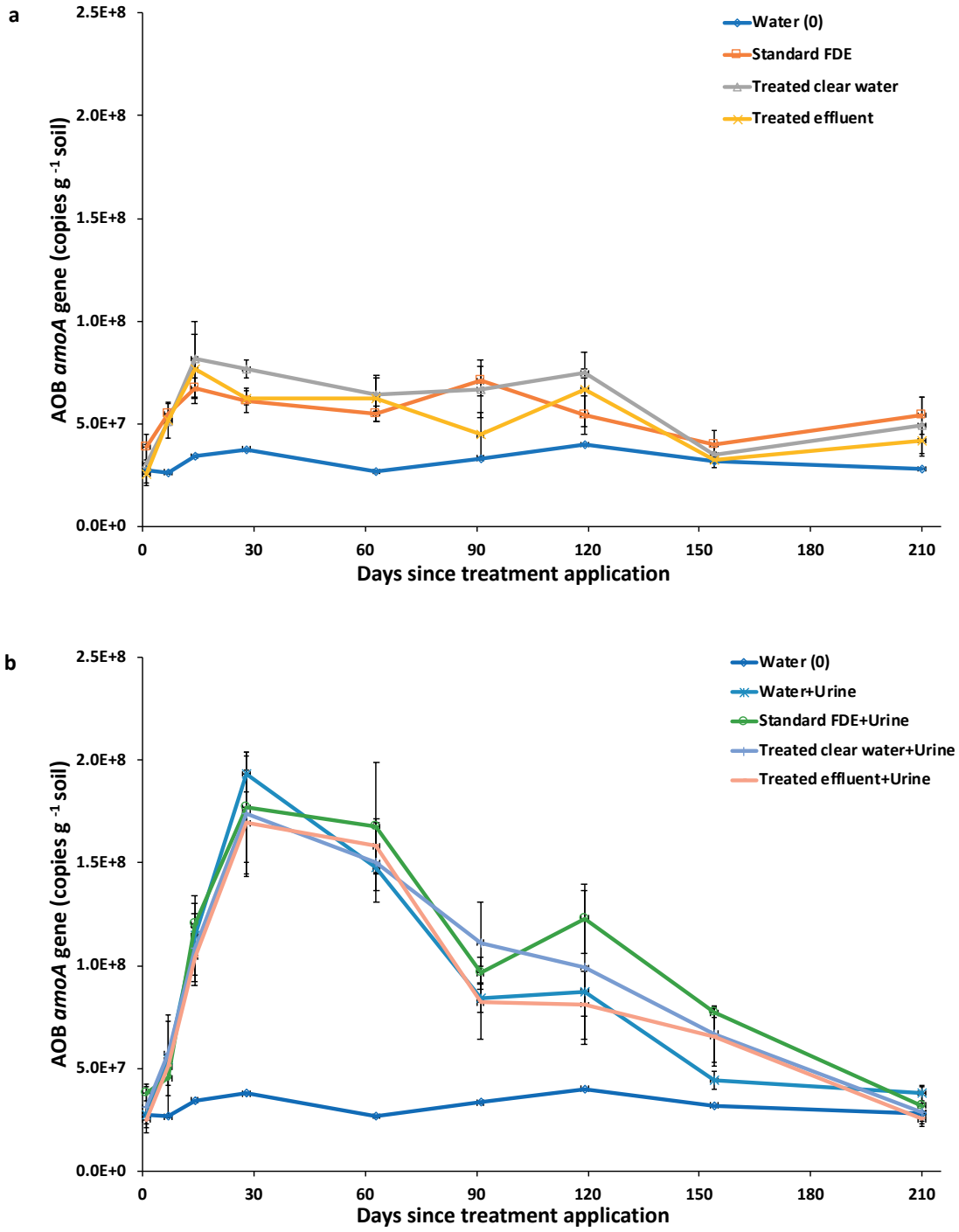

Fig. 4.7 AOB *amoA* gene abundance in the soil. (a): Effluent treatments; (b): Effluents plus urine treatments. The error bars represent the standard error of the mean (n=4).

nirS

Generally speaking, the application of the different effluents did not result in significant changes in the *nirS* gene copy numbers (Fig. 4.9a).

The *nirS* gene copy numbers in the effluent plus urine treatments were similar to those in the effluent without urine treatments between 0 and 90 days of incubation (Fig. 4.9b). However, the *nirS* gene abundance in the urine treatments decreased significantly to levels below those in the control and effluent treatments between 90 and 210 days ($P<0.05$). However, there was no significant difference in *nirS* gene copy numbers among the different effluent plus urine treatments.

nirK

Similar to the patterns of the *nirS* gene, the application of the different effluents did not have a major effect on the *nirK* gene copy numbers (Fig. 4.10a).

The *nirK* gene copy numbers in the effluent plus urine treatments were generally similar to those in the effluent without urine treatment between 0 and 90 days of incubation. However, the *nirK* gene abundance then declined to levels significantly below those in the control and the different effluent treatments between 90 and 210 days ($P<0.05$, Fig. 4.10b). There was no significant difference in *nirK* gene copy numbers among the different effluent plus urine treatments ($P>0.05$).

***nosZ* I**

The application of the effluents did not significantly affect the *nosZ* I gene copy numbers throughout the incubation period (Fig. 4.11a).

The application of urine plus the different effluents resulted in similar *nosZ* I gene copy numbers as those in the effluent without urine treatments between 0 and 90 days of incubation (Fig. 4.11b). However, the *nosZ* I gene copy numbers decreased to below those in the control treatments between 90 and 210 days of incubation. There was no significant difference in *nosZ* I copy numbers among the different effluent plus urine treatments ($P>0.05$).

***nosZ* II**

The changing patterns in *nosZ* II gene copy numbers were very similar to those of *nosZ* I gene copy numbers as described above (Fig. 4.12 a and b).

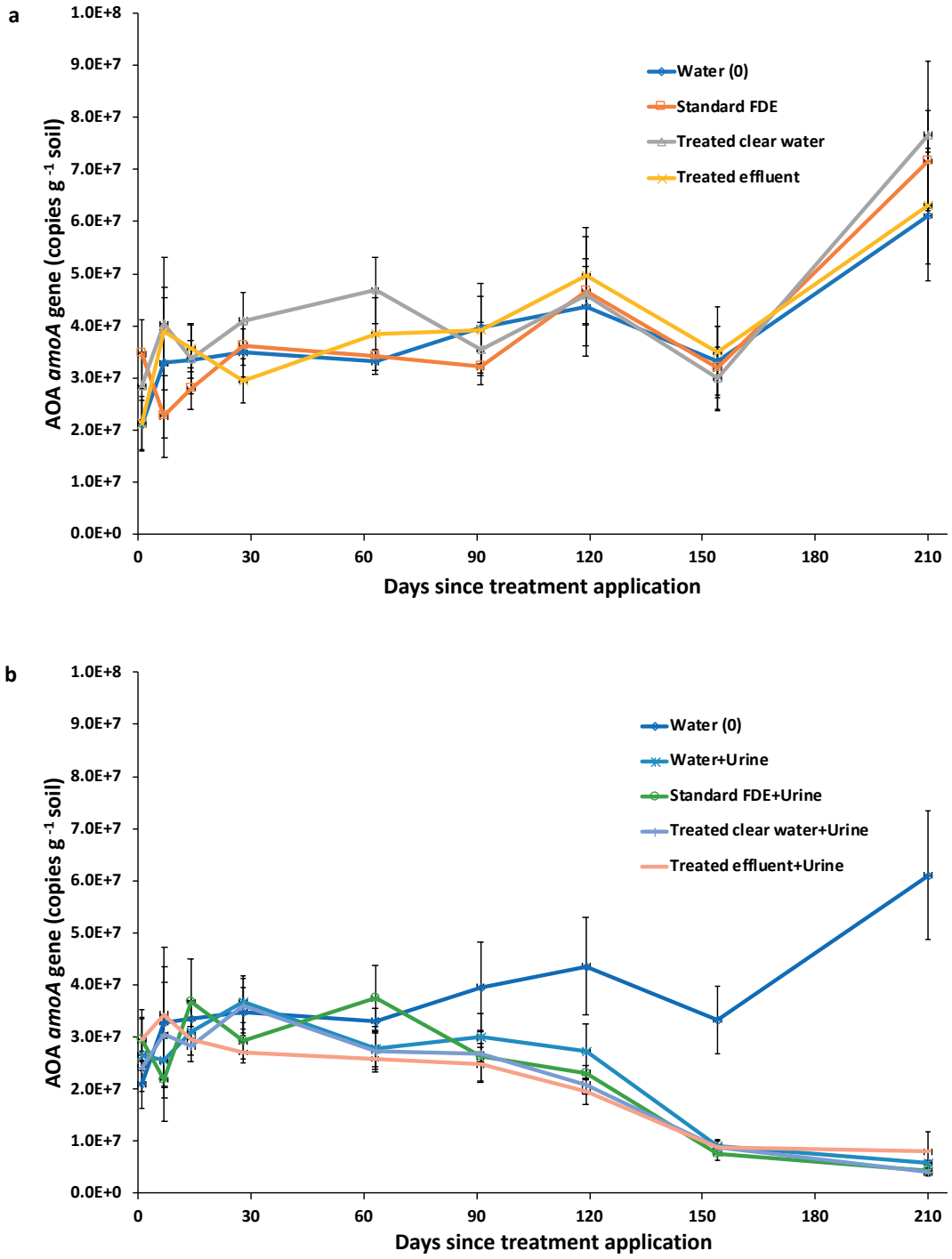

Fig. 4.8 AOA *amoA* gene abundance in the soil. (a): Effluent treatments; (b): Effluents plus urine treatments. The error bars represent the standard error of the mean (n=4).

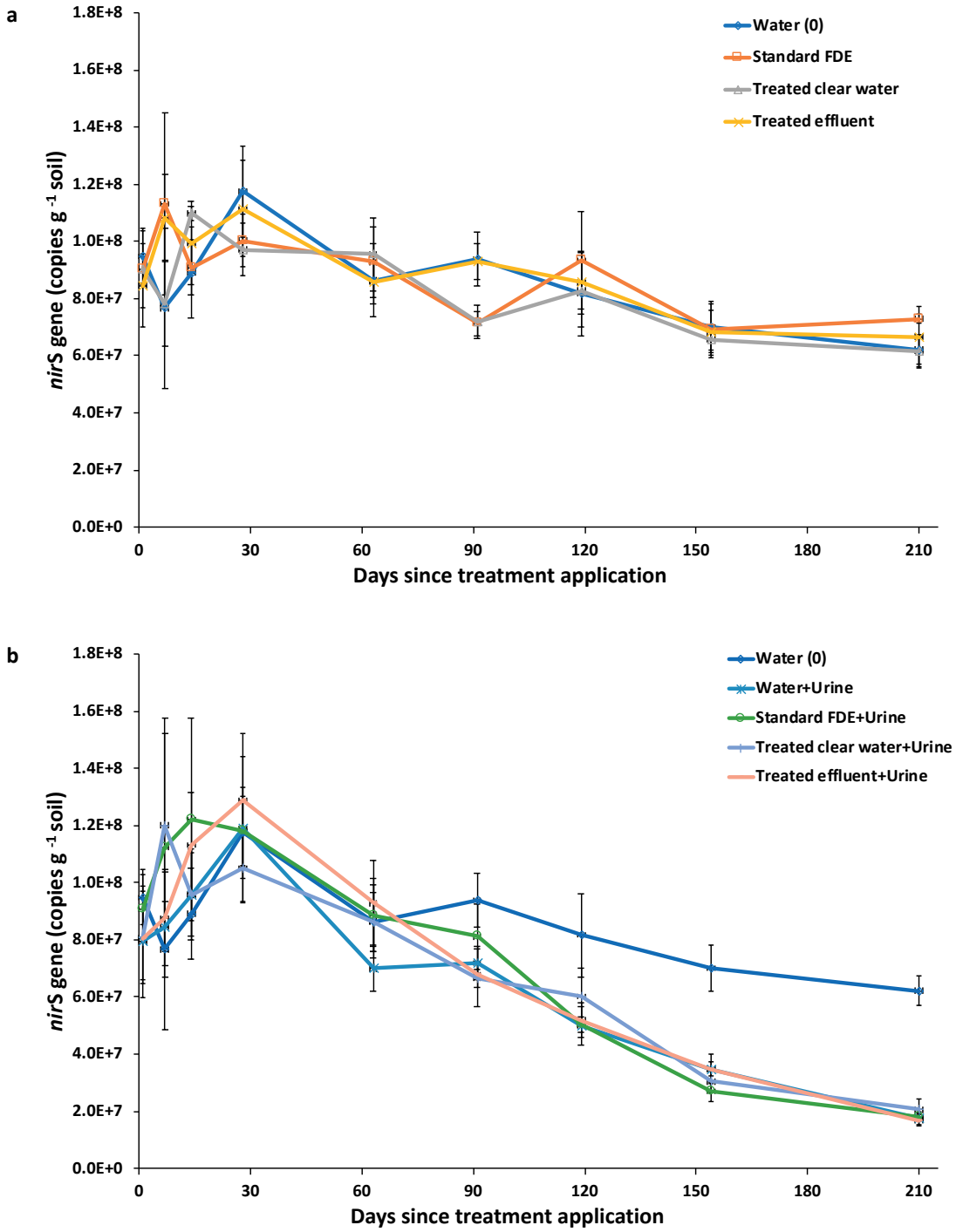

Fig. 4.9 *nirS* gene abundance in the soil. (a): Effluent treatments; (b): Effluents plus urine treatments. The error bars represent the standard error of the mean (n=4).

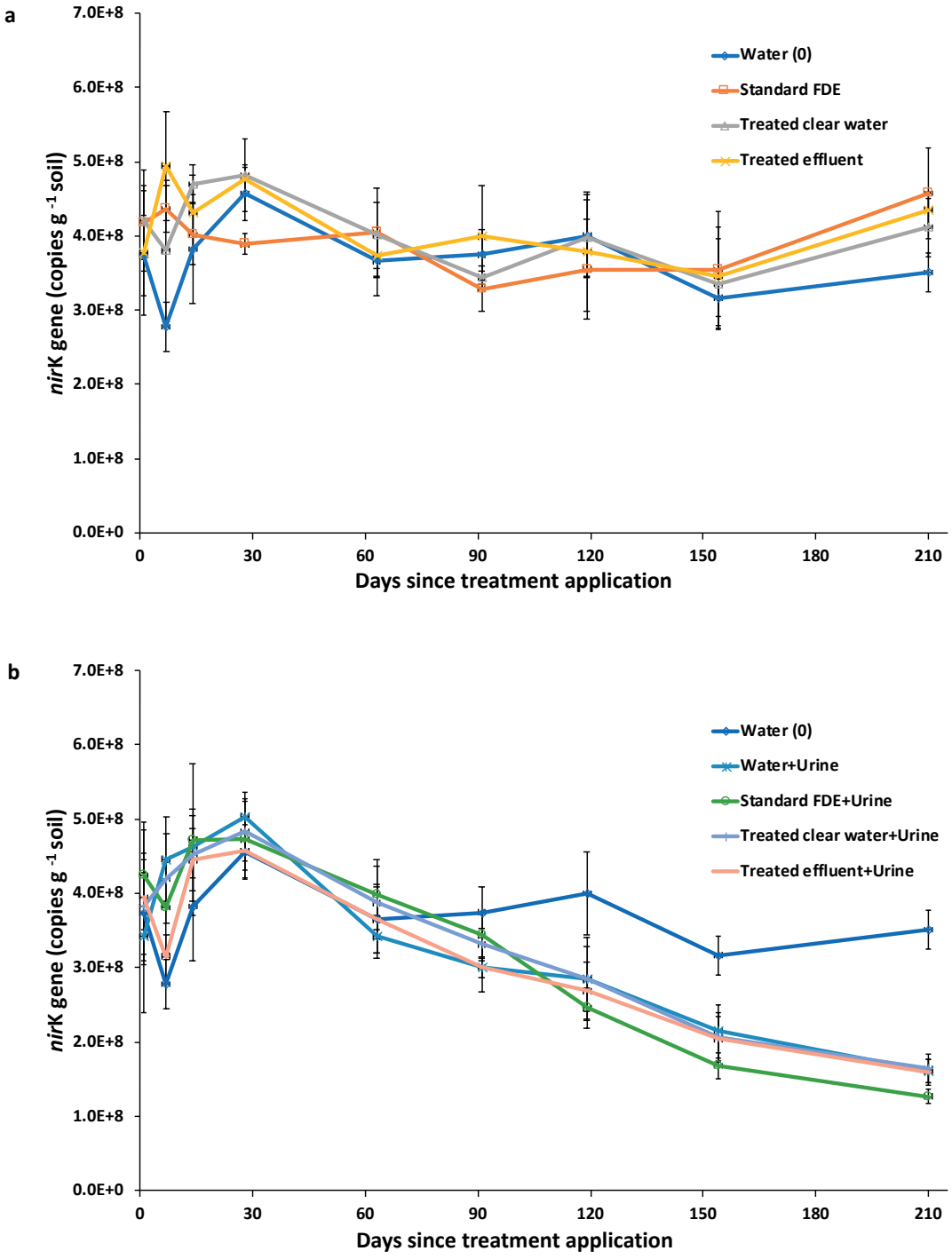

Fig. 4.10 *nirK* gene abundance in the soil. (a): Effluent treatments; (b): Effluents plus urine treatments. The error bars represent the standard error of the mean (n=4).

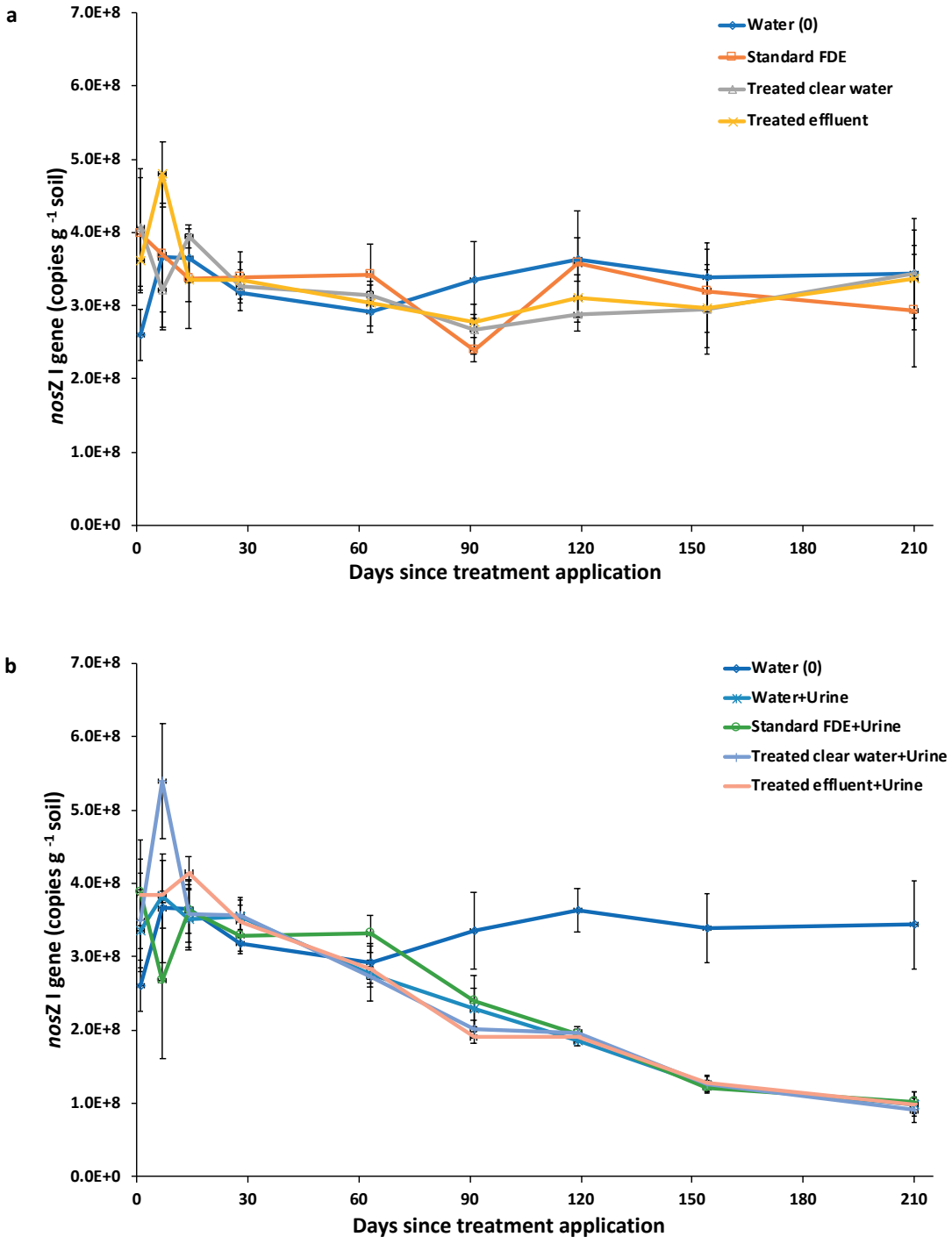

Fig. 4.11 *nosZ* I gene abundance in the soil. (a): Effluent treatments; (b): Effluents plus urine treatments. The error bars represent the standard error of the mean (n=4).

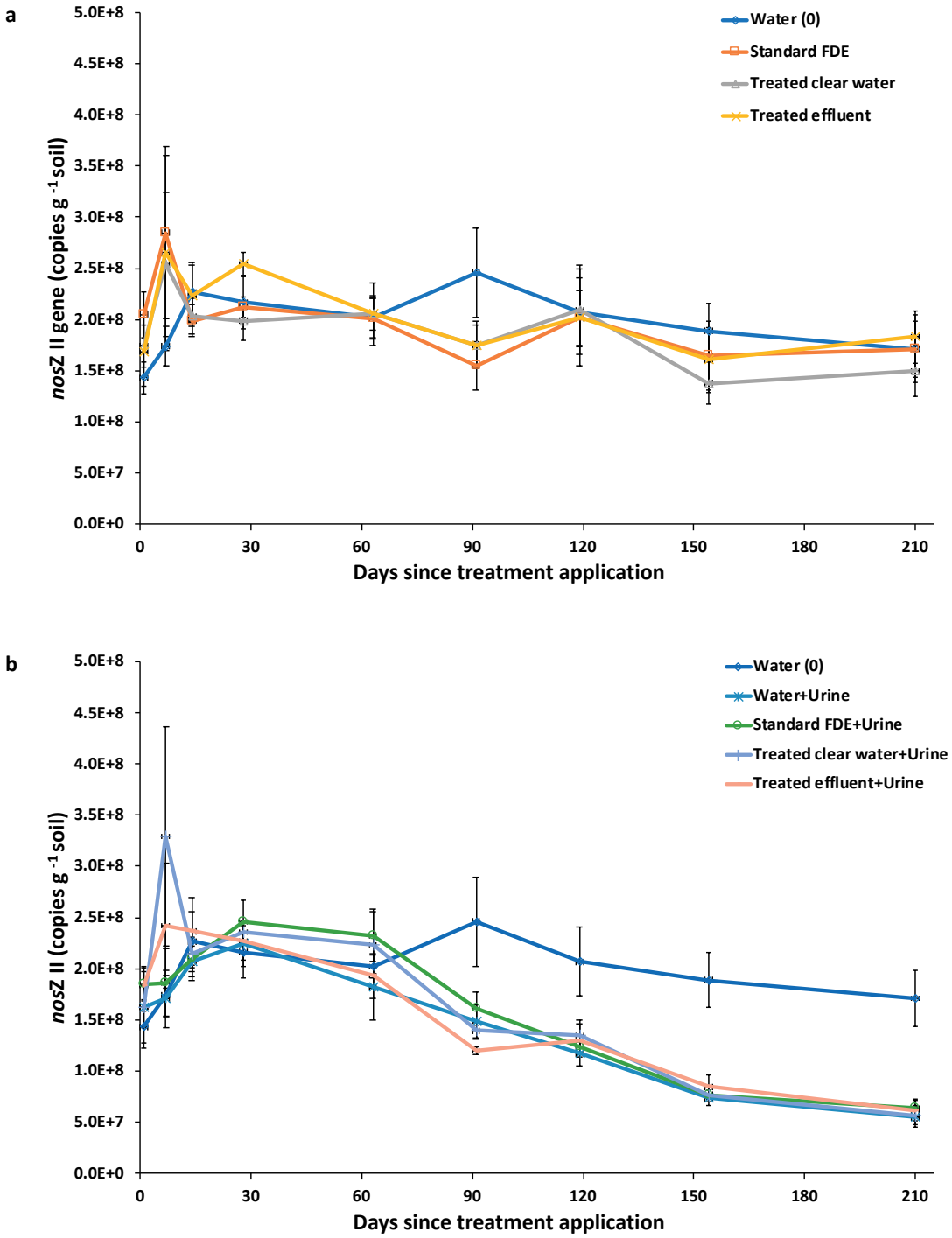

Fig. 4.12 *nosZ* II gene abundance in the soil. (a): Effluent treatments; (b): Effluents plus urine treatments. The error bars represent the standard error of the mean (n=4).

4.4 Discussion

4.4.1 N_2O emissions

FDE and animal urine are major sources of the greenhouse gas N_2O in New Zealand. N_2O accounts for about 20.6% of the agricultural greenhouse gases in New Zealand (Ministry for the Environment, 2017). Therefore, it is important to ensure the effluent treatment technology aimed at recycling water, increasing the storage pond capacity and minimising microbial contamination of waterways do not inadvertently increase N_2O emissions. Results from this incubation study showed that despite the differences in composition of the treated effluent and treated clear water compared with the standard FDE, the N_2O emissions from the three different effluents were similar. This would indicate that the effluent treatment technology that has been developed to recycle the water would not lead to increased N_2O emissions when the treated effluent and treated clear water are applied to the soil. The N_2O emission factors from the three types of effluents, ranging from 0.03% to 0.10%, were lower than the standard 0.25% emission factor used for the 2017 National Greenhouse Gas Inventory Report (Ministry for the Environment, 2017).

The emission factors of the animal urine-N applied together with the different animal effluents, ranging from 0.8% to 1.2% were similar to the 1% EF for animal urine used in the New Zealand greenhouse gas inventory calculations (Ministry for the Environment, 2017). The significantly higher emission factors of the urine-N when co-applied with the different effluents compared with the urine plus water treatment were probably because of an interaction between the organic C in the effluent and urine-N in the soil. It is known that organic C can enhance denitrification thus leading to increased N_2O emissions (de Klein et al., 2001; Di and Cameron, 2003; Cameron et al., 2013). However, it is important to note that the emission factor of treated clear water or treated effluent co-applied with urine-N were the same as that in the standard FDE plus animal urine treatment. The lack of a significant difference in N_2O emissions among the three types of effluents, with or without urine, was probably because the difference in effluent properties between the effluent types was not large enough to lead to different N_2O emissions. More research is needed to verify these results under field conditions. The initial pulse of N_2O emissions straight after effluent and urine application was probably because of a priming effect following the application of the effluents (Fig. 4.2 a and b).

4.4.2 Mineral nitrogen dynamics

Although the NH_4^+-N concentrations declined with time following the application of the different effluents, with or without urine, as a result of nitrification, the nitrification rates were similar in the different effluent treatments (Fig. 4.4). Similarly, the NO_3^--N concentrations in the different effluent treatments also increased in a similar pattern among the effluent only treatments, or among the effluent plus urine treatments (Fig. 4.5). This would indicate that the different effluents produced

similar nitrification rates in the soil. Therefore, the treated clear water and treated effluent from the new effluent treatment technology would result in similar nitrification rates as that from the untreated original effluent when applied to soil.

4.4.3 Soil pH

During the process of nitrification, hydrogen ions (H^+) are released (Equation 2.1 and Equation 2.2 in Chapter 2), therefore, the soil pH decreased following the application of the effluents or animal urine (Fig. 4.6). The pH decline was particularly significant where animal urine was applied because of the high rates of NH_4^+ applied (Fig. 4.6b). Most of the N in animal urine is urea, which upon hydrolysis in the soil releases NH_4^+, leading to increased nitrification accompanied by a pH decrease.

During the first 7 days of the incubation, the soil pH did not decrease significantly but remained around 7.6 following the urine application (Fig. 4.6b). This was because urea hydrolysis was still occurring and the H^+ produced by nitrification was neutralized by the OH^- produced by the urea hydrolysis (Fig. 4.6b). Further nitrification thereafter decreased the pH to between 4.7 and 4.2 after 60 days of the incubation (Fig. 4.6b). However, it is important to note that the pH decreased at the same rate among the different effluent treatments, and among the different effluent plus urine treatments, again, indicating similar nitrification rates among the effluent only treatments, or effluent plus urine treatments.

4.4.4 Functional gene abundance

It has been reported that the growth and activity of AOB and AOA may vary depending on soil and environmental conditions (Dai et al., 2013; Di et al., 2014; Robinson et al., 2014; Di and Cameron, 2017). The AOB *amoA* gene abundance in the different effluent only treatments (including standard FDE, treated clear water and treated effluent) showed a similar trend among the different effluent treatments. This demonstrated that the effluents treated by the new effluent treatment technology had a similar effect on AOB growth in the soil compared with the standard FDE. The NH_4^+ contained in the different effluents therefore stimulated AOB growth, increasing the AOB abundance above those in the control (P<0.05). In contrast, the AOA population abundance did not change significantly until the end of the incubation study in the effluent only treatments. In fact, the AOA abundance decreased in the urine treatments, showing some inhibition effect on AOA abundance by the high rates of NH_4^+ in the soil. These results support those of Di et al. (2009); Di et al. (2010); Wang et al. (2011); Di et al. (2014) who reported that the abundance and activity of AOB increased in response to the addition of NH_4^+, whereas AOA did not grow or declined following high rates of NH_4^+ application. The similar changing patterns of AOB or AOA among the respective effluent only or effluent plus urine treatments, again, demonstrated the similar effect of the three effluents on AOB or AOA populations.

Generally speaking, the application of the three different effluents alone or when applied together with animal urine did not lead to different changing patterns of the denitrifier abundance in the soil among the effluent or effluent plus urine treatments. This would indicate that despite the different compositions of the three types of effluent, the application of the treated effluent or clear water is unlikely to lead to different denitrifier growth compared with the application of the original effluent. Although the total organic C content of the three different effluents were different (Table 3.3), the amounts of soluble organic C were similar, and it is the soluble organic C that is readily available to stimulate microbial activity. This might partly explain the similar denitrifier responses to the application of the different effluents (Miller et al., 2009; Cameron et al., 2013). It was indicated by Paul and Beauchamp (1989) that denitrification was highly correlated with the concentration of water-soluble C in manure and suggested that the water-soluble C might be consumed as a primary source of C by denitrifiers.

However, the denitrifier abundance decreased in the effluent plus urine treatments compared with the Control at the later stages of the incubation (Fig. 4.9, Fig. 4.10, Fig. 4.11 and Fig. 4.12). Soil pH may have played a part in decreasing the denitrifier population abundance in the urine treatments. Optimum soil pH for denitrifying organisms was reported to be between 7 and 8 (Sherlock et al., 1992). In acid soils, the denitrification rate was low (Šlmek and Cooper, 2002). Therefore, as soil pH decreased to below 5.0 after 60 days of incubation and to around 4.4 after 90 days of incubation, this may have limited denitrifier growth and activity in the urine treatments. It has previously been reported that the copy numbers of denitrifiers in acidic soils were significantly lower than those of soil in neutral pH (Čuhel et al., 2010). Denitrification rate has been reported to be slow in acid soils (Fageria and Baligar, 2008), particularly with pH less than 5 (McLaren and Cameron, 1996). Therefore, soil pH may have been an important factor in causing the denitrifier abundance in the urine treatments to decrease below that in the Control.

4.5 Conclusions

The application of the standard FDE, treated clear water and treated effluent did not lead to different N_2O emissions, soil pH, nitrification rate dynamics or different abundance of AOB, AOA and denitrifying functional genes in the soil. This would indicate that the effluents produced by the new effluent treatment technology would have similar N_2O emissions as the standard FDE when applied to the soil, and would have a similar effect on nitrification rate dynamics and the abundance of AOB, AOA and denitrifying populations.

The **hypothesis** that "the treated effluent and treated clear water would have different N_2O emission factors compared with the untreated standard FDE when applied to soil" was therefore rejected. The N_2O emission factors were similar among these three types of effluents. In this research, the emission

factors from the three types of effluents (0.03% to 0.1%) were similar to those reported by van der Weerden et al. (2016a) and lower than the emission factor of FDE (0.25%) used in the New Zealand greenhouse gas inventory calculations (Ministry for the Environment (2017).

When urine was co-applied with standard FDE, treated clear water and treated effluent, the N_2O emissions, soil pH, nitrification rate dynamics and the abundance of AOB, AOA and denitrifying functional genes were also similar among the three effluent types. Therefore, the **hypothesis** that "there would be different interactive effects between the treated effluents and standard FDE and animal urine when they were co-applied to soil" was also rejected. The emission factor of the urine applied ranged from 0.8% to 1.2%, similar to the 1% EF for animal urine used in the New Zealand greenhouse gas inventory calculations (Ministry for the Environment, 2017).

Chapter 5

General conclusions and recommendations for future research

5.1 General conclusions

In New Zealand, 94.8% of the total nitrous oxide (N_2O) emissions came from agriculture (Ministry for the Environment, 2017). Farm dairy effluent (FDE) is the most common form of manure collection and application to New Zealand pastoral soils and N_2O emissions from FDE are the third largest source of N_2O emissions from agriculture. Recently, new FDE treatment technologies have been developed to recycle water, increase the storage pond capacity of the effluent pond on a dairy farm, and reduce contamination of water ways. Thus, it is important to find out if the effluent treated by the latest treatment technology would increase N_2O emissions compared with the standard FDE. In this study, the effects of treated clear water and treated effluent were compared with standard FDE, in terms of the N_2O emissions, mineral N dynamics, soil pH and the abundance of AOB *amoA* gene, AOA *amoA* gene and denitrifying functional genes (*nirS*, *nirK* and *nosZ*).

5.1.1 N_2O emissions

Results showed that the N_2O emissions from soil receiving treated clear water and treated effluent were not significantly different from soil receiving an application of standard FDE. This indicated that the application of treated clear water and treated effluent produced by the new treatment technology would not increase N_2O emissions from soil compared with the application of the original standard FDE. Therefore, the **hypothesis** that "the treated effluent and treated clear water would have different N_2O emission factors compared with the untreated standard FDE when applied to soil" was rejected. The emission factors of the three types of effluents were similar (from 0.03% to 0.10%) and were lower than the 0.25% EF value used for the 2015 National Greenhouse Gas Inventory Report (Ministry for the Environment, 2017).

The co-application of urine with the treated clear water and treated effluent also led to similar N_2O emissions compared with the application of standard FDE plus urine. This, again, demonstrated that the treated clear water and treated effluent produced by the latest technology had similar interactive effects to standard FDE when co-applied with urine. Patches of animal urine are deposited on the grazed pasture soil during grazing. The results from the experiment comparing the different types of effluents co-applied with animal urine illustrated that the treated clear water and treated effluent produced by the new treatment technology did not increase the N_2O emissions even on the urine patches. Thus, the **hypothesis** that "there would be different interactive effects between the treated effluents (treated clear water and treated effluent) and standard FDE and animal urine when they are

co-applied to soil" is rejected. Additionally, the emission factors of urine-N applied with the different animal effluents (ranging from 0.8% to 1.2%) were similar to the 1% EF used for animal urine-N in the New Zealand Greenhouse Gas Inventory calculations (Ministry of Environment, 2017).

5.1.2 Mineral nitrogen

There was no difference in the ammonium-N (NH_4^+-N) concentration and the nitrate-N (NO_3^--N) concentrations in the soil following the application of the standard FDE, the treated clear water and the treated effluent. This indicated that nitrification rate and denitrification rate following the application of the three types of effluents were similar. Additionally, the concentrations of NH_4^+-N and NO_3^--N were also similar among the effluent plus animal urine treatments during the experiment period. This illustrated that there were similar interactive effects among the three different effluents with animal urine on N dynamics in the soils.

5.1.3 Functional gene abundance

Despite the differences in composition between the treated clear water, treated effluent, and the standard FDE, the abundance of the functional genes (including AOB *amoA* gene, AOA *amoA* gene and denitrifiers) varied in a similar pattern among the different effluent treated soils. This would indicate that when the effluents from the new effluent treatment technology are applied on land, they would not have different effects on the functional gene abundance compared with the application of the standard FDE. This also supported the results of the similar N_2O emissions and N dynamics following the application of the three different effluents.

When animal urine was applied with the standard FDE, treated clear water and treated effluent to the soil, the abundance of the functional genes also varied in a similar pattern among the different effluent plus urine treatments. Therefore, there was no significance difference in the AOB *amoA* gene, AOA *amoA* gene and denitrifier abundance in the effluent plus urine treatments. This demonstrated that when the treated clear water and treated effluent produced from the new treatment technology are applied to the soil together with animal urine, they would have a similar effect on AOB, AOA and denitrifiers as that caused by the application of the standard FDE. This would again reject the **hypothesis** that "there would be different interactive effects between the treated effluents (treated clear water and treated effluent) and standard FDE and animal urine when they are co-applied to soil".

In conclusion, the latest effluent treatment technology aimed at recycling water, increasing the effluent storage pond capacity on a dairy farm and reducing the risk of contamination of waterways would not increase the N_2O emissions nor change the N dynamics, soil pH and the abundance of AOB *amoA* gene, AOA *amoA* gene and denitrifiers in grazed pasture soil with, or without, urine application.

5.2 Future research

The research reported in this thesis was conducted under controlled conditions in the laboratory. It is not clear if these results can be directly transferred to field conditions. Therefore, there is a need to conduct similar research under field conditions.

In addition, this is a short-term study and the effluents were only applied to soil once. Therefore, there is a need to conduct long-term studies in the field where the effluents are applied repeatedly over multiple years.

Thirdly, there is also a need to study potential long-term effects of the different effluents on other microbial communities that were not included in this project.

Finally, there is also a need to conduct similar research on other soil types.

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