Effects of cow urine and its constituents on soil microbial populations and nitrous oxide emissions

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A thesis
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by

Janet Bertram

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In all things of nature there is something of the marvellous.

Aristotle 350 BCE
from ‘On the Parts of Animals’

Human history becomes more and more a race between education and catastrophe.

H. G. Wells 1920 CE
from ‘The Outline of History’

What is, is.

Lennier 2260 CE
on Babylon 5

Abstract of a thesis submitted in partial fulfilment of the
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EFFECTS OF COW URINE AND ITS CONSTITUENTS ON SOIL MICROBIAL POPULATIONS AND NITROUS OXIDE EMISSIONS

by Janet Bertram

New Zealand's 5.3 million strong dairy herd returns approximately 106 million litres of urine to pasture soils daily. The urea in that urine is rapidly hydrolysed to ammonium (NH_4^+), which is then nitrified, with denitrification of nitrate (NO_3^-) ensuing. Nitrous oxide (N_2O), a potent greenhouse gas (GHG), is produced via nitrification and denitrification, which are enzyme-catalysed processes mediated by soil microbes. Thus microbes are linked intrinsically to urine patch chemistry. However, few previous studies have investigated microbial dynamics in urine patches. Therefore the objective of these four experiments was to investigate the effects on soil microbial communities of cow urine deposition. Methods used included phospholipid fatty acid (PLFA) analyses of microbial community structure and microbial stress, dehydrogenase activity (DHA) assays measuring microbial activity, and headspace gas sampling of N_2O, ammonia (NH_3) and carbon dioxide (CO_2) fluxes.

Experiment 1, a laboratory study, examined the influence of soil moisture and urinary salt content on the microbial community. Both urine application and high soil moisture increased microbial stress, as evidenced by significant changes in PLFA trans/cis and iso/anteiso ratios. Total PLFAs and DHA showed a short-term (< 1 week) stimulatory effect on microbes after urine application. Mean cumulative N_2O-N fluxes were 2.75% and 0.05% of the nitrogen (N) applied, from the wet (70% WFPS) and dry (35% WFPS) soils, respectively.

Experiment 2, a field trial, investigated nutrient dynamics and microbial stress with plants present. Concentrations of the micronutrients, copper, iron and molybdenum, increased up to 20-fold after urine application, while soil phosphorus (P) concentrations decreased from 0.87 mg kg^{-1} to 0.48 mg kg^{-1}. Plant P was also lower in urine patches, but total PLFAs were higher, suggesting that microbes had utilised the available nutrients. Microbial stress again resulted from urine application but, in contrast to experiment 1, the fungal biomass recovered after its initial inhibition.

Studies published during the course of this thesis reported that hippuric acid (HA) and its hydrolysis product benzoic acid (BA) significantly reduced N_2O-N emissions from synthetic cow urine, thus experiment 3 investigated this effect using real cow urine. Cumulative N_2O-N fluxes were 16.8, 5.9 and 4.7% of N applied for urine (U) alone, U+HA and U+BA, respectively. Since NH_3-N volatilisation remained unchanged, net gaseous N emissions were reduced. Trends in total PLFAs and microbial stress were comparable to experiment 1 results.

Experiment 4 studied HA effects at different temperatures and found no inhibition of N_2O-N fluxes from HA-amended urine. However, mean cumulative N_2O-N fluxes were reduced from 7.6% of N applied at 15–20°C to 0.2% at 5–10°C. Total cumulative N emissions (N_2O-N + NH_3-N) were highest at 20°C (17.5% of N applied) and lowest at 10°C (9.8% of N applied). Microbial activity, measured as potential DHA, increased with increasing temperature.

This work has clearly shown that the stimulation and inhibition of the soil microbial community by urine application are closely linked to soil chemistry and have significant impacts not only on soil nutrient dynamics but also on N_2O-N emissions and their possible mitigation.

Keywords: nitrous oxide, ruminant urine, urine patch, nitrogen dynamics, hippuric acid, benzoic acid, phospholipid fatty acid analysis, soil microbial community, microbial stress, dehydrogenase activity

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Acronyms and abbreviations

AOB	Ammonia-oxidising bacteria
BA	Benzoic acid
BAME	Bacterial acid methyl ester
CEC	Cation exchange capacity
CFE	Chloroform fumigation extraction
CO_2	Carbon dioxide
cy/ω7 ratio	Ratio of cyclopropyl 17:0 (cy 17:0) to 16:1ω7 PLFAs
DHA	Dehydrogenase activity
DI	Deionised
DOC	Dissolved organic carbon
DRP	Dissolved reactive phosphorus
EC	Electrical conductivity
FA	Fatty acid
F/B ratio	Ratio of fungal to bacterial PLFA biomarkers
FAME	Fatty acid methyl ester
G-	Gram-negative bacteria
G+	Gram-positive bacteria
G+/G- ratio	Ratio of Gram-positive to Gram-negative PLFA biomarkers
GC	Gas chromatograph
GHG	Greenhouse gas
HA	Hippuric acid
i/a ratio	Ratio of iso-15:0 to anteiso-15:0 PLFAs
ICP-OES	Inductively coupled plasma optical emission spectrometer
ID	Internal diameter
IRMS	Isotope ratio mass spectrometer
MBC	Microbial biomass carbon
N_2O	Nitrous oxide
NH_3	Ammonia
NH_4^+	Ammonium
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite-oxidising bacteria
PCA	Principal component analysis
PLFA	Phospholipid fatty acid
PO_4^{3-}	Phosphate
qCO_2	Metabolic quotient for CO_2
SOM	Soil organic matter
SO_4^{2-}	Sulphate
t/c ratio	Ratio of 18:1ω9trans to 18:1ω9cis PLFAs
TOC	Total organic carbon
TPF	Triphenyl formazan
TTC	Triphenyl tetrazolium chloride
WFPS	Water-filled pore space
WSC	Water-soluble carbon

Chapter 1
Introduction

Numerous studies have investigated the effects of ruminant urine deposition on pasture soils, but the focus has traditionally been on soil chemistry and gaseous emissions, with little research carried out on the impact of urine on the soil microbial community. Soil microbes are an intrinsic part of the pasture soil system and are responsible for transforming and/or utilising many of the nutrients added in and released by the urine. However, a review of the literature (Chapter 2) revealed a dearth of information about the significance of changes in soil chemistry following urine deposition with respect to soil microbes.

Therefore this study was initially instigated to investigate the broad research area of cow urine effects on soil chemistry and microbiology, and the relationships between these two interconnected components. This was the underlying rationale behind experiments 1 and 2, which were laboratory-based and a field trial, respectively. Consequently these two studies involved numerous analyses of a range of soil parameters encompassing chemistry, microbiology and gaseous emissions. The publication of two papers after the completion of experiment 2 suggested a new study focus, while still coming within the scope of the original research proposal. These papers reported research performed to examine the effects of the natural urinary constituent hippuric acid on nitrous oxide emissions from synthetic cow urine. The results of these experiments indicated that hippuric acid affected microbial function in the urine patch. Following on from the studies documented in these papers, experiment 3 was conceived to examine the effect of hippuric acid and its hydrolysis product, benzoic acid, on nitrous oxide emissions, using real cow urine. Subsequently, experiment 4 was planned to study the inhibitory effect of hippuric acid on nitrous oxide emissions at different temperatures.

Thus the main objectives of the study were to:

1) Investigate soil microbial dynamics by assessing the biomass, activity, and community structure of the microbial population before and after urine application,

2) Determine the effects that urine addition to soil has on the nature and dynamics of existing soil nutrients, and the short-term fate of those nutrients released into soil solution following urine application,

3) Quantify soil gaseous emissions (nitrous oxide, carbon dioxide and ammonia) as a consequence of urine addition,

4) Identify and characterise the relationships between the measured microbial population dynamics (objective 1) and the nutrient changes and fluxes determined in objectives 2 and 3, and

5) Determine the impact of specific urinary compounds on the effects of urine, particularly nitrogen dynamics.

Following this introduction, Chapter 2 reviews the current literature on the chemistry and microbiology of the urine patch, including some of the methods used in this study and previously. Chapter 3 fully details the methods used during the four experiments described and discussed in the following four chapters. Chapter 4 covers an introductory laboratory experiment, which investigated the influence of soil moisture and urinary salt content on the soil microbial community, with a focus on microbial stress. The field trial, detailed in Chapter 5, examined the effects of plants on nutrient dynamics and on the microbial community by comparison with the previous experiment in which plants were absent. Chapter 6 covers the main laboratory experiment examining the effects of hippuric acid and benzoic acid on soil microbial dynamics with a particular focus on nitrogen transformations and nitrous oxide emissions. Chapter 7 details a further laboratory experiment, which examined the effects of hippuric acid inhibition of soil processes across a range of temperatures experienced under field conditions. Finally, Chapter 8 synthesises the discussions of the previous experimental chapters, and presents conclusions and future research recommendations.

Chapter 2
Literature Review

2.1 Introduction

Grasslands have been defined as “terrestrial ecosystems dominated by herbaceous and shrub vegetation and maintained by fire, grazing, drought and/or freezing temperatures” (White et al., 2000). These ecosystems cover up to 50% of the global land surface area (excluding Antarctica and Greenland), and include the 20% of the land surface area that is covered by managed grasslands (pastures) (Snaydon, 1981; White et al., 2000).

Grazing animals are an important component of natural grassland ecosystems, and contribute to nutrient cycling through the deposition of dung and urine (Snaydon, 1981). However, because pastures used for agriculture are often stocked at higher densities than natural systems, the impact of stock is more pronounced (White et al., 2000).

Pastoral farming is a mainstay of New Zealand’s economy, with 7.75 million hectares of pasture land supporting sheep, beef, dairy and deer farming in 2007 (Statistics New Zealand, 2008a). Of this, the 1.7 million hectares of pasture in dairying (Statistics New Zealand, 2008a) supported 5.3 million dairy cattle (Statistics New Zealand, 2008b). With each animal producing approximately 20 L of urine per day (Haynes and Williams, 1993), New Zealand’s dairy herd deposits approximately 106 million L of urine onto pasture every day.

Urine contains compounds that alter the chemical and physical properties of the soil onto which it is deposited, and consequently urine deposition affects soil microbes. Changes in the soil microbial community alter nutrient cycling, including the production of greenhouse gases (GHGs), and can thus influence processes on a global scale.

Greenhouse gases are atmospheric trace gases that can absorb infra-red radiation reflected from the Earth’s surface, thus preventing entrained heat from escaping the atmosphere (Wang et al., 1976). The mean global surface temperature increased by 0.74°C ± 0.18°C between 1905 and 2005 (Trenberth et al., 2007), and the eight warmest years on record occurred in the decade to 2007 (Goddard Institute for Space Studies, 2008), showing the influence of anthropogenic GHG emissions.

The GHG nitrous oxide (N_2O) has a global warming potential (GWP) 298 times that of carbon dioxide (CO_2) over a 100 year time horizon, and its atmospheric concentration is increasing

linearly (0.26% yr^{-1}), rising from 270 ppb in 1750 to 319 ppb in 2005 (Forster et al., 2007). Globally, over 40% of anthropogenic N_2O emissions are agriculturally derived (Denman et al., 2007), and the urine deposited by grazing ruminant animals is a major source of the N_2O emitted from agricultural soils worldwide (Oenema et al., 2005).

In New Zealand 48.5% of the GHG inventory is attributable to agriculture, with N_2O from agricultural soils accounting for approximately one-third of the total (Ministry for the Environment New Zealand, 2007). As a signatory to the Kyoto Protocol, New Zealand has undertaken to reduce anthropogenic GHG emissions for the period of 2008–2012 (Rogner et al., 2007), or to otherwise take responsibility for its emissions through mechanisms such as carbon trading (UNFCCC, 2008). Thus, in addition to potential environmental effects, urine patch chemistry and microbiology could have a major impact on the costs incurred by New Zealand due to its Kyoto Protocol obligations.

2.2 Cow urine

2.2.1 Urine patches

Cattle urinate 8–12 times daily, producing between 1.6 and 2.2 L each time (Haynes and Williams, 1993). The area covered by each urine patch ranges from 0.16 to 0.49 m^2 (Haynes and Williams, 1993). However, the area of affected pasture averages 0.68 m^2 because of the diffusion of urine through the soil and because the roots of plants outside the patch may extend into the affected area (Lantinga et al., 1987). Urine patch size is influenced by a number of factors including wind, slope (During and McNaught, 1961) and the extent of soil compaction (Williams et al., 1990b). Tracer studies have indicated that preferential flow via soil macropores may allow urine to penetrate to a depth of 400 mm, but the majority (ca. 85%) of urine remains in the top 100 mm of the soil column (Williams and Haynes, 1994).

Urine patches are unevenly distributed, with only 4–20% of a pasture receiving urine each year (Snaydon, 1981). Most of the urine is deposited in areas where stock congregate (stock camps) (Hilder, 1966), often near shelter-belts, around water troughs, and on hill farm ridges (Gillingham and During, 1973; Haynes and Williams, 1993).

2.2.2 Chemical nature of cow urine

Cow urine is an aqueous solution of nitrogenous and sulphurous compounds, minerals, and other minor components (Church, 1976). Concentrations of carbon (C) in cow urine are low (Lovell and Jarvis, 1996), and measurements of 3.1–20.4 g C L^{-1} (mean = 9.9) have been made (Roger Cresswell, pers. comm., 2009). The pH of ruminant urine is usually between 8.4 and 8.6

(e.g. Sherlock and Goh, 1984; Haynes and Williams, 1992; Somda et al., 1997), but may be as low as 7.2 (Doak, 1952).

2.2.2.1 Nitrogen

Urination is the main pathway of nitrogen (N) excretion for grazing animals, and the N content of urine ranges from 2–20 g N L^{-1} (e.g. Doak, 1952; Betteridge et al., 1986; Bristow et al., 1992). Up to 40–53% of the dietary-N intake of cattle can be excreted in the urine (Hutton et al., 1967; Betteridge et al., 1986), with urine-N increasing as the N content of the diet increases (Kebreab et al., 2001).

The annual rate of urine-N returned to pasture is a function of many variables including stocking rate, N intake, and water consumption (Barrow, 1987; Haynes and Williams, 1993). Estimates of ca. 200 kg N ha^{-1} yr^{-1} have been made (Richards and Wolton, 1976; Haynes and Williams, 1993). Under the urine patch the N loading may reach 1000 kg N ha^{-1} (Jarvis and Pain, 1990; Haynes and Williams, 1993).

Of the total N excreted in cow urine, 50–93% is in the form of urea, with a range of minor constituents also present (Table 2.1) (Doak, 1952; Bristow et al., 1992).

Table 2.1 Nitrogenous constituents of bovine urine expressed as percentages of total N. Range of percentage values for 10 animals (Bristow et al., 1992) or for 4 animals (Doak, 1952).

Urine constituent	Bristow et al. (1992)	Doak (1952)
	% of total N (range)	% of total N (range)
Urea	60.6 – 93.5	50.3 – 74.2
Hippuric acid	3.4 – 8.0	1.9 – 6.0
Allantoin	2.2 – 11.8	4.0 – 6.4
Uric acid	0.6 – 1.9	ND[a]
Xanthine	0.29 – 0.69	ND[a]
Creatinine	1.75 – 5.48	1.3 – 2.0[b]
Creatine	1.33 – 4.14	
Amino acid	0.28 – 3.67	ND[a]
Ammonia	0.27 – 9.08	0.3 – 0.6

[a] ND = not determined

[b] not determined separately

Hippuric acid

The hippuric acid (HA) content of ruminant urine is a function of dietary intake. For example, cows fed a protein-free diet produced urine significantly lower in HA (mean < 0.6 g HA L^{-1}) than cows on either low protein or normal diets (means 6.7 and 11.9 g HA L^{-1} respectively) (Kreula et al., 1978), and HA concentrations in the urine of starved sheep decreased by 97% over a four day period (Martin, 1969). Pasture maturity has also been shown to affect the HA content of ruminant urine (Martin, 1970).

Hippuric acid is a conjugate of benzoic acid (BA) and glycine (Chesson et al., 1999). Benzoic acid is a known antimicrobial agent (Marwan and Nagel, 1986) and denitrification inhibitor (Her and Huang, 1995), although it can be metabolised by some soil microbes (Philippe et al., 2001; Pumphrey and Madsen, 2008). The presence of HA in urine may further increase the soil pH and enhance urea hydrolysis and ammonia (NH_3) volatilisation (Doak, 1952; Whitehead et al., 1989) (Sections 2.5.1.1 & 2.5.1.2). A reduction in cumulative N_2O emissions of over 50% was measured when concentrations of HA were increased from 3% of total N to 9% in synthetic urine (Kool et al., 2006b). Speculation that BA caused the reduction of N_2O emissions in this study was supported by the findings of a further experiment, in which BA had a similar effect on N_2O emissions and denitrification as HA (van Groenigen et al., 2006) .

2.2.2.2 Other urine chemical constituents

Urine is the primary excretion route of both sulphur (S) and potassium (K^+) in ruminants. The amount of S excreted in the urine increases as the dietary S intake increases (Barrow and Lambourne, 1962; Bird, 1971, 1972; Kennedy, 1974), and concentrations between 350 and 810 mg S L^{-1} have been measured (Ledgard et al., 1982; Haynes and Williams, 1991; Kear and Watkinson, 2003). Urinary-S is a mixture of organic and inorganic forms, with the proportion of inorganic sulphate (SO_4^{2-}) increasing as the amount of S in the diet increases (Kennedy, 1974). Kear and Watkinson (2003) found that 33% (205 mg S L^{-1}) of urinary-S in fresh dairy cow urine was in the form of SO_4^{2-}.

Concentrations of K^+ in cattle urine are also affected by dietary intake and have been measured at 2.9–10.1 g K^+ L^{-1} (Richards and Wolton, 1976; Betteridge et al., 1986). Potassium amounts to 60–70% of the total urinary cation content (Haynes and Williams, 1993), and the proportion of K^+ expelled in urine is 33–81% of the total consumed (Hutton et al., 1967; Safley et al., 1984; Betteridge et al., 1986).

Concentrations of magnesium (Mg^{2+}) and calcium (Ca^{2+}) in urine are usually very low (< 1 g L^{-1}) (Haynes and Williams, 1993), and only 12–22% of the Mg^{2+} and ca. 3% of the Ca^{2+}

consumed are excreted in dairy cow urine (Hutton et al., 1965, 1967; Safley et al., 1984). The majority of the sodium (Na^+) consumed by ruminants (56–59%) is excreted in the urine (Hutton et al., 1967; Safley et al., 1984), although actual concentrations are comparatively low (e.g. 1.24 g Na^+ L^{-1} (Richards and Wolton, 1976)).

The major anions in urine are chloride (Cl^-) (20–50%) and bicarbonate (HCO_3^-) (40–70%) (Haynes and Williams, 1993). Urine is also the principal means of excretion of boron (B^-) (Owen, 1944; Green and Weeth, 1977) and iodide (I^-) (Miller et al., 1965; Barry, 1983).

In ruminants, phosphorus (P) is predominantly excreted in the dung, and urine generally contains much less than 0.1 g P L^{-1} (Betteridge et al., 1986; Wu et al., 2000; Knowlton and Herbein, 2002). Trace elements from the diet are not absorbed by the digestive tract so are expelled primarily in the dung; these include copper (Cu), zinc (Zn), manganese (Mn), iron (Fe), (Safley et al., 1984; Underwood and Suttle, 1999), cadmium (Cd) (Smith, 1984) and lead (Pb) (Reichlmayr-Lais and Kirchgessner, 1984). Excretion of molybdenum (Mo) via the urine increases when the diet is high in S (Barrow, 1987).

The lack of P and trace elements in cow urine may be limiting factors to microbial growth and nutrient cycling in urine patches, which could lead to increased competition for resources between microbes and plants. However, there appears to be a lack of work examining interactions between nutrient dynamics and microbial communities in urine patches.

2.3 Urine effects on pasture plants and soil chemistry

2.3.1 Urine and pasture plants

Pasture growth is usually enhanced by urine deposition, probably due to the addition of N and/or K^+ (During and McNaught, 1961; Ledgard et al., 1982; Haynes and Williams, 1993). The enhanced response is dominated by the grass component of the pasture, and clover growth and N fixation may both be reduced as a result of urine application (Ledgard et al., 1982; Fraser et al., 1994).

Several studies have shown rapid N uptake by plants, with up to 12% of the urine-N detected in plant material after 29 days (Williams and Haynes, 1994) and 43% after 1 year (Fraser et al., 1994). Plants in the urine patch contained more N and less Mg^{2+} and Ca^{2+} than plants outside the urine patch (Williams and Haynes, 1994). The P content of pasture (usually 0.21–0.63% dry weight) may increase after urine or urea application, where existing soil solution P is low (Reid et al., 1966; Williams et al., 1999a; Shand et al., 2002; Soder and Stout, 2003).

Urine deposition may also cause scorching of pasture (Richards and Wolton, 1975; Williams et al., 1999a), which has been attributed to root damage caused by high NH_3 concentrations (Richards and Wolton, 1975; Ritchey et al., 2003), elevated plant N content (Richards and Wolton, 1975), and high urine N concentration (Lantinga et al., 1987).

2.3.2 Effects of urine on soil chemistry

2.3.2.1 Soil moisture and pH

Urine deposition increases the soil moisture content (e.g. Bol et al., 2004), with application rates estimated at 5 L m^{-2} in a bovine urine patch (Williams and Haynes, 1994). Urine addition may also reduce moisture evaporation from the soil (Shand et al., 2000).

Following urine deposition, the pH in the top 20–40 mm of soil generally increases by 1–3 pH units (e.g. Doak, 1952; Sherlock and Goh, 1984; Haynes and Williams, 1992; Shand et al., 2002). This occurs because most of the urinary urea is retained in the top soil layers, where it undergoes hydrolysis, thus creating basic conditions (Section 2.5.1.1). The soil pH usually returns to its original value after 2–3 months, although it may remain elevated for longer if NH_3 volatilisation and nitrification are retarded by low soil temperatures and/or very low soil moisture conditions (Holland and During, 1977; Sherlock and Goh, 1984; Somda et al., 1997).

2.3.2.2 Nitrogen availability

Following urine deposition the mineral N concentration in the soil may increase to over 400 mg N kg^{-1} (Haynes and Williams, 1992; Williams and Haynes, 1994). The majority of the N is initially in the form of urea-N (Table 2.1), which is rapidly hydrolysed to ammonium (NH_4^+-N) (Section 2.5.1.1) and which may subsequently be volatilised as NH_3-N (Section 2.5.1.2). The remainder of the NH_4^+-N generated then undergoes nitrification to form nitrate (NO_3^--N) (Section 2.5.1.3), whereupon denitrification can occur (Section 2.5.1.4). The dinitrogen gas (N_2-N) and nitrous oxide (N_2O-N) thus produced may be lost to the atmosphere, while urea and nitrate (NO_3^--N) may move down the soil profile by macropore flow and/or leaching (Haynes and Williams, 1993; Clough et al., 1998b). Both NH_4^+-N and NO_3^--N may be utilised by plants, and isotope studies have found 22–78% of the urea-N in urine to be retained in living plant biomass, while 20–30% remained in the soil organic matter (SOM) (Clough et al., 1998b; Leterme et al., 2003). In studies using sheep urine, an increase in dissolved organic N (DON) concentrations was attributed solely to sources within the soil (Shand et al., 2000).

The fate of urinary-N is somewhat dependent on soil characteristics, with recovery of urine-^{15}N being higher in a sandy loam soil than in a sandy soil (Sørensen and Jensen, 1996). This greater

loss of ^{15}N from the sandy soil corresponded with a higher net rate of urine-N mineralisation and lower N uptake by plants (Sørensen and Jensen, 1996). In another study, urine-^{15}N recovery was in the order: clay (81%) > silty loam (77%) > sandy loam (71%) (Clough et al., 1998b). Therefore added urine-N is likely to be less available to plants or soil microbes in soils containing high proportions of sand.

2.3.2.3 Carbon availability and soil organic matter

The C content of a soil may increase due to the addition of urinary compounds (Section 2.2.2) or the release of C from SOM due to the increased soil pH, and through the release of organic intracellular solutes by soil microorganisms as they try to maintain cell integrity or as microbial cells lyse (Kieft et al., 1987; Monaghan and Barraclough, 1993; Halverson et al., 2000; Shand et al., 2002). Although an increase in the soluble C content of the soil has been measured following urine deposition (Monaghan and Barraclough, 1993; Shand et al., 2000), the continued elevation of dissolved organic C (DOC) measured by Petersen et al. (2004b) indicated that this C may not be readily available to microbes. Studies have shown that 20–50% of the urea-derived C may be accounted for several days after urine application in the top 200 mm of soil, in above-ground plant material, and as emitted CO_2-C (Bol et al., 2004; Petersen et al., 2004a).

The application of sheep urine to a pasture soil created increased concentrations of DOC (as well as dissolved organic N and dissolved organic P) in conjunction with a pH increase, and the dark colour of the soil solution suggested that humic matter had been solubilised (Shand et al., 2000; Shand et al., 2002). However, studies relating nutrient release in urine patches to changes in the soil microbial community are lacking.

2.3.2.4 Soil solution nutrients

Nutrients are added to the soil solution both directly from the urine and indirectly via changes in the soil resulting from urine deposition (e.g. Monaghan and Barraclough, 1993; Shand et al., 2000). The majority of the urine is retained in the top 100 mm of the soil as demonstrated by bromine tracer studies (Williams and Haynes, 1994). The electrical conductivity (EC) in the top 25 mm of urine-affected soil shows the greatest increase, and the ionic strength of the soil solution in this zone may increase to be 6–8 times that of untreated soil (Haynes and Williams, 1992).

The ions detected in the soil solution immediately after urine deposition are consistent with those measured in the urine (Section 2.2.2.2) (Haynes and Williams, 1992). Concentrations of

HCO_3^-, Cl^- and SO_4^{2-} diminish with time, and NO_3^- becomes the dominant anion as nitrification proceeds (Williams and Haynes, 1994). Nitrification also causes a decline in the concentration of NH_4^+, which forms following urea hydrolysis (Williams and Haynes, 1994) (Sections 2.5.1.3 & 2.5.1.1).

Concurrent with the increase in NO_3^- are increases in concentrations of the counter-ions Ca^{2+} and Mg^{2+}, predominantly sourced from soil exchange sites through displacement by urinary K^+, Na^+ and NH_4^+, and by hydrogen ions (H^+) released during nitrification (Haynes and Williams, 1992; Williams and Haynes, 1994; Early et al., 1998). Over time, the Ca^{2+} and Mg^{2+}, as well as some K^+ and Na^+, are redistributed by leaching with NO_3^- through the soil profile (Holland and During, 1977; Haynes and Williams, 1992). However, concentrations of exchangeable K^+ may remain elevated, as it is strongly adsorbed by the soil cation exchange complex (CEC) (Williams et al., 1990a; Haynes and Williams, 1992; Early et al., 1998). In soils with a large proportion of pH-dependent negative charge sites, the capacity of the CEC to retain K^+ is enhanced by the increase in pH following urine deposition (Williams et al., 1988; Haynes and Williams, 1993).

Concentrations of PO_4^{3-}-P in soil solution generally range between 0.1 and 1.0 mg kg^{-1} (Plante, 2007), but may increase with increasing pH, as occurs in a urine patch, although this depends on how the P is bound in the soil (Gahoonia et al., 1992; Hartikainen and Yli-Halla, 1996). Solubilisation of humic material caused by an increased pH has been suggested as the source of higher concentrations of dissolved organic P after sheep urine was applied to pasture soil (Shand et al., 2000; Shand et al., 2002). This could potentially also result in increased concentrations of trace elements and other metals, which are bound in the SOM.

2.4 Soil microorganisms and urine deposition

2.4.1 Soil microbes

One gram of soil may contain over one billion individual microorganisms and up to 4000 species (Torsvik et al., 1990; Torsvik and Øvreås, 2002). The typical soil microbes (bacteria, fungi, protozoa and algae) constitute 90% of the total soil biomass (Liesack et al., 1997). Bacteria and fungi are the most important in terms of abundance, biomass and soil function (Richards, 1994; Wollum, 1999) and are the only groups investigated in this research.

Bacteria are prokaryotes of the domains Archaea and Bacteria (Woese et al., 1990; Alexander, 1999), and are vital to the functioning of the soil system, carrying out primary production, decomposition, nutrient cycling, and humus formation (Dalal, 1998; Alexander, 1999). The

most common soil bacteria are the Gram-positive (G+) and Gram-negative (G-) groups of the domain Bacteria (Alexander, 1999). Gram-positive bacteria have only an inner membrane and are stained by the Gram-staining technique, while G- bacteria have an additional outer membrane that does not stain (Harwood and Russell, 1984).

Fungi are the most abundant soil microbes by mass, and may decompose organic matter, form symbiotic relationships with plants or be pathogenic to plants (Morton, 1999; Thorn and Lynch, 2007).

Microorganisms are an intrinsic part of all soil ecosystems, and soil microbial diversity influences the stability, productivity, and resilience of ecosystems by affecting soil and plant quality (Torsvik and Øvreås, 2002). The heterogeneous nature of soil, even over distances of <1 mm, ensures the uneven distribution of soil microbes (Nannipieri et al., 2003). There may be zones of high microbial activity ('hot spots') where conditions are suitable, but overall the availability of nutrients and energy sources for soil microbes is low (Nannipieri et al., 2003). Since microbes are dependent on water, they are largely restricted to soil aggregates comprising water-retaining clay minerals and SOM (Stotzky, 1997). Variations in temperature and moisture affect the size of the microbial biomass directly, as well as indirectly by affecting substrate availability (Dalal, 1998).

2.4.1.1 Microbial communities in pasture soils

The microbial biomass content of a temperate grassland soil has been estimated at 1–4 t ha^{-1} and 2–5 t ha^{-1} for the bacterial and fungal populations, respectively (Killham, 1994). The microbial biomass in an unimproved grassland soil was found to be higher than in a pasture soil, and this was attributed to the higher SOM content in the grassland soil (Williams et al., 1999a), since soil organic C content is correlated to microbial biomass (Schutter and Dick, 2001; Nannipieri et al., 2003). The lower biomass in the pasture soil was probably due to the fungal biomass being smaller (e.g. Bardgett et al., 1996; Grayston et al., 2001). In contrast, the bacterial biomass is often higher in pasture soils than in natural grassland soils, accounting for the higher microbial respiration rate measured in pasture soils (Grayston et al., 2001), as bacteria have a higher respiration rate than fungi (Anderson and Domsch, 1975).

The use of fertilisers to increase soil nutrient concentrations (Haynes and Williams, 1993) can reduce the microbial biomass, alter microbial community structure, and increase the soil pH (Grayston et al., 2001; Clegg et al., 2003; Grayston et al., 2004). An increased soil pH may

accelerate SOM degradation (van Bergen et al., 1998). However, the soil pH may decrease under long-term pasture, and lime is often used to preclude this (Haynes and Williams, 1993).

2.4.2 Assessment of urine-induced microbial community changes

Few studies have investigated the direct effects of urine on soil microbes. Changes in soil conditions caused by urine deposition (Section 2.3.2) can stimulate or inhibit microbial populations (e.g. Lovell and Jarvis, 1996; Williams et al., 1999a). There are a variety of methods available to assess these changes.

2.4.2.1 Measuring the microbial biomass

Microbial biomass is frequently assessed using chloroform fumigation techniques (Dalal, 1998). Chloroform fumigation incubation (CFI) involves inoculating a fumigated soil with unfumigated soil and measuring the CO_2 emitted over a 10 day incubation period (Jenkinson and Powlson, 1976). This method has the disadvantages of requiring a long incubation time and being unusable in acid soils or soils with recently added substrate (Jenkinson et al., 2004). These problems are overcome by using the chloroform fumigation extraction (CFE) method, in which the C, N, P, or S content of comparable samples of fumigated and unfumigated soil is measured, and the difference is attributed to the microbial biomass lysed by the chloroform (Saggar et al., 1981; Brookes et al., 1982; Brookes et al., 1985; Vance et al., 1987).

Phospholipid fatty acids (PLFAs) and adenosine triphosphate (ATP) are good measures of the living microbial biomass, as they comprise a constant proportion of the biomass (Jenkinson, 1988; Zelles et al., 1992; Contin et al., 2001) and degrade rapidly on cell death (Holm-Hansen and Booth, 1966; Hill et al., 2000). Phospholipid fatty acid analysis (Section 3.3.1) allows the total microbial biomass to be determined, and is well correlated with microbial biomass C (MBC) determined by CFE (Bailey et al., 2002). Furthermore, since the bacterial and fungal biomasses can be measured separately, the fungal/bacterial (F/B) ratio can be calculated (Frostegård and Bååth, 1996), allowing the effects of soil management or soil fertility on the microbial community to be investigated (e.g. Bardgett and McAlister, 1999). The fungal PLFA biomarker is normally strongly correlated to ergosterol, another fungal biomarker (Seitz et al., 1979; Frostegård and Bååth, 1996), although this is not always the case (Frostegård and Bååth, 1996; Högberg, 2006).

It should be noted that while biomass studies may show differences between microbial communities, or changes in a community over time, they do not directly correspond with

phenotypic, genotypic or functional analyses. In addition, the size of the microbial biomass is variable and does not directly correlate with microbial activity (Grayston et al., 2001).

The effects of urine on the microbial biomass

The soil microbial biomass may increase following urine deposition (e.g. Lovell and Jarvis, 1996; Williams et al., 1999a), or remain unchanged (e.g. Williams et al., 2000; Nunan et al., 2006; Rooney et al., 2006). Part of this effect may be due to the increased soil pH, as the highest MBC was measured at pH > 7.0 when analyses were carried out on one soil type varying from pH 3.7–8.3 (Aciego Pietri and Brookes, 2008). When urine addition does enhance the microbial biomass, the effect can differ in terms of magnitude and timing (Williams et al., 1999a). An increase in MBC and microbial biomass N (MBN) seen by Lovell and Jarvis (1996) after urine application appeared to be an effect of soil wetting, as the same increase was observed when only water was added to the soil. Measurements of total PLFAs by Petersen et al. (2004a) showed an increase in the biomass 4 days after urine application (10 or 17 g urea-C m^{-2}), which persisted after 14 days in the high urea treatment but not in the low urea treatment. In another study the application of urine (10 g N L^{-1}) resulted in an immediate increase in total PLFAs, followed by a slight decline and then another increase after three days (Petersen et al., 2004b).

Increases in the soil microbial biomass have been measured in areas visited more frequently by stock, e.g. under intensively grazed pastures and stock camps (Haynes and Williams, 1999; Ingram et al., 2008). Patra et al. (2005) found that the bacterial biomass was higher under an intensively grazed pasture than under a lightly grazed pasture, while the fungal biomass was unchanged.

2.4.2.2 Measuring microbial activity

Microbial activity can be measured by quantifying rates of respiration or enzyme activities (Hill et al., 2000). Microbial respiration is assessed by measuring emissions of CO_2 from the soil surface. This can be measured as basal (natural) respiration, or as substrate-induced respiration (SIR) following glucose addition (Anderson and Domsch, 1978). Measurement of SIR allows the size of the active biomass to be ascertained, by relating it to MBC (Anderson and Domsch, 1978), and using selective inhibitors the relative contributions of bacteria and fungi to the overall respiration rate can be determined (Anderson and Domsch, 1975; Laughlin and Stevens, 2002). The metabolic quotient for CO_2 (qCO_2), measuring CO_2 emitted per unit biomass, is calculated by dividing the basal respiration rate by the microbial biomass (Anderson and Domsch, 1986).

Enzyme assays measure total or specific enzyme activity in the soil. Several commonly-used assays are non-specific and are performed by measuring the rate of degradation of a substrate. These methods include dehydrogenase activity (DHA) (Thalmann, 1968; Alef, 1995), fluorescein diacetate hydrolysis (Schnürer and Rosswall, 1982; Green et al., 2006b) and arginine ammonification (Alef and Kleiner, 1986). Specific assays may also be carried out for particular enzymes or for groups of enzymes responsible for certain processes (e.g. denitrification enzyme activity (DEA) and nitrification enzyme activity (NEA)). Incorporation of a substrate (e.g. thymidine) can also be used as an indicator of microbial community activity (Bååth, 1992).

The effects of urine on microbial activity

Microbial basal CO_2 respiration usually increases following the addition of urine (e.g. Kelliher et al., 2005), and real cow urine has been observed to have a greater effect than synthetic urine (Lovell and Jarvis, 1996). However, the response can vary between soil types and a decrease in basal respiration has also been measured after urine application (Williams et al., 2000). Intensive grazing, stock camps, and high rates of urea application can also produce enhanced microbial respiration rates (Haynes and Williams, 1999; Petersen et al., 2004b; Ingram et al., 2008).

Enzyme activity often increases following urine deposition. Patra et al. (2005) observed higher NEA, DEA, and nitrogenase activity under heavily-grazed pasture than under lightly-grazed pasture. However, while Carter et al. (2006) saw an increase in NEA after urine addition, there was no effect on DEA in their experiment, and Petersen et al. (2004b) measured a decrease in DEA after urea application (5–10 g urea-N L^{-1}). Haynes and Williams (1999) found that many enzymes were more active under a stock camp than under the general pasture, although DHA was unaffected, whereas Rooney et al. (2006) measured a higher DHA when synthetic sheep urine was applied at a rate > 500 kg N ha^{-1} than at lower application rates.

Incorporation of thymidine by microbial cells showed that the microbial community generally adapted to a pH increase from 4.9 to 7.5, similar to that measured in a urine patch, within 8 d, although periods of up to 100 d were required in some cases (Pettersson and Bååth, 2003).

2.4.2.3 Measuring microbial diversity

The term 'biodiversity' encompasses genetic, functional, and taxonomic aspects, with reference to the number of species (richness) and the abundance of each species (evenness) (Torsvik and Øvreås, 2002; Nannipieri et al., 2003). In eukaryotic organisms, species are defined as "groups of interbreeding natural populations which are reproductively isolated from other such groups"

(Embley and Stackebrandt, 1997). However, this definition is not relevant to prokaryotic species because gene transfer and recombination can occur between different types of bacteria. Therefore a bacterial species is commonly described as a group of bacterial strains with similar features and differing considerably from other strains (Staley and Krieg, 1984). Microbial diversity can be assessed at the species or community level, by studying genotypic (genetic), phenotypic (observable), or functional (metabolic) characteristics.

Microbial community structure

Measurements of genetic diversity assess the complexity in a microbial community by comparing proteins and nucleic acids to determine the amount and diversity of genetic information (Torsvik and Øvreås, 2002). Genotype-based analyses have allowed bacteria to be classified into genomic species, and studies using DNA: DNA hybridisation have shown that species defined by this analysis are often also phenotypically similar (Goodfellow et al., 1997). The wide array of molecular techniques now available includes: denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), ribosomal intergenic spacer analysis (RISA), and 16S-rRNA analysis. Genetic analyses were not carried out as part of this study; refer to Hill et al. (2000) and Kirk et al. (2004) for further discussion of these methods.

Phenotypic diversity has traditionally been the most widely studied aspect of diversity, and uses physical features to classify bacteria. Analyses were customarily carried out by culturing samples on nutrient media, but it has been estimated that only 1% of soil microbes can be cultured (e.g. Torsvik and Øvreås, 2002), and it is unknown if the other 99% of the microbial cells are organisms unrelated to the culturable cells, or are phylogenetically similar but inactive (Rondon et al., 1999). Therefore culturing techniques may not give a true representation of the natural community. In addition, culturing cannot distinguish between bacteria that are phenotypically similar but genotypically different (Alexander, 1999).

More recent studies into phenotypic diversity have used PLFA analysis and molecular methods. These can incorporate isotope studies, by assessing which groups have assimilated isotopically-labelled substrates (e.g. Petersen et al., 2004a; Patra et al., 2005). Using PLFA analysis, different taxonomic groups can be identified by the presence of signature fatty acids (FAs) (Table 3.3). Analysis of PLFAs is described in Section 3.3.1.

Microbial community function

Functional diversity is the result of genetic diversity operating in conjunction with environmental effects and interspecies interactions, and can be defined as ‘the numbers, types,

activities, and rates at which a suite of substrates are utilised by the bacterial community' (Zak et al., 1994). Microbial communities with unequal use of substrates (low catabolic evenness) are less tolerant of environmental stress (less resilient) than those with high catabolic evenness (Degens et al., 2001; Torsvik and Øvreås, 2002).

The presence of inactive cells makes it difficult to gauge the functional diversity of a microbial community using traditional plating techniques. Modern methods of assessing community level physiological profiles (CLPP) show the patterns of substrate use of microbial communities. Biolog assays use microplates comprising 96 wells containing various C sources with tetrazolium dye. Following inoculation with suspended soil solutions, reduction of the dye, shown by the development of colour in a well, indicates utilisation of that substrate (Garland and Mills, 1991). Specific plates are available for G- and G+ bacteria and for fungi (Garland and Mills, 1991; Kirk et al., 2004).

Functional diversity cannot be directly correlated with the genotypic or phenotypic diversity of a microbial community due to 'functional redundancy', wherein a number of microbial types are capable of carrying out the same role, allowing a system to remain active under changing conditions (Hill et al., 2000; Nannipieri et al., 2003).

The effects of urine on microbial diversity

Williams et al. (2000) used culturing to show an increase in bacterial numbers for at least 5 weeks after urine application (500 kg N ha^{-1}), while fungal numbers were unaffected. Also using culturing techniques, Patra et al. (2005) found that soil from intensively grazed pasture generally supported higher populations of heterotrophs, NH_3-N oxidisers, nitrite (NO_2^--N) oxidisers, and denitrifiers than soil from lightly grazed pasture. Biolog analyses demonstrated that urine addition stimulated substrate use, particularly by the bacteria that use rhizosphere C (Williams et al., 2000; Nunan et al., 2006).

Phospholipid fatty acid analysis has shown higher concentrations of branched G+ biomarkers after urine application (500 kg N ha^{-1}), and higher concentrations of cyclic G- biomarkers in intensively grazed pasture when compared with lightly grazed pasture (Patra et al., 2005; Nunan et al., 2006). These changes in the microbial PLFA community composition were supported by data from DGGE and RISA analyses (Patra et al., 2005; Nunan et al., 2006). Also using RISA, Rooney et al. (2006) found that the plant species present could affect the timing of urine-induced community changes

With DGGE, Mahmood and Prosser (2006) discovered that the NH_3-oxidising community in a urine patch was predominantly *Nitrosospira* species, and that more of their DNA was present when NO_3^--N production was greatest. Using both DGGE and 16S-rRNA analyses, it was found that urine application altered the bacterial soil community but not the archaeal community (Nicol et al., 2004).

The rapid pH rise that can occur in a urine patch can be simulated experimentally, and increasing the pH of a bacterial suspension from pH 4 to 7, by adding alkali, resulted in increases in microbial diversity and bacterial cell numbers after anaerobic incubation for 30 h (Blosl and Conrad, 1992).

2.4.2.4 Measuring microbial stress

It has been suggested that G- bacteria may be more resistant to stress than G+ bacteria owing to the presence of cyclopropyl PLFAs in their membranes and to their additional external membranes (Kaur et al., 2005). Therefore the ratio of G+ to G- bacteria (G+/ G-) can indicate whether a microbial community is under stress. In addition to cyclopropyl FAs, the membranes of G- bacteria also contain monounsaturated PLFAs, while iso- and anteiso-branched PLFAs are found predominantly in the membranes of G+ bacteria (Federle, 1986; O'Leary and Wilkinson, 1988; Wilkinson, 1988; Zelles, 1997). Accordingly examples of these PLFAs are used as biomarkers of the two groups (Table 3.3).

Several other PLFA biomarker ratios have been used to evaluate microbial stress. An increase in the ratio of cy17:0 to 16:1ω7 (cy/ω7) indicates higher stress, since cis-monounsaturated FAs (e.g. 16:1ω7) are converted to cyclopropyl FAs by transmethylation when cells become dormant (Kaur et al., 2005), as may occur under conditions of stress. Cyclopropyl FAs are more stable and help the microbial cell to resist degradation (Kaur et al., 2005). Another stress indicator is a lower ratio of iso15:0 to anteiso15:0 (i/a). Environmental stress may cause cell membranes to become more fluid and thus potentially more permeable (Kaur et al., 2005), and an increase in anteiso FAs is believed to make the cell membrane more rigid, to protect the cell in hyperosmotic conditions (Chihib et al., 2003). A higher ratio of trans/cis 18:1ω9 (t/c) also indicates increased microbial stress, with the transformation of unsaturated cis fatty acids to trans fatty acids by isomerisation creating a more stable molecule and reducing membrane permeability (Kaur et al., 2005).

An increase in the qCO_2 (Section 3.4.3.1), which indicates a reduction in microbial community size in conjunction with increased CO_2 respiration, can denote an increasingly stressed soil

community, with energy being diverted to cell maintenance rather than growth under stressful conditions (Odum, 1985). An increased qCO_2 is considered to be a good indicator of adverse environmental conditions, i.e. stress or disturbance (Wardle and Ghani, 1995).

Rupturing of the cell membrane can be detected by propidium iodide (PI) staining of the cell's nucleic acids, which is analysed by fluorescence detectors (Baatout et al., 2007).

The effects of urine on microbial stress

Only two studies were found that directly investigated the effects of urine on microbial stress using PLFA stress ratios. One of these studies was a field trial, in which cow urine amended with ^{13}C-labelled urea (5.6 or 9.5 g N L^{-1}) was applied to a sandy loam pasture soil (Petersen et al., 2004a). The other study was a laboratory experiment, in which ^{15}N-labelled urea solutions (5 or 10 g N L^{-1}) were applied to repacked cores of the same sandy loam soil (Petersen et al., 2004b). In these studies, the cy/ω7 and t/c ratios indicated a reduction in microbial stress following urine deposition, showing that the microbial communities were stimulated by the addition of urine (Petersen et al., 2004a; Petersen et al., 2004b). However, a decline in the i/a ratio 4 d after urine was applied at a rate of 400 kg N ha^{-1}, suggested that the microbial community in this treatment was under salt stress (Chihib et al., 2003; Petersen et al., 2004a).

Phospholipid fatty acid analysis has also been used to investigate the response of the microbial community to stresses associated with urine deposition, as detailed below. Osmotic stress, which may increase in the soil after urine application due to the added salt, can also be induced by removing moisture. An increased t/c ratio was observed when water activity was reduced (a_w = 0.99) after the addition of NaCl to a microbial culture (Heipieper et al., 1996), while desiccation of microbial cells in porous media resulted in an increase in both the t/c and cy/ω7 ratios (Kieft et al., 1994). The t/c ratio also increased when microbial cells were starved (Guckert et al., 1986). This may occur if the nutrients supplied by urine application become exhausted. Rietz and Haynes (2003) found that the qCO_2 increased by a factor of 7 when the EC increased from 0–5 dS m^{-1}.

Urine deposition usually causes a large increase in the soil pH, followed by a decrease below the original value after nitrification has begun (Section 2.5.1.3). Two hour incubations of *Pseudomonas* cultures at different pHs did not cause alterations in the t/c ratios of cell membranes in the pH range of 6–11, but there was a peak in the t/c ratio between pH 4 and pH 6 (Heipieper et al., 1996). No changes were seen in t/c or cy/ω7 ratios of the soil microbial community when the pH changed from 4.8 to 5.9 after liming soil for > 1 year (Treonis et al., 2004), while a shift to more G- and fewer G+ biomarkers was seen in soil where the pH was

increased from 4 to 7 by liming 5–6 years earlier (Frostegård et al., 1993b). Soil bacteria incubated in nutrient broth for 2 h at pHs ranging from 2–12 showed increasing loss of membrane potential at a pH ≤ 5 or ≥ 9, as shown by PI fluorescence staining (Baatout et al., 2007).

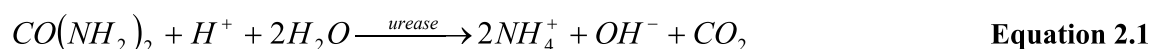
No studies were found that measured microbial stress as a response to enhanced free NH_3-N concentrations, although this is clearly an inhibitory effect as discussed in Sections 2.5.1.3 and 2.5.1.5.

2.5 Urine effects on nutrient cycling

2.5.1 Nitrogen cycling

2.5.1.1 Urea hydrolysis

The majority of the urinary-N voided onto soil is urea (Table 2.1), which undergoes rapid hydrolysis to NH_4^+-N in the topsoil (Equation 2.1) (e.g. Sherlock and Goh, 1984; Haynes and Williams, 1993), and may be completely hydrolysed within 2 d (Holland and During, 1977; Petersen et al., 1998). Hydrolysis of urea is optimal at pH > 6.5, and causes a localised increase in the soil pH (Equation 2.1) (Haynes and Sherlock, 1986; Jarvis and Pain, 1990).



$$CO(NH_2)_2 + H^+ + 2H_2O \xrightarrow{urease} 2NH_4^+ + OH^- + CO_2$$ **Equation 2.1**

Urea hydrolysis is catalysed by the enzyme urease, which is ubiquitous in the pasture environment (Bremner and Mulvaney, 1978; Hoult and McGarity, 1986). Urease exists within the microbial biomass (intracellular) and in the bulk soil (extracellular) (Vlek et al., 1980). Approximately 60% of urease activity in the soil is believed to be associated with extracellular enzymes bound to soil colloids (Pettit et al., 1976). Rates of urease activity increase with increasing temperature (Moyo et al., 1989).

A fraction of the NH_4^+-N is converted to NH_3-N (Equation 2.2), with the relative proportions of each being dependent on the soil pH, temperature, and NH_3-N volatilisation rate (Haynes and Sherlock, 1986). Between 20 and 80 g m^{-2} of ammoniacal-N (NH_4^+-N + NH_3-N) is found within a urine patch (Oenema et al., 1997). The highest concentration is in the top 15 mm of soil, corresponding with findings that 75% of urea remains at that depth, although ammoniacal-N can move deeper into the soil after rainfall (Holland and During, 1977).

The concentration of ammoniacal-N in the soil usually remains elevated for at least 2-3 weeks after urine deposition, depending on the nitrification rate (e.g. Ball et al., 1979; Whitehead and

Bristow, 1990). The peak soil NH_4^+-N concentration often occurs within 24 h of urine deposition, although concentrations remain elevated for several days (Sherlock and Goh, 1984; Lovell and Jarvis, 1996; Petersen et al., 1998).

Figure removed due to copyright. For the original figure please refer to: Wrage, N., G.L. Velthof, M.L. van Beusichem, and O. Oenema. 2001. Role of nitrifier denitrification in the production of nitrous oxide. Soil Biology and Biochemistry 33:1723-1732.

Figure 2.1 Transformations of mineral N in soil (modified from Wrage et al., 2001).

2.5.1.2 Ammonia volatilisation

Although urea hydrolysis (Equation 2.1) is a biochemical reaction, NH_3-N formation (Equation 2.2) is a chemical reaction, which requires an alkaline pH (Court et al., 1964; Saggar et al., 2004). The high pH of a urine patch, in conjunction with the increase in the soil NH_4^+-N concentration, provides ideal conditions for NH_3-N production (Haynes and Sherlock, 1986; Haynes and Williams, 1993), and as the pH rises the amount of NH_4^+-N converted to NH_3-N increases (Bates and Pinching, 1950; Court et al., 1964). The NH_3-N in soil solution is in equilibrium with atmospheric NH_3-N, and can be volatilised (Whitehead, 1995a).

$$NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O \quad \textbf{Equation 2.2}$$

Although most of the NH_3-N emitted following urination is formed from urea-N, the minor N components of urine (Table 2.1) also contribute (Whitehead et al., 1989; Bussink and Oenema, 1998). The rapid hydrolysis of urea (Section 2.5.1.1) results in a flush of NH_3-N soon after urine deposition onto soil, whereas formation of NH_3-N from the minor constituents is often delayed (e.g. Holland and During, 1977; Petersen et al., 1998).

The proportion of total urinary-N volatilised as NH_3-N is normally 10–40% (e.g. Ball et al., 1979; Whitehead et al., 1989; Whitehead and Raistrick, 1991; Bol et al., 2004), although measurements from 3 to 52% have been made (Petersen et al., 1998). Sherlock and Goh (1984) measured NH_3-N emissions of 9–42% of total urinary-N, noting seasonal variations, while Whitehead and Raistrick (1992) detected between 23% and 39%, with emissions higher on bare soil and lower in the presence of ryegrass. This was despite Doak's (1952) supposition that interception of urine by pasture plants may be a major contributor to NH_3-N emissions.

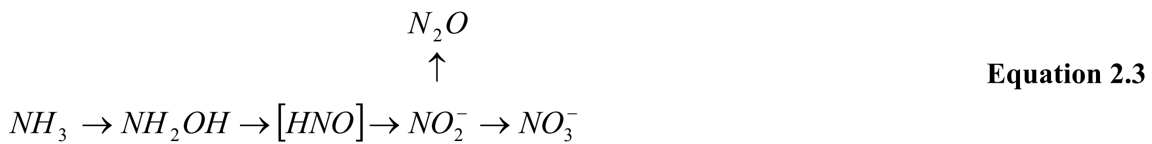
The pH and CEC are the main soil factors that determine the extent of NH_3-N volatilisation (Whitehead and Raistrick, 1993; Zhenghu and Honglang, 2000). However, NH_3-N volatilisation may correlate better with the soil pH after urine deposition than with the initial soil pH (Whitehead and Raistrick, 1993). Although the high pH in a urine patch increases the NH_3-N: NH_4^+-N ratio, potentially enhancing NH_3-N volatilisation, it may also increase the negative surface charge of the CEC, so that more NH_4^+ is adsorbed, which could reduce NH_3-N volatilisation (Haynes and Williams, 1993).

Soil moisture and temperature can also affect losses of NH_3-N. Irrigation may reduce NH_3-N volatilisation, as water application within 24 h of urine deposition was found to inhibit emissions of NH_3-N (Whitehead and Raistrick, 1991). Black et al. (1987) measured a 92% reduction in NH_3-N emissions when 16 mm of water was applied within 3 h of urea application, and this effect diminished as the time to watering increased. Ammonia volatilisation may rise significantly due to temperature increases in the range of 4–20°C (Whitehead and Raistrick, 1991). At higher temperatures, however, biochemical reactions such as nitrification are also enhanced, so that NH_4^+-N uptake will increase while NH_3-N volatilisation declines (Whitehead and Raistrick, 1991). This may explain the lack of difference in volatilisation rates over a range of soil temperatures from 8 to 18°C in experiments by Holland and During (1977).

2.5.1.3 Nitrification

Nitrification is a two-step process, and was traditionally believed to be carried out by ammonia-oxidising bacteria (AOB) which transform NH_3-N to NO_2^--N, (Equation 2.4) while nitrite-oxidising bacteria (NOB) transform the NO_2^--N to NO_3^--N (Figure 2.1 and Equation 2.3) (Kowalchuk and Stephen, 2001). The production of hydrogen ions during this process (Equation 2.4) reduces the soil pH. The NOB comprise four phylogenetic groups and include members of the *Nitrobacter* and *Nitrosospira* genera (Poly et al., 2008), which are chemolitho-autotrophic, obligately aerobic members of the G- bacterial phylum Proteobacteria (Head et al., 1993; Kowalchuk and Stephen, 2001). Recent molecular studies have shown that the principal

NH_3-N oxidisers in terrestrial ecosystems may be members of the Archaea (Leininger et al., 2006; Prosser and Nicol, 2008), and that fungi may also be important NH_3-N oxidisers in grassland soils (Laughlin et al., 2008).



$$NH_3 \rightarrow NH_2OH \rightarrow [HNO] \rightarrow NO_2^- \rightarrow NO_3^- \quad (\uparrow N_2O)$$ **Equation 2.3**

$$NH_3 + 3[O] \xrightarrow{6e^-} NO_2^- + H_2O + H^+$$ **Equation 2.4**

Urine deposition enhances nitrification through the addition of urea and moisture to the soil. By increasing the pH, urea hydrolysis can enhance conditions for nitrification, which is optimal at a pH > 7 (Allison and Prosser, 1993; Villaverde et al., 1997), although there is some repression of nitrification and nitrifier growth at high pHs (> 8), coinciding with an increase in the concentration of free NH_3-N (Antoniou et al., 1990; Villaverde et al., 1997; Park et al., 2007).

The NH_4^+-N supplied from urea hydrolysis (Equation 2.1) supports an increase in the population of nitrifying bacteria following urine deposition, as shown by the incorporation of urea-^{13}C into PLFAs commonly found in AOB and NOB (Petersen et al., 2004a). However, because NOB can be inhibited by high levels of NH_3-N (> 20 mg N L^{-1}) or by high pH alone , addition to the soil of high-N urine may result in NO_2^--N accumulation and a decrease in NO_3^--N production (Doak, 1952; Monaghan and Barraclough, 1992). The higher rate of NH_3-N production at high pHs (Section 2.5.1.2) may be the reason that nitrification is repressed at a pH > 8 (Doak, 1952; Villaverde et al., 1997). Both *Nitrosomonas* and *Nitrobacter* can be inhibited by high concentrations of NO_2^- at a pH ≤ 7.5 (Hunik et al., 1992, 1993). In addition, *Nitrobacter* can be inhibited by NO_3^- at pH 6.5–7.5 and by NH_4^+ at pH 6.5 (Hunik et al., 1993).

Nitrification is also inhibited by P deficiency, trace element toxicity, pesticides, and high Cl^- concentrations (Haynes, 1986; Roseberg et al., 1986). However, high concentrations of Cl^- are unlikely to be present in pasture soils with a medium to high pH (Roseberg et al., 1986; Monaghan and Barraclough, 1992), and Cl^- concentrations in a urine patch would probably not be high enough to inhibit nitrification, as Monaghan and Barraclough (1992) estimated that concentrations > 2000 mg Cl^- kg^{-1} soil would be required. *Nitrosomonas* were found to be inhibited by Cl^- and Na^+ salts at concentrations of 100 mmol L^{-1}(Hunik et al., 1992).

The rate of nitrification is greatest at soil moisture potentials of -10 to -33 kPa, but can occur at the permanent wilting point of -1500 kPa (Malhi and McGill, 1982; Haynes, 1986). The

reaction is non-existent or very slow in saturated soil (0 kPa) because oxygen levels are too low for bacterial oxidation to occur (Malhi and McGill, 1982; Haynes, 1986). Monaghan and Barraclough (1992) found that nitrification was inhibited at a soil moisture potential of ≤ -1000 kPa and was absent at -2000 kPa. However, since urease is still active in waterlogged soil (Vlek et al., 1980), urea hydrolysis will still occur, and a large proportion of the added N may be volatilised as NH_3-N (Vlek and Craswell, 1979).

Wetting of a dry soil, as occurs with urination, can result in a flush of microbial growth and an increase in NO_3^--N (Campbell and Biederbeck, 1982). This is probably due to nitrification, but upward movement of NO_3^--N with evaporating water may also contribute.

The optimum temperature range for nitrification in soil is usually 25–35°C (Alexander, 1977; Haynes, 1986; Whitehead, 1995b) but indigenous microbial communities adapt to local conditions (Mahendrappa et al., 1966; Malhi and McGill, 1982), and nitrification has been measured in frozen soils (Malhi and Nyborg, 1979).

2.5.1.4 Denitrification

During denitrification NO_3^--N and NO_2^--N are reduced to N_2-N and N_2O-N (Figure 2.1 and Equation 2.5).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad \textbf{Equation 2.5}$$

Genes for denitrification have been detected in Archaea and G+ bacteria, but are most common in the G- bacteria of the phylum Proteobacteria (Philippot, 2002). These normally aerobic bacteria are able to utilise NO_3^--N and NO_2^--N as electron acceptors when oxygen is unavailable (Firestone, 1982; Robertson and Groffman, 2007). The different reductase enzymes used for each step of Equation 2.5 are repressed by oxygen (Firestone, 1982; Drury et al., 1991; Dendooven and Anderson, 1994). Therefore bacterial denitrification is predominantly carried out in wet soils, but may also occur in anaerobic microsites in dry soils (Whitehead, 1995c; Müller et al., 2004). Because N_2O-N reductase is particularly sensitive to oxygen, the proportion of N_2O-N produced increases in marginally anaerobic conditions, although the N_2: N_2O ratio is highly variable (Whitehead, 1995c).

The supply of organic C may determine the rate and extent of denitrification (Firestone, 1982; Haynes and Sherlock, 1986). In addition, a ready supply of NO_3^--N is necessary, and the greatest reaction rates occur at pH 7–8 (Bremner and Shaw, 1958; Haynes and Sherlock, 1986). Although early studies indicated that denitrification could only occur in soil at ≥ 60% of its

water-holding capacity (Bremner and Shaw, 1958), more recent studies have shown that NO_3^--N reduction can take place in soils under largely aerobic conditions (Müller et al., 2004). In general though, N_2O-N production increases with increasing soil moisture content (Linn and Doran, 1984), and at high moisture contents (e.g. 70% water-filled pore space (WFPS)) may be attributed entirely to denitrification (Bateman and Baggs, 2005). Urine patches can thus provide ideal conditions for denitrification via the addition of N, C and moisture, and through the resulting increase in soil pH. Observations have shown that the rate of denitrification in soil may increase by over 0.6 g N m^{-2} d^{-1} following urine deposition (de Klein and van Logtestijn, 1994).

Rates of denitrification are highest at 60–70°C (Nõmmik, 1956; Bremner and Shaw, 1958; Keeney et al., 1979), although non-biological reactions probably contribute at these high temperatures (Firestone, 1982; Haynes and Sherlock, 1986). Soil temperatures may cause fluctuations in denitrification rates, with the highest rates usually occurring in the afternoon on a diurnal scale and in summer on a yearly scale (Rolston et al., 1978; Haynes and Sherlock, 1986), assuming suitable conditions, e.g. water availability. However, a clear relationship is not always seen (Ginting and Eghball, 2005).

Recent studies have indicated that fungi also play a denitrifying role in grassland soils. A reduction in N_2O-N production of 70–89% was seen in the presence of fungal inhibitors compared with a 20–23% reduction due to bacterial inhibitors (Laughlin and Stevens, 2002; Spokas et al., 2006). The major product of fungal denitrification is N_2O-N (Shoun et al., 1992; Spokas et al., 2006), so this pathway is important with respect to GHG emissions.

Nitrifier denitrification

In nitrifier denitrification (Figure 2.1), ammoniacal-N is transformed by autotrophic AOB to NO_2^--N, which is then reduced. Both *Nitrosospira* and *Nitrosomonas* have genes that code for enzymes involved in denitrification (Casciotti and Ward, 2005; Garbeva et al., 2006), and both can produce N_2O-N from NO_2^--N (Schmidt et al., 2004; Shaw et al., 2006). Up to 30% of N_2O-N production may originate from this pathway when a high concentration of organic N is coupled with low organic C availability and low oxygen pressure (Wrage et al., 2001). It is conceivable that these conditions might be found within a urine patch.

2.5.1.5 Nitrous oxide

Nitrous oxide (N_2O) is formed during both nitrification and denitrification (Figure 2.1 and Equations 2.3 & 2.5). The relative importance of these two pathways in N_2O-N production is largely dependent on the soil moisture content. Nitrification dominates production where soil

moisture is low (e.g. Bol et al., 2004), while denitrification is more important in moist soils (e.g. Monaghan and Barraclough, 1993; Bateman and Baggs, 2005). Therefore, the addition of moisture to soil by urine deposition is likely to enhance denitrification rates. Increased N_2O-N emissions were detected when urine was applied to either compacted or dung-affected soil, and also in stock camps, where the combination of urine deposition and soil compaction means more soil pores are saturated (van Groenigen et al., 2005).

In addition to the soil moisture content, the rate and extent of N_2O-N production is affected by soil type (de Klein et al., 2003), and by levels and forms of inorganic N. The inhibition of NO_2^--N oxidation during nitrification, by high concentrations of NH_3-N (Section 2.5.1.3), leads to an increase in N_2O-N emissions (Monaghan and Barraclough, 1993; Oenema et al., 1997). In contrast, decreased N_2O-N emission rates were observed when very high concentrations of urinary N were added to the soil (Clough et al., 2003b). Application of the nitrification inhibitor, dicyandiamide (DCD), can reduce N_2O-N emissions from urine patches, with a 72% decrease seen in one experiment (Di and Cameron, 2008).

The proportion of urinary-N emitted as N_2O varies widely, having been measured at between 0.1% (Di et al., 2007) and 13.3% (Kool et al., 2006b). The higher values are generally seen in laboratory studies under controlled temperature regimes. However, it is usually < 3% (de Klein et al., 2001), and the International Panel on Climate Change (IPCC) default emission factor is 2% (IPCC, 2006). Nitrous oxide fluxes are higher and more immediate when urine is applied to soil, than when urea alone is applied (Sherlock and Goh, 1983), possibly due to enhanced urea hydrolysis in the presence of hippuric acid (Section 2.2.2.1).

2.5.2 Carbon cycling

Carbon is present at only low concentrations in urine (Section 2.2.2). During urea hydrolysis the C is transformed into CO_2-C (Equation 2.1), although a small proportion of the urea-C is assimilated into the microbial biomass (Petersen et al., 2004a).

2.5.2.1 Carbon dioxide

The rapid flush of CO_2-C within 24 h of urine deposition results largely from urea hydrolysis (Bol et al., 2004), while microbial respiration may remain elevated for a number of weeks (Lovell and Jarvis, 1996). The proportion attributed to urea hydrolysis has been estimated at 40–87% (Bol et al., 2004; Ambus et al., 2007). The increased microbial respiration rate following urination is probably due to soil C being solubilised rather than to the small amount of C added in the urine (Lovell and Jarvis, 1996). Some of the methane (CH_4) that forms under a urine

patch will be utilised by methanotrophic bacteria and oxidised to CO_2-C (Lovell and Jarvis, 1996) (Section 2.5.2.2). This could occur at the interface between the dry soil and the urine-wetted soil.

2.5.2.2 Methanogenesis

Urine deposition results in only minor emissions of CH_4, especially when compared with CH_4 emissions from dung (Jarvis et al., 1995). Urine addition may make the soil environment more suitable for CH_4 production by (1) creating anaerobic zones through the addition of moisture, (2) increasing the amount of DOC through the increase in pH, and (3) inhibiting methanotrophic bacteria through the production of NH_4^+-N by nitrification (Lovell and Jarvis, 1996). However, these effects appear to be inconsequential when considered against total CH_4 emissions from pasture. This is due in part to the utilisation of CH_4 by methanotrophic soil bacteria in aerobic zones, such that measurements of CH_4 production after urine application were still ≤ 0.1 μg m^{-2} min^{-1} despite an increase in methanogenesis (Lovell and Jarvis, 1996; Li and Kelliher, 2007). In fact, the rate of oxidation of CH_4 by pasture soils may exceed its emission from urine (Lovell and Jarvis, 1996). Because CH_4 emissions from urine are so low, it was not measured for this study, and CH_4 production will not be considered further.

2.6 Summary

Managed pastures, which constitute ca. 20% of the Earth's land surface, support artificially high densities of grazing animals, so that natural nutrient cycling is enhanced by increased inputs of excrement. In New Zealand, pastoral agriculture supports 5.3 million dairy cows, which produce 106 million L of urine every day. Urine deposition affects soil chemistry, soil microbiology, and gaseous emissions. The gases produced include CO_2 and N_2O, which are greenhouse gases (GHGs) and contribute to the increase in the Earth's surface temperature. Emissions of N_2O have increased since 1750 at an average rate of 0.26% yr^{-1}, with 40% of global N_2O production coming from agriculture. In New Zealand, 48.5% of the GHGs produced are agriculturally sourced, and of this one-third is N_2O. Under the Kyoto Protocol, New Zealand has committed to reducing GHG emissions, or otherwise taking responsibility for excess emissions through schemes such as carbon trading. Thus urine patch chemistry and microbiology could have an impact on New Zealand's economy and reputation.

The influence of a bovine urine patch on the soil may extend laterally to 0.68 m^2 and vertically to 0.40 m depth, through diffusion, plant root uptake, and macropore flow. Usually, however, ca. 85% of the urine remains in the top 100 mm of soil. Bovine urine may contain 2–20 g N L^{-1}, with the average rate of N returned to pasture being estimated at 200 kg ha^{-1} yr^{-1}. However, the

concentration of N in a urine patch may be as high as 1000 kg ha^{-1} or > 400 mg kg^{-1} of soil. Of the urinary-N, 50–93% is in the form of urea and 2–8% is contained in hippuric acid. Hippuric acid is a conjugate of benzoic acid and glycine, and its concentration in urine depends on dietary intake. Hippuric acid may increase urea hydrolysis and NH_3-N volatilisation, and reduce denitrification and N_2O-N emissions. Urine also contains S, cations (K^+, Na^+) and anions (Cl^-, HCO_3^-), but only trace amounts of P.

The urinary urea is hydrolysed in the pasture environment by the urease enzyme to produce NH_4^+-N. This process is rapid and is optimal at a pH of > 6.5. In alkaline conditions, some of the NH_4^+-N will be transformed to NH_3-N, and 10–40% of the total urinary-N may be volatilised from the soil in this form. During nitrification, ammonia oxidisers transform the NH_3-N to NO_2^--N, and nitrite oxidisers then transform the NO_2^--N to NO_3^--N. The nitrifiers include chemoautotrophic Proteobacteria, Archaea, and fungi. The NO_3^--N produced during nitrification is then reduced to N_2-N via NO_2^--N and N_2O-N. Denitrification is carried out by Archaea, fungi and G+ bacteria, but predominantly by G- Proteobacteria. The reductase enzymes involved are repressed by oxygen to varying degrees, so denitrification occurs either in wet soils or in saturated microsites in dry soils. Nitrous oxide is produced during both nitrification, which dominates at low soil moisture, and denitrification, which dominates at high soil moisture.

Pasture growth is often enhanced by urine deposition, but scorching of plant roots can occur if concentrations of NH_3-N are high. Urine deposition increases the soil moisture content, and causes the soil pH to increase by 1–3 pH units. An increase in C concentration in the urine patch may be sourced from urine constituents, through dissolution of soil organic matter, or through release of microbial cell constituents. Between 40 and 87% of the CO_2-C produced by microbial respiration following urine deposition comes from urea hydrolysis.

Bacteria and fungi are the predominant soil microbes in terms of mass, abundance and soil function. They are largely limited to zones where water, nutrients, and energy sources are available, and the microbial biomass is usually well correlated with soil organic C content. The biomass in grassland soils has been estimated at 3–9 t ha^{-1}. The soil microbial community can be measured in terms of biomass, activity, structure and function. In addition, microbial stress can be assessed. Urine application often causes an increase in the microbial biomass, as well as increased CO_2-C respiration and enzyme activity. Urine addition also stimulates substrate use, and alters the bacterial community. Microbial stress may decrease directly after urine application, but conditions in the urine patch later may cause increased microbial stress.

In the course of this review of the literature, it has become apparent that there are a lack of studies linking nutrient dynamics and soil microbial communities in urine patches. Therefore the focus of this study will be the connections between these two components of the soil system following urine deposition.

Chapter 3
Materials and Methods

3.1 Urine collection and analyses

Specific details of the urine collection for each experiment are included in the Materials and Methods sections in each chapter. For all experiments, the urine was collected from multiple dairy cows one day prior to application. The urine was bulked and analysed for total-N, potassium (K^+), urea, and hippuric acid (HA) concentrations, as required. Urinary-N was quantified on a Continuous Flow Isotope Ratio Mass Spectrometer (IRMS) (PDZ Europa Ltd, Crewe, UK) by pipetting 10 µL of urine onto a bed of chromosorb-W inside a tin capsule. Samples were analysed in triplicate. Urinary K^+ was analysed by HPLC on a Waters 432 Conductivity Detector (Millipore Corp., Milford, MA, USA) using a universal cation 7 µm column (100 mm length x 4.6 mm ID) with 3 mM methanesulphonic acid at 1 mL min^{-1} as the mobile phase. Urea was analysed, using a kinetic UV assay, at Gribbles Veterinary Pathology (7 Halkett St, Addington, Christchurch). The HA and benzoic acid (BA) concentrations in the urine were measured using a modified version of the method of Yue-dong (1998). In short, the urine was diluted with deionised (DI) water before storage at -20°C and was further diluted (up to 2000-fold) with DI water before analysis by HPLC using a Waters 409E Programmable Multiwavelength UV/Vis Detector at 220 nm. The HPLC was fitted with a GraceSmart RP18 5 µm column (150 mm length x 4.6 mm ID) and samples (10 µL) were analysed in a mobile phase of KH_2PO_4 and CH_3OH (99:1) at a pH of 6.5.

3.2 Soil collection and analyses

3.2.1 Soil collection and storage

The soil collection processes for the three incubation experiments (Chapters 4, 6 and 7) are detailed in the Materials and Methods sections of those chapters. The soils were sieved within 48 h of collection and were stored at field moisture content before and after sieving in plastic bags at 4°C, until being packed into cores. During pre-incubation, the soil was adjusted to the experimentally required moisture level. Key soil properties of the three soils used are presented in Table 3.1.

Table 3.1. Key properties of the soil types used during this study.

Soil type	Total N ($mg\ g^{-1}$)	Total C ($mg\ g^{-1}$)	Olsen P ($\mu g\ mL^{-1}$)	CEC ($meq\ 100g^{-1}$)
Templeton silt loam	2.6	34.0	25	13.0
Wakanui silt loam	2.8	30.0	20	15.4
Temuka silt loam	3.1	32.3	37	17.5

3.2.2 Gravimetric soil moisture content

A sub-sample of 5–10 g of field-moist soil was weighed into a metal dish of known weight and placed in a 105°C oven for 24 h. After being allowed to cool, the samples were reweighed and the gravimetric soil moisture content was calculated using Equation 3.1 (Topp and Ferré, 2002):

$$\theta_g = \frac{M_w}{M_s}$$ **Equation 3.1**

where;
θ_g = gravimetric soil moisture content (g water g^{-1} oven dry soil)
M_w = mass of water (g) (mass of field moist soil (g) - mass of oven dry soil (g))
M_s = mass of oven dry soil (g)

3.2.3 Soil pH & Electrical conductivity

Soil surface pH was measured in experiments 1, 3 and 4 (Chapters 4, 6 and 7) using a Hanna HI 9025C portable pH meter fitted with a soil surface probe (Broadley-James Corporation, Irvine, CA, USA), after moistening the soil with one drop of DI water. For experiments 2 and 4 (Chapters 5 and 7), the pH of air-dried bulk soil was measured using the method of Blakemore et al. (1987). This involved adding 25 mL of DI water to 10 g of air-dried soil (ratio 2.5:1) and shaking orbitally for 30 min at 130 rev min^{-1}. The mixture was then left to settle for a minimum of 12 h before measuring the pH with a SevenEasy pH meter (Mettler Toledo, Columbus, OH, USA).

Electrical conductivity (EC) was determined using the method of Blakemore et al. (1987). Air-dried soil was shaken with DI water at a 1:5 soil: water ratio for 30 min at 20°C. The mixture was then centrifuged at 1500 rev min^{-1} (270 x *g*) for 5 min. Conductivity was measured immediately, using a Radiometer Copenhagen CDM 83 electrical conductivity meter that was calibrated using a 0.01 M KCl solution. The conductivity readings in $\mu S\ cm^{-1}$ were divided by 1000 for conversion into $dS\ m^{-1}$ (Miller and Curtin, 2008).

3.2.4 Inorganic nitrogen

Inorganic-N was extracted with a solution of 2 M KCl (Mulvaney, 1996) at a 1:10 ratio for 1 h, using either 2 g soil (experiments 1, 2 & 3) or 10 g soil (experiment 4). The extractant was then centrifuged at 2000 rev min^{-1} (480 x *g*) for 10 min, and filtered (Whatman No. 41) into 30 mL screw-top plastic containers before storage at 4°C until analysis. The ammonium (NH_4^+-N), nitrate (NO_3^--N) and nitrite (NO_2^--N) analyses were performed on an Alpkem FS3000 twin channel Flow Injection Analyser (FIA) (Alpkem, College Station, TX, USA). Blank samples, (filtered KCl solution), were analysed at the same time. Equation 3.2 was used to calculate the inorganic-N concentration of each sample:

$$N_s = \frac{(N_e \times V)}{M_s}$$ **Equation 3.2**

where;

N_s = inorganic N content (mg kg^{-1} dry soil)

N_e = inorganic N concentration of extract (mg L^{-1})

V = volume of solution (KCl + soil moisture) (L)

M_s = mass of oven dry soil (kg)

3.2.5 Dissolved reactive phosphorus

The method used was that of Ohno and Zibilske (1991). Dissolved reactive P (DRP) is the most bioavailable form of P in the environment, and is largely inorganic orthophosphate (Pote and Daniel, 2000).

3.2.5.1 Reagents and standards

Polyvinyl alcohol (PVA; 1 g) was heated in 100 mL of DI water until dissolved, and then allowed to cool. To another 350 mL of DI water, 1.2 g of boric acid (H_3BO_3) and 36.18 g of ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$) were added. A 5 M solution of sulphuric acid (H_2SO_4) was prepared by adding 140 mL of 18 M H_2SO_4 to 360 mL of chilled DI water. After cooling, this was placed in a 1 L volumetric flask and 0.23 g of malachite green was added. After adding the PVA and boric acid/molybdate solutions, the reagent volume was made up to 1 L. The reagent was stored in a dark plastic bottle.

The stock P standard (100 mg L^{-1}) was prepared by dissolving 0.44 g of potassium dihydrogen phosphate (KH_2PO_4) in 100 mL of DI water, with 1 mL of toluene as a preservative, and then making the volume up to 1 L with DI water. This was stored at 5°C for up to 12 months. The

working P standard (10 mg L^{-1}) was made by diluting 100 mL of the stock standard to 1 L with DI water. This was stored at 5°C for up to 6 months. Analytical standards (0–200 μg L^{-1}) were prepared with the samples, by diluting aliquots of the working standard (0–1000 μL) to volume with DI water in 50 mL volumetric flasks. The standards were then transferred to 100 mL conical flasks and 10 mL of malachite green reagent was added. Absorbance was read at 635 nm after 1 h on a Shimadzu UVmini-1240 UV-Vis spectrophotometer (Shimadzu Scientific Instruments, Sydney, Australia).

3.2.5.2 Method

Air-dried soil (7 g) was extracted with 7 mL of DI water in a plastic centrifuge tube by shaking orbitally for 1 h. The samples were then centrifuged for 15 min at 15 000 rev min^{-1} (26 890 x *g*) and filtered through 0.45 μm cellulose nitrate membrane filters (Advantec MFS Inc., Pleasanton, CA, USA). In a volumetric flask, 3 mL of the filtered extract and 0.6 mL of malachite green reagent were mixed. After 1 h the mixture was transferred to a cuvette and the absorbance read as for the standards.

3.2.6 Water soluble carbon

Concentrations of water soluble carbon (WSC) were determined by extracting field-moist soil (2 g) with 20 mL of DI water (1:10 ratio), using a modified version of the method of Ghani et al. (2003). The extraction containers (70 mL screw-top plastic vials) were shaken end-over-end for 30 min. The solutions were then left to stand until the solid matter had settled (experiment 1) or were centrifuged at 8000 rev min^{-1} (7650 x *g*) for 5 min (experiments 2 & 3). Following this the solutions were filtered (Whatman No. 42), as was an additional 20 mL DI water blank. The filtered samples were analysed for WSC using a Shimadzu Total Organic Carbon Analyser TOC 5000A (Shimadzu Oceania Pty Ltd, Sydney, Australia) fitted with a Shimadzu ASI-5000A autosampler. The total organic carbon (TOC) content of the samples (in mg L^{-1}) was calculated by subtracting the inorganic carbon (IC) from the total carbon (TC) for each sample. Equation 3.3 was used to convert TOC to WSC:

$$WSC = \frac{(TOC \times V)}{M_s}$$ **Equation 3.3**

where;

WSC = water soluble carbon content (mg kg^{-1} dry soil)

TOC = total organic carbon concentration of extract (mg L^{-1})

V = volume of solution (DI water + soil moisture) (L)

M_s = mass oven dry soil (kg)

3.2.7 Ion exchange chromatography

A subsample of the WSC extracts (Section 3.2.6) was analysed for cations (experiment 1) and anions (experiments 1 and 2), after being passed through a 0.20 µm membrane filter (Advantec MFS Inc., Pleasanton, CA, USA). Ionic concentrations were converted into $cmol_c$ kg^{-1} dry soil using Equation 3.4 (modified from Sumner and Miller (1996)):

$$M^{a+} = \left(\frac{M^{n+} \times V \times n \times C_{cm}}{M_s \times A} \right)$$ **Equation 3.4**

where;

M^{a+} = ionic concentration ($cmol_c$ kg^{-1} dry soil)

M^{n+} = ionic concentration (g L^{-1})

V = volume of extraction solution (L)

n = valence of ion (mol_c mol^{-1})

C_{cm} = conversion factor mol_c to $cmol_c$ [100 $cmol_c$ mol_c^{-1}]

M_s = mass oven dry soil (kg)

A = atomic weight (g mol^{-1})

3.2.7.1 Cations

Cations (calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^+) and K^+) were analysed by HPLC with a Waters 432 Conductivity Detector linked to a Waters 717 Plus Autosampler, equipped with an Alltech Universal Cation 7µm column (100 mm length x 4.6 mm ID). The eluent was 3 mM methanesulphonic acid at a flow rate of 1 mL min^{-1}.

3.2.7.2 Anions

Anions (chloride (Cl^-), phosphate (PO_4^{3-}) and sulphate (SO_4^{2-})) were analysed on a Dionex DX-120 Ion Exchange Chromatograph (Dionex Corp., Sunnyvale, CA, USA) fitted with a Dionex AAS50 Autosampler. The system was suppressed with an Anion Self-Regenerating Suppressor

(ASRS®-Ultra). The ions were separated on a Dionex Ion-Pac® AS9-SC column (250 mm length x 4 mm ID), and the eluent was a weak sodium carbonate/ sodium bicarbonate (2 mM Na_2CO_3/ 0.75 mM $NaHCO_3$) solution at a flow rate of 2 mL min^{-1}.

3.2.8 Hippuric acid

The free fraction of HA in the soil following urine application was measured using the method of Baziramakenga et al. (1995). Air-dried soil (5 g) was weighed into a 35 mL centrifuge tube and mixed with 10 mL of DI water. After leaving the tubes at room temperature for 16 h, they were centrifuged at 15 000 rev min^{-1} (26 890 x *g*) for 15 min and the contents were then filtered through 0.45 µm cellulose nitrate membrane filters. The solutions were frozen without further amendment until analysis as for urinary hippuric acid (Section 3.1).

3.2.9 Trace element analyses

Trace elements and other metals were extracted with DI water, to ensure that the trace element concentrations measured were from the exchangeable (bioavailable) fraction rather than from the bound fraction (Fedotov et al., 2007). The extractions were performed with 20 mL of DI water and 5 g of field moist soil. The extraction tubes were shaken end-over-end for 2 h, followed by centrifugation at 10 000 rev min^{-1} (11 950 x *g*) for 10 min. Samples were filtered (Whatman No. 52) and stored at 4°C for up to a week or at -18°C for longer periods. All equipment was acid-washed, rinsed and air-dried prior to use. The stock standard for each element, made by diluting the appropriate 1000 µg mL^{-1} standard solution (Spectrosol® grade, BDH) to the required concentration with DI water, was used to construct a standard curve. Each standard was made up in a 100 mL volumetric flask with DI water, and included 1.45 mL of 16 M nitric acid (HNO_3) to stabilise the solution and prevent microbial growth. The extracts were analysed on a Varian 720-ES Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (Varian, Melbourne, Australia). A standard curve was created prior to each run and a reference standard was run after every 20 samples.

3.3 Microbial analyses

3.3.1 Phospholipid fatty acid (PLFA) analysis

The method used was based on the lipid extraction method of Bligh and Dyer (1959) with modifications by White et al. (1979) and Frostegård et al. (1991). The nomenclature used for fatty acids (FAs) was as described by Ratledge & Wilkinson (1988) and Frostegård et al. (1993b; 1993a). Each name consists of the total number of carbon atoms in the fatty acid molecule, then the number of double bonds, after a colon. The location of the first double bond from the methyl end of the molecule is indicated by ω and the position number. Multiple double bonds are assumed to be 3 chain positions apart (i.e. separated by a methylene group) (Ratledge and Wilkinson, 1988). Anteiso and iso branching are indicated by the prefixes a and i, respectively, while c and t suffixes denote cis and trans configurations, and cy refers to cyclopropyl fatty acids. An n-OH prefix indicates the presence of a hydroxyl group and its position from the carboxyl end of the molecule (Ratledge and Wilkinson, 1988). The structures of some of the major fatty acid methyl esters (FAMEs) analysed are presented in the Appendix.

3.3.1.1 Reagents and standards

All chemicals and solvents used were analytical reagent (AR) grade. The 0.15 M citrate buffer (Frostegård et al., 1991) comprised 31.5 g of citric acid (BDH) per 1 L of DI water. Sodium hydroxide pearls (Merck) were used to raise the pH to 4.0, and 20 mL of chloroform (Merck) was added to prevent microbial growth. Bligh and Dyer extractant (Bligh and Dyer, 1959) was made by combining 66 mL of chloroform with 132 mL of CH_3OH (Merck) and 53 mL of citrate buffer (ratio 1: 2: 0.8 v/v/v). The 0.2 M KOH solution was freshly made for each use, by dissolving 0.28 g of KOH pearls (BDH) in each 25 mL of CH_3OH required. The 1 M acetic acid solution was prepared by diluting 15.01 g of 100% acetic acid (Merck) to 250 mL of DI water, and microbial growth was inhibited by adding 10 mL of chloroform and storing at 4°C. Two internal standards were made by adding either methyl tridecanoate (13:0; 5.650 mg) or methyl nonadecanoate (19:0; 5.775 mg) to 25 mL of hexane (Merck) to make standards of 226.7 μg mL^{-1} and 230.8 μg mL^{-1}, respectively. These diluted working standards were stored at 5°C for a maximum of 3 months, while the remaining undiluted standards (Sigma-Aldrich) were stored at -18°C. The qualitative bacterial acid methyl ester (BAME) standard was made up by diluting Supelco BAME CP Mix 47080-U (10 mg mL^{-1}) 1:9 in ethyl acetate and was stored at -18°C.

3.3.1.2 Equipment cleaning

All equipment used for PLFA analysis was first washed with phosphorus-free detergent and rinsed thoroughly with tap water. This was followed by 3–4 rinses with reverse osmosis (RO)

water and 2 final rinses with DI water, and then air drying at 60–80°C. Dried glass tubes and vials plus Pasteur pipettes were placed in a muffle furnace at 450°C for 4 h to remove any traces of residual organic matter. Centrifuge and culture tube lids were rinsed with acetone before use. Both 35 mL screw-capped glass centrifuge tubes (Kimble/Kontes, Vineland, NJ, USA) and 50 mL Teflon® centrifuge tubes (Nalge Nunc, Rochester, USA) were used. The Teflon® tubes underwent washing and tap water rinsing, and were then soaked for 12–24 h in bleach (4.0% w/v available chlorine) followed by RO and DI rinsing and air drying.

3.3.1.3 Extraction

The gravimetric water content (θ_g) of the soil was determined the day before the extraction was undertaken (Section 3.2.1). Blank samples underwent all procedures, but without soil. Fresh soil (1.5 g (Grayston et al., 2001)) was weighed out into centrifuge tubes and citrate buffer was added to bring the total water content of soil plus buffer to 1.5 mL. Next 1.9 mL of chloroform, 3.8 mL of CH_3OH and 2 mL of Bligh and Dyer extractant were added and the mixture was vortexed for 1 min and then left to stand. After 2 h the tubes were centrifuged at 2500 rev min^{-1} (750 x *g*) for 10 min at 20°C and the supernatant was collected and transferred into a clean centrifuge tube. The soil pellet was then rinsed with 2.5 mL of Bligh and Dyer extractant by vortexing and centrifuging again, and this liquid was added to the original supernatant. Blank tubes were also re-rinsed with extractant. Following this, 3.1 mL of chloroform and 3.1 mL of buffer were added to the supernatant and the tubes were vortexed for 1 min and left to stand overnight. The next day, 3 mL of the lower chloroform phase was transferred by Pasteur pipette into clean scintillation vials and evaporated under a stream of N_2 until completely dry, using an Evap-o-Rac manifold (Cole-Parmer, Vernon Hills, IL, USA). The dried samples could then be stored at -18°C until the fractionation step was undertaken.

3.3.1.4 Lipid fractionation

The fractionation step separated the sample into different classes of lipids. Silicic acid columns (IST, Hengoed, UK) were placed over glass culture tubes and rinsed (x 3) with 0.1 mL of chloroform. Flow rates were checked by adding 1 mL of chloroform to the column; if the chloroform took more than 2 min to drain, the column was discarded. Three times the dried lipid material (from the extraction step) was dissolved in 0.1 mL of chloroform, vortexed for 10 seconds and transferred onto the column. The sample was left to set onto the column matrix for at least 2 min. Elution chemicals were added down the side of the column to avoid disturbing the fatty acids. Neutral lipids were eluted with 5 mL of chloroform and then glycolipids with 5 mL of acetone (LabServ), with both of these fractions being discarded. The

columns were then placed over clean centrifuge tubes, and the polar lipids were eluted with 5 mL of CH_3OH. The eluent was evaporated under N_2 in a water bath at 37°C. If necessary the tubes were stored at -18°C prior to methylation.

3.3.1.5 Methylation

Mild alkaline methanolysis was used to transform the phospholipids into fatty acid methyl esters (FAMEs), as this method of esterification is rapid and the yield is quantitative (Gutnikov, 1995; Berdeaux et al., 1999). The 13:0 and 19:0 internal standards (60 µL of each) were added to each tube and incorporated by adding 1 mL of toluene (Univar): CH_3OH solution (1:1 v/v) and vortexing for 30 seconds. Following this, 1 mL of fresh 0.2 M KOH was added and the tubes were vortexed for 30 seconds, before incubation in a 37°C water bath for 15 min. After this time, 2 mL of hexane: chloroform (4:1 v/v), 0.3 mL of 1 M acetic acid and 2 mL of DI water were added to the tubes, which were vortexed for 1 min. Following centrifugation for 5 min, the upper organic phase was transferred to a clean scintillation vial. The original mixture was rinsed again with 2 mL of the hexane: chloroform solution and the upper layer was added to the same scintillation vial, while the lower layer was discarded. Two mL of the organic phase was then transferred to a new clean scintillation vial (to give a quantitative final amount) and evaporated under N_2. The residue, containing the FAMEs to be analysed, was stored at -18°C.

3.3.1.6 Gas Chromatographic separation

After removal from storage, each sample was twice dissolved in 100 µL of ethyl acetate (BDH), vortexed for 20 seconds, and transferred by Pasteur pipette to a 250 µL insert in a labelled 2 mL gas chromatograph (GC) vial. The capped vials were placed on the GC autosampler for analysis. The qualitative BAME standard mixture (diluted as specified in Section 3.3.1.1) was analysed with each run. The run sequence was as follows: BAME – control 1 – first half of samples – BAME – second half of samples – control 2 – BAME. The GC analyses were carried out on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu, Tokyo, Japan) fitted with a Shimadzu AOC-20s autosampler. The column was a Restek Rtx®-5MS, which was 30 m in length x 0.25 mm ID x 0.25 µm stationary phase (Restek Cat. # 12623). The sample (1 µL) was injected at high pressure (240 kPa held for 5 min) in a splitless injection with the inlet at 250°C. The column programme started at 150°C and increased at 1.5°C per min to 200°C, then at 4°C per min to 240°C. At this point the peaks of interest had eluted and the temperature was ramped up to 300°C at 30°C per min and held for 7 min to remove any remaining residue from the column. This programme took 52.3 min and column flow was 1 mL min^{-1}. Detection was by Flame Ionisation Detector (FID) at 310°C.

3.3.1.7 Analysis of GC data

The FAs in the samples were identified by comparison with retention times for the BAME standard using 'GCsolution' software (version 2.21.00SUI, © 2000-2003 Shimadzu). The list of FAMEs identified in each sample was transferred onto a spreadsheet. The internal standards (13:0 and 19:0) were identified in each sample and the retention time of each listed FAME peak was compared with those of the internal standards. If these differences were the same as those in the BAME standard, the sample peak was identified as the BAME constituent.

Quantification (Microbial biomass)

The peak area data were corrected by subtracting the maximum blank value for that compound, and were then converted to nmole values by comparison with the quantitative 19:0 internal standard (Tunlid et al., 1989). The nmole values could then be converted to units of nmoles g^{-1} dry soil. The total microbial biomass (nmoles g^{-1} dry soil) was quantified by totalling the values for the constituents of the BAME standard, as listed in Table 3.2 below. Since C13:0 and C19:0 were added as internal standards, they were not included in this process.

Table 3.2 Constituents of the Bacterial Acid Methyl Ester (BAME) standard used for peak identification and for quantification and comparison of microbial groups.

FAME type	FAMEs present in BAME standard
Straight chain	11:0, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0
Hydroxylated	2-OH 10:0, 2-OH 12:0, 3-OH 12:0, 2-OH 14:0, 3-OH 14:0, 2-OH 16:0
Monounsaturated	16:1ω7c, 18:1ω9c, 18:1ω9t
Polyunsaturated	18:2ω6
Terminally branched	i-15:0, a-15:0, i-16:0, i-17:0
Cyclopropyl	cy-17:0, cy-19:0

Community structure analysis

Several analyses were carried out to determine the treatment effects on microbial community structure (Experiments 1, 2 & 3). The fungal/bacterial (F/B) ratio was calculated using the biomarkers listed in Table 3.3 below (Bardgett et al., 1996; Frostegård and Bååth, 1996), as was the ratio of Gram-positive to Gram-negative (G+/G-) bacteria. Both ratios indicate changes in the microbial community structure over time and between treatments. The degree of microbial stress was shown by calculating the following ratios for each treatment: i15:0 / a15:0 (i/a), cy17:0 / 16:1ω7 (cy/ω7), and 18:1ω9t / 18:1ω9c (t/c) (Section 2.4.2.4) (Guckert et al., 1986;

Chihib et al., 2003; Petersen et al., 2004a; Kaur et al., 2005). Although these biomarkers are specifically bacterial biomarkers, any stress indicated was presumed to be affecting the entire microbial community. Therefore these biomarker ratios are referred to as microbial stress ratios throughout this thesis.

Table 3.3 Signature fatty acid methyl ester (FAME) biomarkers used to identify specific bacterial groups for microbial community structure analysis.

Biomarkers for:	FAME biomarkers used	References
Bacterial biomass	i-15:0, a-15:0, 15:0, i-16:0, i-17:0, cy-17:0, 17:0, cy-19:0	(Tunlid et al., 1989; Frostegård et al., 1993b; Bardgett et al., 1996; Grayston et al., 2001; Nannipieri et al., 2003)
Fungal biomass	18:2ω6	(Federle, 1986; Frostegård and Bååth, 1996)
Gram+ bacteria	i-15:0, a-15:0, i-16:0, i-17:0	(O'Leary and Wilkinson, 1988; Zelles, 1997)
Gram– bacteria	16:1ω7c, cy-17:0, cy-19:0	(O'Leary and Wilkinson, 1988; Zelles, 1997)
Microbial stress	i-15:0, a-15:0	(Chihib et al., 2003; Petersen et al., 2004a)
Microbial stress	cy-17:0, 16:1ω7c	(Guckert et al., 1986; Petersen et al., 2004a; Kaur et al., 2005)
Microbial stress	18:1ω9t, 18:1ω9c	(Guckert et al., 1986; Kaur et al., 2005)

In addition to the analyses above, principal component analyses (PCA) were carried out on the relative percentages of the FAMEs (mol%), calculated by dividing the quantity of each individual FAME (nmole) by the total quantity of FAMEs (nmole) in each sample (Dytham, 1999; Hamer et al., 2007).

3.3.2 Dehydrogenase enzyme activity

Microbial activity was assessed with a non-specific enzyme assay that determined the dehydrogenase activity (DHA) in the soil (Thalmann, 1968; Alef, 1995), by measuring the rate of reduction of triphenyltetrazolium chloride (TTC) to triphenyl formazan (TPF).

3.3.2.1 Reagents

The 100 mM Tris-HCl buffer was made by dissolving 12.1 g of Tris (hydroxymethyl)-aminomethane (BDH) in 700 mL of DI water, adjusting the pH to 7.6 with HCl, and then adding DI water to make 1 L. The TTC solution was made by dissolving 0.7 g of TTC (Sigma)

in 100 mL of Tris buffer, and the TPF stock standard was prepared by dissolving 50 mg of TPF (Fluka) in 100 mL acetone (Analar grade). Standards (0–50 µg TPF mL^{-1}) were made by pipetting aliquots of the TPF stock standard into 50 mL volumetric flasks, adding 8.3 mL of Tris buffer, and making this up to 50 mL with acetone.

3.3.2.2 Method

All procedures were carried out under diffuse light because of the light sensitivity of TTC and TPF. Samples of field-moist soil (5 g) were mixed with 5 mL of TTC solution in 82 mL glass tubes, which were sealed with glass stoppers and then incubated in the dark for 24 h at 30°C. Each run included a blank containing only 5 mL of TTC solution. After the 24 h incubation, 40 mL of acetone was added to each tube and the contents were thoroughly mixed. The tubes were then incubated in the dark for a further 2 h, with shaking at half-hourly intervals. After this time, the solutions were filtered (Whatman No. 41) and the absorbance was measured (546 nm) on a Varian Cary 50 UV-Vis spectrophotometer (Varian Australia Pty Ltd, Melbourne, Australia) fitted with a dip probe. The absorbance reading for the blank was subtracted from the absorbance reading for each sample. Equation 3.5 was used to calculate DHA (Alef, 1995):

$$DHA = \frac{(TPF \times V)}{M_s}$$ **Equation 3.5**

where;
DHA = dehydrogenase activity (µg TPF g^{-1} dry soil)
TPF = concentration of TPF (µg mL^{-1})
V = volume of solution (TTC + acetone) (mL) [V = 45]
M_s = mass of oven dry soil (g)

3.3.3 Chloroform fumigation extraction

The chloroform fumigation extraction (CFE) method used for determining microbial biomass C (MBC) was based on that of Vance et al. (1987).

3.3.3.1 Reagents

The 0.5 M potassium sulphate extractant was prepared by dissolving 87.1 g of K_2SO_4 per litre of DI water. All chloroform used for the extraction was first purified to remove ethanol, a stabiliser that is a source of C (Jenkinson et al., 2004). The purification was carried out by shaking 200 mL of chloroform (Analar grade) with 400 mL of 5% H_2SO_4 in a 1 L separating funnel. The lower acid layer was discarded and the process was repeated twice more, followed

by a final rinse with 400 mL of DI water. The purified chloroform was stored in a Schott bottle with anhydrous Na_2SO_4 at 5°C until required for analyses.

3.3.3.2 Extraction

Duplicate sub-samples of moist soil (5 g) were weighed out. One sample was placed in a glass beaker and fumigated (as described below) and the other was put into a 70 mL plastic screw-top vial and extracted immediately with 20 mL of K_2SO_4 (1:4 soil to extractant ratio). The plastic containers were shaken end-over-end for 30 min and the contents were then filtered (Whatman No. 41) into 30 mL plastic vials and stored at 4°C for analysis within one week.

3.3.3.3 Fumigation

The beakers to be fumigated were positioned in a vacuum box located in a fume cupboard. A beaker containing 25 mL of purified chloroform and 2–3 boiling chips was placed at the bottom of the vacuum box. The box was evacuated using a vacuum pump until the chloroform was boiling, at which point the box was sealed and the pump disconnected. The box was then covered and the samples left to fumigate in the dark for at least 12 h. After this time, the vacuum box was evacuated and flushed (x 3) with fresh air to remove any remaining chloroform vapour. The beakers were then removed and the samples underwent extraction with K_2SO_4 as described above.

3.3.3.4 Calculation of microbial-C

Both the fumigated and unfumigated sub-samples were analysed for TOC as described in Section 3.2.6 above and the measured TOC was then converted to units of mg WSC kg^{-1} dry soil using Equation 3.3. For each soil sample the C content of the unfumigated treatment was subtracted from that of the fumigated treatment and the MBC was calculated by dividing the difference by the k_{EC} value of 0.45 (Jenkinson et al., 2004). This k_{EC} value is a constant that accounts for the efficiency of the soil microbial biomass extraction. The difference between fumigated and unfumigated sub-samples was assumed to be due to the release of organic C from microbial cells lysed by the chloroform treatment (Jenkinson, 1966, 1976).

3.4 Headspace gas sampling and analysis

The intact soil cores were placed into 0.5 L Mason jars sealed with metal screw-top lids that were pre-fitted with septa pierced by 3.8 cm 16 gauge hypodermic needles (Precision-Glide, Becton-Dickinson, NJ, USA). Each needle was topped with a 3-way stopcock (Baxter Healthcare Corp., IL, USA) to which a 20 mL glass syringe was attached. The syringe was flushed twice with ambient air before each sampling and twice with headspace air when attached to the stopcock, after which a gas sample was removed. Separate samples were taken for analysis for N_2O-N or CO_2-C. For each sample, 10 mL of headspace gas was transferred to pre-evacuated 6 mL Exetainer® vials (Labco Ltd, High Wycombe, UK), allowing the vials to be over-pressurised to prevent diffusion of external air into the sample. The vials were reduced to ambient pressure immediately prior to analysis using a double-ended needle to release the extra pressure into a beaker of water. Samples of ambient air to be used for initial gas concentration readings were collected at each sampling location before headspace gas sampling commenced. The temperature throughout sampling was 21–22°C, except in experiment 4 when core temperatures were the same during gas sampling as for incubation.

3.4.1 Gas chromatographic analysis

The gas samples were analysed on an automated SRI 8610 gas chromatograph (GC) (SRI Instruments, Torrance, CA, USA) interfaced with a Gilson 222XL liquid autosampler, configured as in Clough et al. (1998a) and similar to the configuration used by Mosier and Mack (1980). To enable gas analyses a purpose-built double concentric injection needle replaced the liquid sample sipper on the autosampler, allowing rapid purging of the gas sample for injection. A 1 m long pre-column preceded a 6 m long analytical column, both 3 mm OD stainless steel packed with Haysep Q. An automated 10-port gas-sampling valve on the GC sent the oxygen-free N_2 carrier gas (40 mL min^{-1}) through both the pre-column and analytical column in series (in inject mode) or back-flushed the pre-column. At the posterior end of the analytical column a 4-port gas-sampling valve was synchronised to send the gas stream to the detector.

3.4.2 Nitrous oxide

Nitrous oxide concentrations were quantified with a ^{63}Ni electron capture detector (ECD) at 360°C. Each batch of N_2O samples was preceded by a series of standards (1.2, 2.5, 5.8 and 9 ppm) and interspersed with 1.2 ppm reference standards. Samples for N_2O were taken at 0.5, 1 or 2 h after the jars were sealed. Emissions were calculated using Equation 3.6:

$$F_{N2O} = \frac{((C_1 - C_0) \times V_h \times C_L \times P \times MW_{N2O-N} \times C_{\mu g})}{(R \times T \times t \times A_s)}$$ **Equation 3.6**

where;

F_{N2O} = N_2O flux (µg N_2O-N m^{-2} h^{-1})

C_1 = N_2O concentration at time 1 (µL L^{-1})

C_0 = N_2O concentration at time 0 (µL L^{-1})

V_h = headspace volume [0.460 L]

C_L = conversion factor µL to L [0.000001 L μL^{-1}]

P = atmospheric pressure [1 atm]

MW_{N2O-N} = molecular weight of N_2O-N [28.01 g mol^{-1}]

$C_{\mu g}$ = conversion factor g to µg [1000000 µg g^{-1}]

R = universal gas constant [0.0821 L atm K^{-1} mol^{-1}]

T = temperature in Kelvin (K)

t = sampling time (h)

A_s = soil surface area [2.29 x 10^{-3} m^2]

3.4.2.1 Temperature coefficient (Q_{10})

The temperature coefficient (Q_{10}) is a measure of the rate of change of a biological or chemical reaction over a 10°C temperature increment. This was calculated for N_2O-N production using Equation 3.7 (Kirschbaum, 1995; Eskandari, 2009):

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{(T_2 - T_1)}\right)}$$ **Equation 3.7**

where;

Q_{10} = temperature coefficient

R_2 = rate of change of N_2O-N concentration at T_2

R_1 = rate of change of N_2O-N concentration at T_1

T_2 = higher temperature in range calculated

T_1 = lower temperature in range calculated

3.4.3 Carbon dioxide

The gas sample was separated on the GC column and the CO_2 was subsequently methanised by a Ni catalyst at 380°C. The methane (CH_4) thus produced was measured with a flame ionisation detector (FID). Preceding each batch of samples a series of CO_2 standards (0.2, 0.5, 1, 2 and 3%

v/v) were analysed, and used to create a standard curve. Interspersed with the samples were 1% reference standards. Equation 3.8 was used to calculate emissions of CO_2 in µg m^{-2} h^{-1}:

$$F_{CO2} = \frac{((C_1 - C_0) \times V_h \times C_L \times P \times MW_{CO2-C} \times C_{\mu g})}{(R \times T \times t \times A_s)}$$ **Equation 3.8**

where;

F_{CO2} = CO_2 flux (µg CO_2-C m^{-2} h^{-1})

C_1 = CO_2 concentration at time 1 (µL L^{-1})

C_0 = CO_2 concentration at time 0 (µL L^{-1})

V_h = headspace volume [0.460 L]

C_L = conversion factor µL to L [0.000001 L μL^{-1}]

P = atmospheric pressure [1 atm]

MW_{CO2-C} = molecular weight of CO_2-C [12.01 g mol^{-1}]

$C_{\mu g}$ = conversion factor g to µg [1000000 µg g^{-1}]

R = universal gas constant [0.0821 L atm K^{-1} mol^{-1}]

T = temperature in Kelvin (K)

t = sampling time (h)

A_s = soil surface area [2.29 x 10^{-3} m^2]

3.4.3.1 Metabolic quotient for CO_2

The metabolic quotient for CO_2 (qCO_2; µg CO_2 mg^{-1} MBC h^{-1}) was calculated by dividing the basal respiration rate (µg CO_2 h^{-1}) by the microbial biomass (mg MBC) (Anderson and Domsch, 1986).

3.4.4 Ammonia

Ammonia (NH_3-N) volatilisation was measured during the N_2O-N and CO_2-C sampling periods in experiments 3 and 4 by suspending an acid trap at the top of each sealed Mason jar. The method used was based on the diffusion method of Brooks et al. (1989) normally used to measure ^{15}N enrichment of NH_4^+-N, but modified by using phosphoric acid as in Robacker and Bartelt (1996). This short-term collection method was tested by incubating a basic ammonium sulphate solution of known concentration for 1 h with 91–94% (± 0.8) recovery of NH_4^+-N. The acid traps consisted of a strip of Whatman No. 41 filter paper moistened with one drop (ca. 23 µL) of orthophosphoric acid. After 1 h the acid traps were removed and placed into individual 30 mL screw-top plastic containers. Ten mL of DI water was added, the containers were shaken, and the solution was analysed by FIA for NH_4^+-N. Assuming that all the NH_3-N

evolved in the 1 h sampling period was trapped and converted to NH_4^+-N by the acid trap, NH_3-N fluxes were calculated using Equation 3.9:

$$N_f = \frac{(N_e \times V)}{(t \times A_s)} \qquad \textbf{Equation 3.9}$$

where;
N_f = NH_3-N flux (mg m^{-2} h^{-1})
N_e = NH_3-N concentration of extract (mg L^{-1})
V = volume of extractant (L)
t = sampling time (h)
A_s = soil surface area [2.29 x 10^{-3} m^2]

These hourly fluxes were converted into per day values, which were then integrated to yield the total cumulative NH_3-N flux over the sampling time (Equation 3.10). This allowed the NH_3-N loss as a percentage of N applied to be calculated.

$$N_{fc} = \Sigma \left((P_S \times P_1) + (0.5 \times (P_S \times (P_2 - P_1))) \times A_s \right) \qquad \textbf{Equation 3.10}$$

where;
N_{fc} = NH_3-N cumulative flux (mg)
P_S = sampling period (d)
P_1 = flux at start of sampling period (mg m^{-2} d^{-1})
P_2 = flux at end of sampling period (mg m^{-2} d^{-1})
A_s = soil surface area [2.29 x 10^{-3} m^2]

3.5 Isotope tracer analyses

3.5.1 Addition of isotope

Urea, enriched with ^{15}N (98.0 atom% $^{15}N_2$-urea; Isotec, Miamisburg, Ohio), was added to the urine to allow isotope trace analysis of urea-N, the dominant N form applied in urine, on selected sampling days (experiments 1 and 3). The final ^{15}N enrichment of the urine was between 5 and 6 atom%. All isotopically labelled samples were analysed on a Continuous Flow Isotope Ratio Mass Spectrometer (IRMS) (PDZ Europa Ltd, Crewe, UK).

3.5.2 Gas IRMS samples

3.5.2.1 Nitrous oxide -^{15}N sampling

The 15 mL headspace N_2O-^{15}N samples were removed at the same time as the GC sample for headspace N_2O-N, and were transferred to pre-evacuated (-1.0 atm) 12 mL Exetainers®. This ensured that the Exetainers® were over-pressurised, to prevent sample contamination. Analyses were carried out as in Section 3.5.2.4.

3.5.2.2 Nitrite-^{15}N analyses

Reagents and equipment

A 1 M solution of hydrochloric acid (HCl) was prepared by diluting 4.15 mL of 37% HCl in DI water to make 50 mL final volume. A hydroxylamine solution (0.04 M NH_2OH) was made by dissolving 0.278 g of hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$) in DI water to make 100 mL final volume. The glass medical flat bottles (108 mL) that were used were acid-washed (1 M HCl) and the screw caps and liners were washed with phosphorus-free detergent, before several RO water rinses and one final DI water rinse, and then air-drying.

Method

The method used was based on that of Stevens and Laughlin (1994), using the reaction below (Equation 3.11) to produce N_2O:

$$HNO_2 + NH_2OH \rightarrow N_2O + 2H_2O$$ **Equation 3.11**

A sufficient amount of the KCl extract (Section 3.2.4) to give a final concentration of 75 µL L^{-1} of N_2O in the headspace was transferred to a flat bottle, and the total volume of solution was made up to 50 mL by adding additional 2 M KCl. Then 1 mL HCl and 0.5 mL NH_2OH were added and the bottles were capped and placed on an orbital shaker at 120 rev min^{-1} for 16 h at 20°C. After this time 15 mL headspace gas samples were transferred to 12 mL Exetainers® for analysis by IRMS as in Section 3.5.2.4. Each batch of samples included a control containing a measured volume of KNO_2 solution of known enrichment in place of the soil extract.

The ^{15}N enrichment of the NO_2 was calculated using Equation 3.12 (Stevens and Laughlin, 1994):

$$Atom\%\ excess\ {}^{15}N\ in\ NO_2 = (2 \times Atom\%\ excess\ {}^{15}N\ in\ N_2O) - Atom\%\ excess\ {}^{15}N\ in\ NH_2OH$$

Equation 3.12

3.5.2.3 Nitrate-^{15}N analyses

Reagents and equipment

Sulphamic acid (2g NH_2SO_3H) was dissolved in 100 mL DI water to make a 0.2 M solution. The buffer was prepared by dissolving 136 g of sodium acetate trihydrate ($CH_3COONa{\cdot}3H_2O$) in DI water and adding 57.3 mL acetic acid (CH_3COOH) and additional DI water to make 1 L of 1 M: 1 M solution. Copper sulphate solution (0.04 M) was made by dissolving 10.0 g $CuSO_4{\cdot}5H_2O$ in DI water to make 1 L final volume, and 6 M hydrochloric acid (HCl) was made by diluting 516 mL of 12 M HCl with DI water to make 1 L final volume. Cadmium/copper (Cd/Cu) reductors were prepared by placing 10 mm lengths of solid Cd rod in 6 M HCl for 1 min and rinsing with DI water, followed by 1 min exposure to 0.04 M $CuSO_4$ and further rinsing with DI water (Keeney and Nelson, 1982).

Method

The method was based on that of Stevens and Laughlin (1994), using the reaction in Equation 3.11 to create N_2O. An aliquot of the KCl extract, sufficient to give a final concentration of 75 $\mu L\ L^{-1}$ of N_2O in the headspace, was pipetted into the flat bottle and the volume made up to 50 mL with 2 M KCl. Sulphamic acid (2.5 mL) was then added and each bottle was capped and shaken for 5 seconds. This converted the existing NO_2-N into N_2-N, ensuring that only NO_3^--N would contribute to the measured N_2O. Following this, the pH was raised from 1.7 to 4.7 by adding 5 mL of buffer solution and a Cd/Cu reductor was placed in each bottle. The bottles were then recapped and shaken horizontally at 120 rev min^{-1} for 2 h at 20°C. Headspace gas samples (15 mL) were then transferred to 12 mL Exetainers® and analysed by IRMS as in Section 3.5.2.4. Every batch of samples included a control containing a measured volume of KNO_3 solution of known enrichment in place of the soil extract. The ^{15}N enrichment of the NO_3 was calculated using Equation 3.13 (Stevens and Laughlin, 1994):

$$Atom\%\ excess\ ^{15}N\ in\ NO_3 = Atom\%\ excess\ ^{15}N\ in\ N_2O \qquad \textbf{Equation 3.13}$$

3.5.2.4 IRMS analysis

Immediately prior to analysis, the Exetainers® were reduced to ambient pressure using a double-ended needle to release the extra pressure into a beaker of water. The gas samples were automatically injected into the TGII trace gas system and the N_2O in the gas sample was concentrated by cryo-trapping and focusing. The gas was then relayed in the He carrier flow to the IRMS. Each run was bracketed by N_2O standards (35 $\mu L\ L^{-1}$) and interspersed with 2 standards after every 9 samples analysed.

3.5.3 Liquid IRMS samples

3.5.3.1 Ammonium-^{15}N analyses

Reagents and equipment

A 2.5 M solution of potassium hydrogen sulphate ($KHSO_4$) was prepared by adding 7 mL of concentrated sulphuric acid (H_2SO_4) and 22 g of potassium sulphate (K_2SO_4) to DI water to make 100 mL final volume. Heavy magnesium oxide (MgO) powder was heated in a muffle furnace at 600°C for 2 h, cooled, and then stored in an airtight container. Lengths of stainless steel wire (7 cm) were bent to allow them to be wedged across the top of a 120 mL plastic screw-top container. A 5 mm disk, cut from glass filter paper with a hole punch, was threaded onto each wire and 10 mL of 2.5 M $KHSO_4$ was pipetted onto the disk.

Method

The method used for NH_4-^{15}N diffusion was based on that of Brooks et al. (1989), and entailed creating a basic environment to convert all NH_4^+ to NH_3 gas, which was then trapped on the acidified filter paper. Sufficient KCl soil extract to provide a total of 50 µg NH_4-N was pipetted into each container and 0.2 g MgO was added. Immediately a wire and acidified disk were inserted across the top of the container and the lid was replaced. The contents of each container were carefully swirled with a glass bead so that the ingredients would mix without splashing the disk. The containers were incubated at room temperature (20–25°C) for 6 days. After this time the wires were removed with tweezers and placed into a drying rack, with the end of the wire in contact with the tweezers being put into the hole in the rack. This ensured that the disks could be pushed off the uncontaminated end of the wire when dry. After 12–24 h in a 50°C oven the dried disks were removed and placed into individual tin capsules for IRMS analysis.

IRMS analysis

The encapsulated diffusion disks were placed into an autosampler tray atop a GSL automated Dumas system and the samples were dropped in sequence into a combustion tube and combusted at 1000°C in an oxygen atmosphere. The gases generated were carried onto a GC column for separation and then into the IRMS for analysis. Each run commenced with a series of reference and calibration standards, all using peach leaves standard (NIST 1546; 2.13‰ vs. N_2 in air), which had been calibrated against IAEA N-1 (0.4‰ vs. N_2 in air). In addition, two reference standards were included after every 10 samples, and the first sample of every nine was analysed in duplicate.

3.6 Statistical analysis

Statistical analysis of data was performed using GenStat Release 11.1 (© 2008, VSN International Ltd.). Data were tested for normality before performing analyses of variance (ANOVAs), with 95% confidence limits ($P < 0.05$) used to indicate levels of significance. The calculated N_2O-N and CO_2-C emissions were log transformed (ln + 1) for statistical analysis, to ensure normal distribution. Least significant differences (LSD) were calculated where differences were indicated. Correlations were carried out between the data sets collected for each experiment.

Principal component analysis (PCA) was carried out on the PLFA data to determine which of the measured variables explained the greatest variation in the data (Dytham, 1999). The concentrations of individual PLFAs were divided by the total concentration of PLFAs to convert them into mol% data. These values were then analysed by PCA to generate principal components (axes). The first principal axis explained the most variation, with the variation explained by subsequent axes declining sequentially, as shown by eigenvalues. A components matrix indicated the variables that corresponded best with the axes, with higher numbers (either positive or negative) showing the best match. In this way it was possible to see which variables (in this case individual PLFAs) were the most important in relation to trends in the data. Principal component scores, generated for each sample, allowed the data to be plotted on the axes. Only the data for the first two principal components were plotted against each other. The graphs indicated differences between data due to treatment or time effects. Graphing was carried out using SigmaPlot Version 8.02 (© 1986-2001, SPSS Inc.).

Chapter 4
Effects of soil moisture and urinary salt concentrations on the soil microbial community

4.1 Introduction

Despite the large number of studies into urine patch chemistry, there have been few investigations on the effects of ruminant urine on soil microbiology. This controlled laboratory experiment monitored the effects of soil moisture content and urinary salt concentration on the soil microbial community and associated nutrient dynamics in the urine patch.

The soil moisture content affects microbially mediated N dynamics, with denitrification occurring in anaerobic sites while nitrification requires aerobic conditions (Monaghan and Barraclough, 1992; Bateman and Baggs, 2005). Therefore the soil moisture treatments chosen for this study were 35% water-filled pore space (WFPS) to favour nitrification and 70% WFPS to favour denitrification (Sections 2.5.1.3 & 2.5.1.4).

The EC of the soil solution increases greatly following urine deposition (Haynes and Williams, 1992), and the increased osmotic pressure may cause microbial cells to lyse or to release organic compounds to counter the pressure changes (Section 2.3.2.3). Microbial stress can be detected by monitoring specific PLFAs, which increase when the cell membrane responds to environmental stresses (Table 3.3). The analyses carried out for this experiment therefore consisted of measurements of microbial community structure by PLFA, as well as of microbial biomass and activity. In addition, soil chemical variables and gas fluxes were measured to determine if treatment effects on soil microbes corresponded to changes in nutrient cycling.

The hypotheses tested were:

1) that the addition of urine with a higher salt content would stress the soil microbial community,

2) that altering the soil moisture content would cause measurable changes in the soil microbial community, and

3) that any changes in microbial stress or microbial community structure would be reflected in changes in nutrient dynamics.

4.2 Materials and Methods

4.2.1 Experimental design and treatments

The experiment was conducted using a 2 x 3 factorial design, with five replicates. The factors were soil moisture at two levels (wet and dry) and cow urine at three levels (no urine, low salt urine and high salt urine), giving a total of six treatments (Table 4.1). Soil moisture in the dry treatments was maintained at 29% and 35% WFPS in the no urine and urine-amended cores respectively. In the wet treatments the soil moisture was 58% and 70% WFPS in the no urine and urine-amended cores respectively. Salt treatments were imposed by varying the urinary K^+ concentration, and were 10.4 mg K^+ L^{-1} (as collected; see below) and 15 mg K^+ L^{-1} in the low and high salt treatments respectively. There were a total of 210 soil cores, allowing destructive sampling on seven occasions between days 0 and 44, and these were arranged in five randomised blocks.

Table 4.1 Summary of treatments used in Experiment 1. The experiment was a 2 x 3 factorial design with 2 levels of soil moisture and 3 urine treatments.

Moisture	Urine		
	No urine	Low salt urine (10.4 mg K^+ L^{-1})	High salt urine (15 mg K^+ L^{-1})
Dry (WFPS)	Dry control (DC) (29%)	Dry + low salt (DLS) (35%)	Dry + high salt (DHS) (35%)
Wet (WFPS)	Wet control (WC) (58%)	Wet + low salt (WLS) (70%)	Wet + high salt (WHS) (70%)

4.2.2 Soil collection & soil core preparation

A Templeton silt loam soil (Typic Immature Pallic; New Zealand Soil Classification (Hewitt, 1998)) was collected from the Lincoln University Dairy Farm (LUDF) (43°38.4S, 172°26.6E) from a depth of 0–10 cm. The pasture was a 5-year old ryegrass/clover and received 200 kg N ha^{-1} yr^{-1} as superphosphate and urea. The soil was sieved (4 mm), and aggregates > 4 mm, stones and organic matter were discarded. A 4 mm mesh was used so that aggregates large enough to support fungal hyphae were retained, while reducing the number of roots, which also contain the 18:2ω6 PLFA used as a fungal biomarker (Harwood and Russell, 1984). Sieved soil was packed to a depth of 60 mm in poly-vinyl chloride (PVC) tubes (70 mm high x 50 mm ID), at a bulk density of 0.89 g cm^{-3}. The tubes had nylon mesh (< 0.5 mm) attached to their bases to prevent soil loss. Prior to urine application, the soil cores were incubated at 21°C for 5 d.

During this incubation, the cores were maintained at 29 or 58% WFPS depending on their experimental water treatment, to allow equilibration at this soil moisture content. Following urine application each soil core was covered with pierced clear plastic, permitting gas diffusion between the soil core headspace and the atmosphere, while reducing moisture loss. Soil cores were then incubated in a temperature-controlled laboratory (21°C) until sampling. Moisture levels were maintained by pipetting DI water slowly onto the soil surface 3 times a week to maintain constant soil core weights (Table 4.1).

4.2.3 Urine collection, amendment and application

Urine was collected from Friesian dairy cows at a local dairy farm 24 h before application and was immediately stored at 4°C until use. The urine contained 4.3 g N L^{-1} and 10.4 g K^+ L^{-1} (Section 3.1). Amendment with urea prior to application took the total urine-N concentration to 15.0 g N L^{-1}, which is in the upper range of N concentrations found in bovine urine (Bristow et al., 1992). This additional urea was ^{15}N-enriched (98.0 atom% $^{15}N_2$-urea; Isotec, Miamisburg, Ohio) and produced a final urine-^{15}N enrichment of 5.36 atom%. For the high salt treatment extra K^+ was added in the form of reagent grade potassium chloride (KCl; BDH). Urine was applied at a rate equivalent to 750 kg N ha^{-1} by pipetting 9.8 mL onto each soil core. This took the soil cores to their experimental soil moisture content levels.

4.2.4 Soil sampling

A sub-set of 30 soil cores (5 replicates of 6 treatments) was destructively sampled on seven occasions between day 0 (urine application) and day 44, following headspace gas sampling for N_2O-N and CO_2-C and measurement of the soil surface pH (Sections 3.4 and 3.2.2). The top 20 mm of the soil (ca. 40 g) was then removed and mixed well for approximately 10 seconds in a small resealable plastic bag. This soil depth was expected to show the greatest effects of urine application, particularly with respect to the potential changes in EC, pH and NH_3-N volatilisation at the soil surface (Sherlock and Goh, 1984; Haynes and Williams, 1992). Sub-samples of this mixed soil were immediately taken for analyses that required field moist soil, and the remainder was air-dried at 25°C for a minimum of 48 h.

4.3 Results

4.3.1 Soil pH

The pH in the urine treatments increased immediately following urine application (Figure 4.1), but declined after day 1 to be lower than the control treatments by day 29. On days 0–15 the pH was higher in all the urine treatments than in the control treatments ($P < 0.001$), whereas on

days 29 and 44 it was higher in the control treatments ($P < 0.01$). The pH decline in the wet urine treatments was greater than in the dry urine treatments, regardless of salt treatment. The pH of the wet urine treatments was higher than that of the dry urine treatments on days 3 and 15 ($P < 0.05$), but on days 29 and 44 the pH of the dry treatments was higher ($P < 0.001$). The salt treatment only affected soil pH on day 0 ($P < 0.001$) and day 3 ($P < 0.05$), when the pH was higher in the low salt treatments.

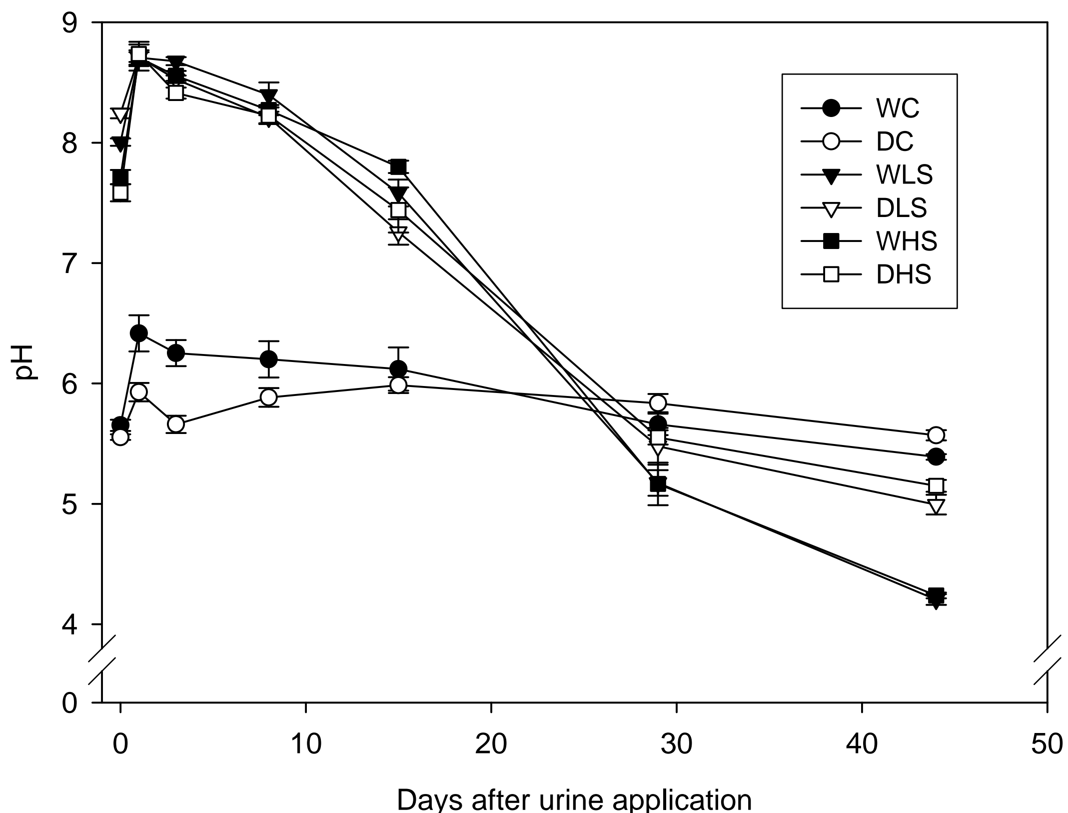


Figure 4.1 Mean soil pH of all treatments measured in the top 20 mm of bare soil cores after urine application (Error bars = SEM; n = 5).

4.3.2 Inorganic N

Soil NH_4^+-N concentrations had increased by day 1after urine application and gradually declined thereafter (Figure 4.2a). Salt treatments had no effect on NH_4^+-N concentrations in the urine treatments throughout the experiment ($P > 0.05$). Within the urine treatments, the wet treatments had higher concentrations of NH_4^+-N than the dry treatments on days 1–8 ($P \leq 0.01$). There was no difference between the wet and dry treatments on day 15, but concentrations were then lower in the wet treatments on days 29 and 44 ($P < 0.001$).

Rates of change in concentrations of NH_4^+-N remained close to zero throughout the experiment in both the control treatments (Table 4.2). In the urine treatments, NH_4^+-N concentrations increased more rapidly in the wet treatments on days 0–1 ($P < 0.001$). After day 1, mean rates of NH_4^+-N decrease were higher in the wet treatments until days 29–44, when they were higher in the dry treatments ($P < 0.001$).

Soil NO_2^--N concentrations remained low in the control treatments (< 0.06 mg kg^{-1}), but increased after urine application, peaking on day 1 in the dry urine-treatments and on day 8 in the wet urine treatments (Figure 4.2b). Nitrite concentrations were significantly higher in the urine treatments than in the control treatments on all days in the wet soils ($P < 0.001$), but only on days 1 and 3 in the dry soils ($P < 0.001$). On day 1, the concentration of NO_2^--N in the DHS treatment was significantly higher than in all the other treatments ($P < 0.001$), whereas the concentration in the DLS treatment did not differ from those in the wet urine treatments. After day 1, NO_2^--N concentrations were higher in the wet urine treatments than in the dry urine treatments ($P < 0.01$). There was no difference due to salt treatments on any day.

Nitrate-N concentrations increased rapidly in the urine treatments after day 3 (Figure 4.2c). The salt treatment did not affect soil NO_3^--N concentrations, but the differing soil moisture regimes did ($P \leq 0.001$), on all days except day 8 ($P = 0.05$). Within the urine treatments, soil NO_3^--N concentrations were initially higher in the dry treatments (days 1 and 3) but after this they were higher in the wet treatments. Differences between the control treatments followed a similar trend, with the DC having significantly higher NO_3^--N concentrations than the WC until day 8 ($P < 0.001$) and the reverse occurring thereafter ($P < 0.01$).

On days 0–1 the rates of change in the NO_3^--N concentrations were highest in the control treatments (Table 4.2). Within the urine treatments, the rate of NO_3^--N change was higher in the DLS treatment on days 0–1 and in the DHS treatment on days 1–3 compared to the other treatments ($P < 0.001$). The rate of change was then lowest in the DHS treatment between days 3 and 15. At the same time the rate was higher in the wet treatments than in the dry treatments, but the difference was only significant for the WLS treatment ($P < 0.001$). From days 15–44 the rates of change in NO_3^--N concentration did not differ due to treatment.

In all the urine treatments, NH_4^+-N had a strong negative correlation with NO_3^--N ($r \leq -0.94$, $P < 0.01$) and a strong positive correlation with pH ($r \geq 0.91$, $P < 0.05$). There was a very strong negative correlation between NO_3^--N and pH ($r \leq -0.95$, $P < 0.01$) in all treatments except the DC.

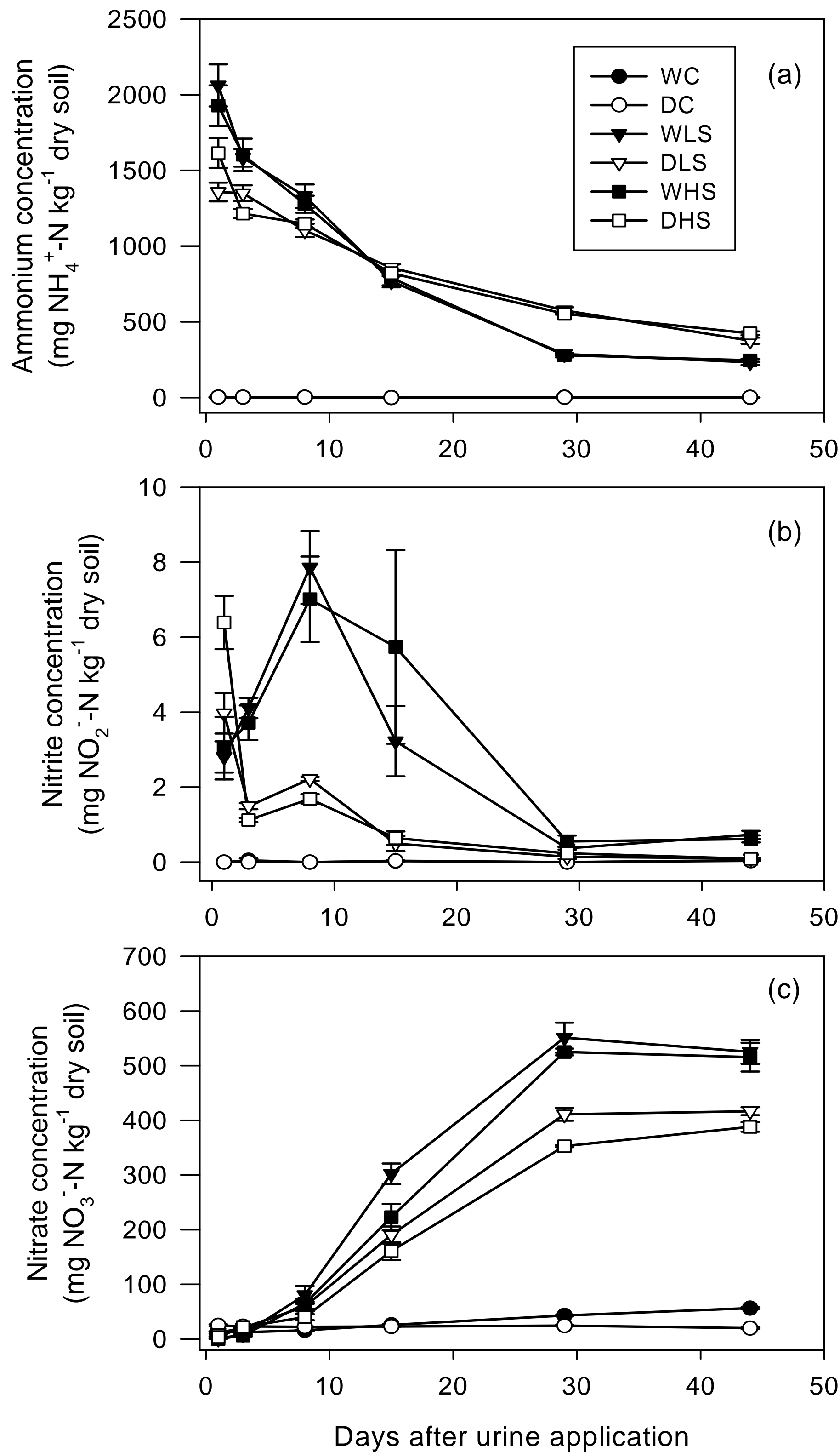


Figure 4.2 Mean soil (a) NH_4^+-N, (b) NO_2^--N, and (c) NO_3^--N concentrations determined in the top 20 mm of bare soil cores following urine application (Error bars = SEM; n = 5). NOTE differing scales on y axes.

Table 4.2 Rates of change in the soil NH_4^+-N and NO_3^--N concentrations (mg N kg^{-1} dry soil d^{-1}) in the top 20 mm of bare soil cores. Negative rates indicate declining concentrations.

	Time (days)					
	0-1	1-3	3-8	8-15	15-29	29-44
	NH_4^+-N (mg N kg^{-1} dry soil d^{-1})					
Treatment						
WC	3	0	0	0	0	0
WLS	2062	-239	-51	-80	-34	-4
WHS	1928	-163	-65	-69	-37	-2
DC	2	0	0	0	0	0
DLS	1358	-4	-49	-35	-20	-13
DHS	1615	-200	-13	-47	-19	-9
significance	***	*	*	***	***	***
LSD (0.05)	268.2	187.3	42.89	21.17	6.03	3.32
df = 29, n = 5						
	NO_3^--N (mg N kg^{-1} dry soil d^{-1})					
Treatment						
WC	13.6	-0.6	0.7	1.5	1.2	0.9
WLS	1.0	3.6	14.5	31.6	17.8	-1.7
WHS	0.0	3.2	11.7	22.6	21.6	-0.6
DC	25.0	-0.9	-0.2	0.1	0.1	-0.3
DLS	11.3	4.5	7.8	18.7	15.8	0.4
DHS	3.5	9.2	3.7	17.2	13.7	2.4
significance	***	***	***	***	NS	NS
LSD (0.05)	3.64	2.93	4.81	12.81		
df = 29, n = 5						

* $P < 0.05$ *** $P < 0.001$
** $P < 0.01$ NS = not significant

4.3.3 Water soluble C and soil moisture content

Water soluble carbon (WSC) concentrations increased immediately after the addition of urine (Figure 4.3), and remained higher than in the control treatments until day 15 ($P < 0.001$). The wet urine treatments had higher WSC concentrations than the dry urine treatments over the first four sampling days ($P < 0.05$). There were no significant differences due to salt treatments. In all the urine treatments WSC had a strong positive correlation with pH ($r \geq 0.93$, $P < 0.01$) and NH_4^+-N ($r \geq 0.96$, $P < 0.01$), and a strong negative correlation with NO_3^--N ($r \leq -0.93$, $P <$ 0.01).

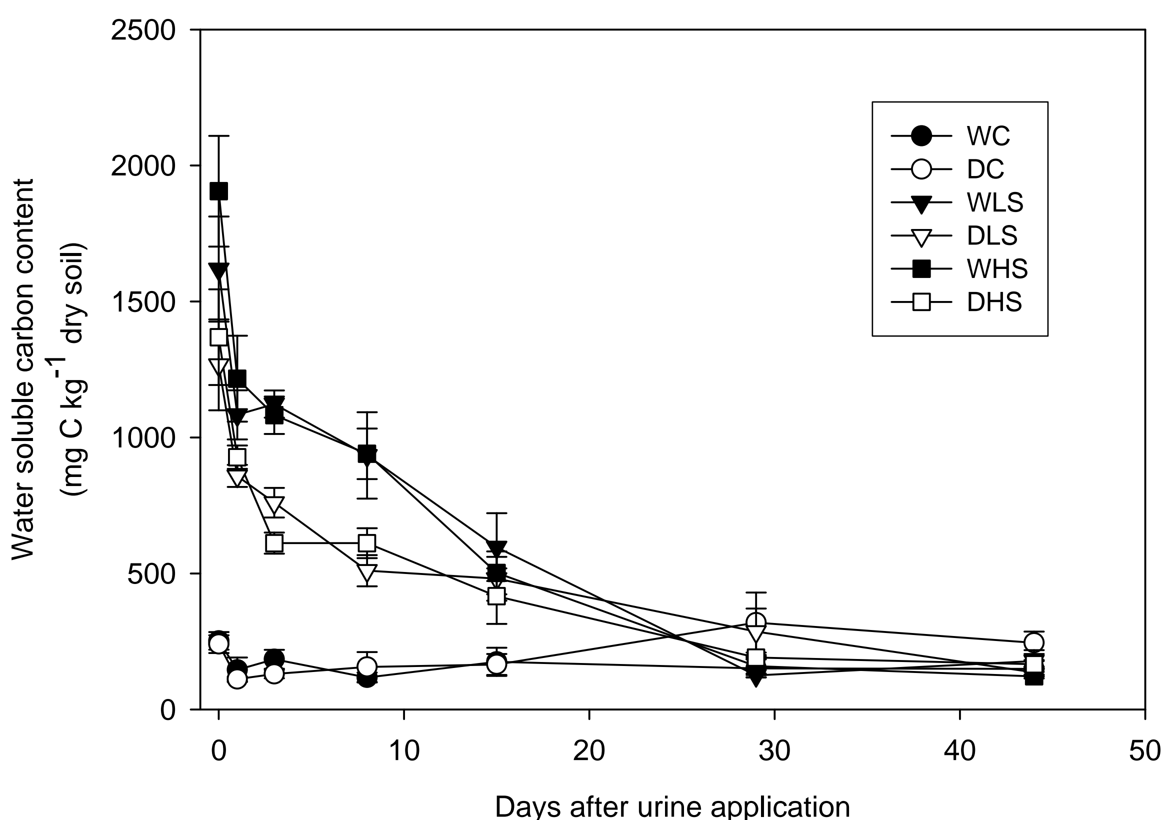


Figure 4.3 Mean concentration of water soluble carbon determined in the top 20 mm of bare soil cores following urine application (Error bars = SEM; n = 5).

The gravimetric moisture contents in the urine treatments remained stable over the course of the experiment (Figure 4.4), averaging 0.50 and 0.28 g water g^{-1} dry soil (66 and 37% WFPS) for the wet and dry treatments respectively. The difference between the two moisture treatments was significant on all sampling occasions because the cores were maintained at the initial high and low moisture contents. The moisture contents of the control treatments were lower than those of the urine treatments at each moisture level ($P < 0.05$) and averaged 0.44 and 0.21 g

water g^{-1} dry soil (59 and 28% WFPS) for the wet and dry treatments respectively. The salt treatments had no effect on soil moisture.

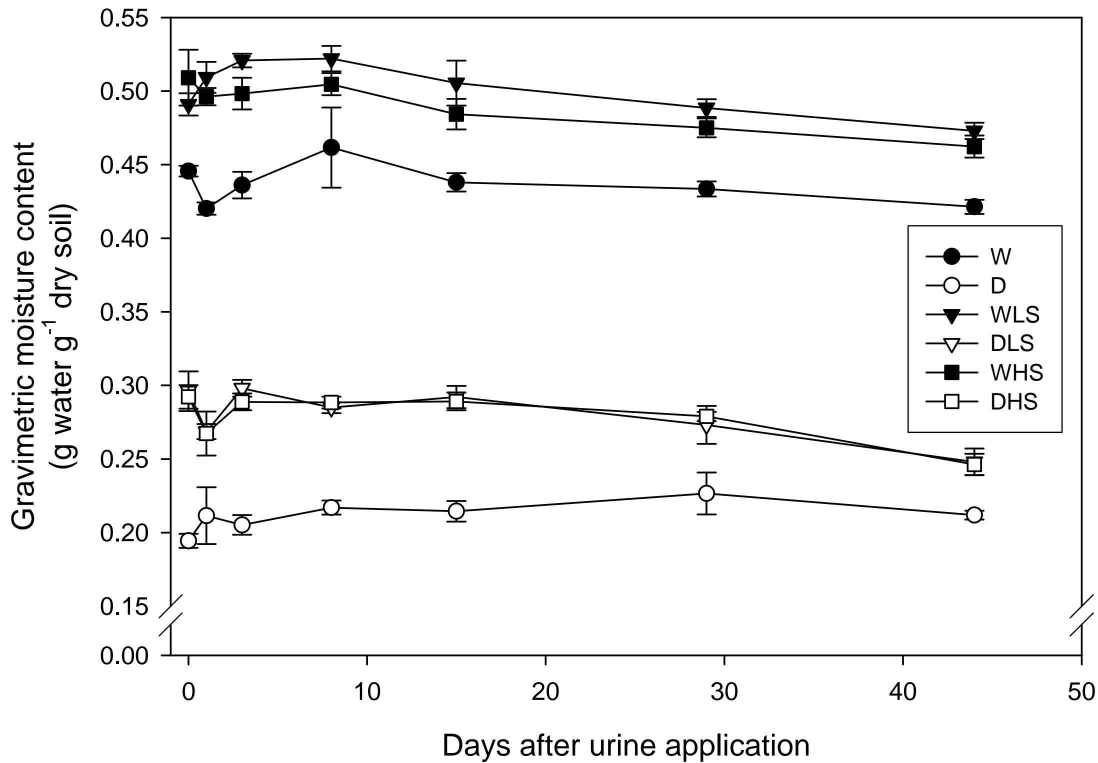


Figure 4.4 Mean gravimetric moisture content measured in the top 20 mm of bare soil cores during the experiment (Error bars = SEM; n = 5).

4.3.4 Electrical conductivity and ionic concentrations

Urine application increased electrical conductivity (EC) from day 1 ($P < 0.001$) (Figure 4.5) and it remained high throughout the experiment in the urine treatments. The mean EC values of the low and high salt treatments were 6–15 times higher and 6–21 times higher than the control values, respectively. In the control soils EC averaged 0.10 dS m^{-1} throughout the experiment. In the urine treatments, EC peaked on day 1, being highest in the WHS soil at 1.80 dS m^{-1}. The mean EC was consistently higher in the high salt treatments than in the low salt treatments, and the difference varied between 0.09 and 0.50 dS m^{-1}. The soil moisture content did not affect the EC.

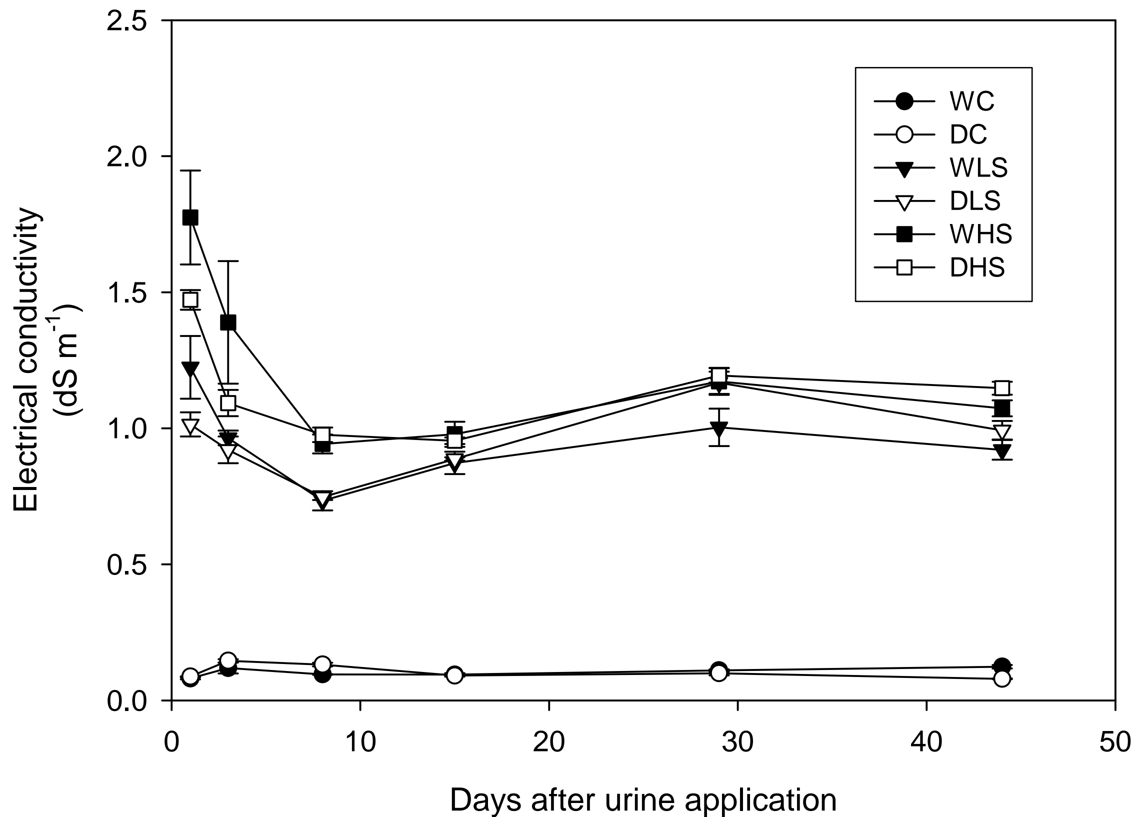


Figure 4.5 Mean electrical conductivity determined in the top 20 mm of bare soil cores following urine application (Error bars = SEM; n = 5).

Concentrations of both K^+ and Na^+ were initially high following urine amendment, but decreased between day 1 and day 15, and then remained constant (Figures 4.6a & 4.6b). The highest mean concentrations were 4.1 $cmol_c\ kg^{-1}$ for K^+ and 1.7 $cmol_c\ kg^{-1}$ for Na^+ in the WHS treatment on day 1. The urine treatments had higher concentrations of both cations than the control treatments on all sampling occasions ($P < 0.001$). Within the urine treatments, mean K^+ concentrations were higher in the high salt treatments than in the low salt treatments on all days, while mean concentrations of both cations were higher in the wet treatments than in the dry treatments throughout the experiment.

In contrast to K^+ and Na^+, concentrations of both Mg^{2+} and Ca^{2+} were initially low in the urine treatments, remaining below 0.05 $cmol_c\ kg^{-1}$ and 0.36 $cmol_c\ kg^{-1}$ respectively until day 15 (Figures 4.6c & 4.6d). However, concentrations of both divalent cations had increased by day 29 and remained high until day 44. In the urine treatments, both Mg^{2+} and Ca^{2+} reached their highest concentrations by day 29 in the wet treatments, whereas concentrations in the dry treatments continued to increase throughout the experiment, yet remained lower than in the wet treatments. After urine amendment, Mg^{2+} concentrations were significantly higher in the wet

treatments than in the dry treatments on all days except day 15, and Ca^{2+} concentrations were significantly higher in the wet treatments on all days. There was no difference in concentrations of either divalent cation due to the salt treatments.

There were strong correlations between Ca^{2+} and Mg^{2+} ($r = 1.00$, $P < 0.001$) and K^+ and EC ($r \geq 0.79$) in all the urine treatments, and K^+ was strongly correlated with both Na^+ ($r \geq 0.92$, $P < 0.01$) and Cl^- ($r \geq 0.83$, $P < 0.05$) in the high salt treatments.

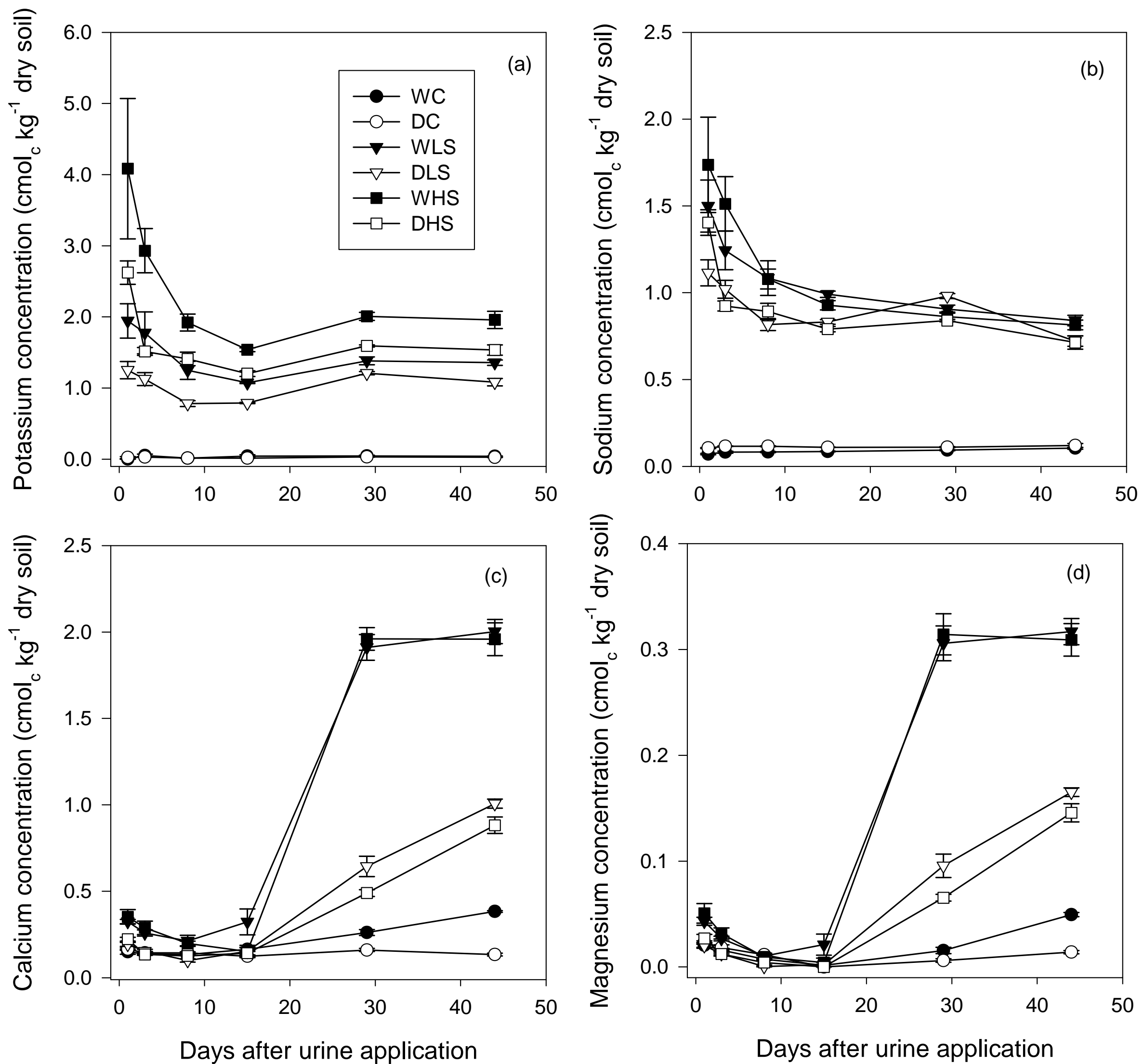


Figure 4.6 Mean concentrations of water-extractable cations (cmol$_c$ kg^{-1} dry soil) measured in the top 20 mm of bare soil cores following urine application; (a) potassium, (b) sodium, (c) calcium, and (d) magnesium (Error bars = SEM; n = 5). NOTE differing scales on y axes.

Concentrations of Cl^- and SO_4^{2-} were initially high in the urine treatments and decreased rapidly over the first 8 d (Figures 4.7a & 4.7b), but remained higher in the urine treatments than in the control treatments throughout the experiment ($P < 0.01$). Chloride concentrations were higher in

the high salt treatments than in the low salt treatments throughout the study ($P < 0.01$), while SO_4^{2-} concentrations did not differ between the high and low salt treatments. Within the urine treatments, the soil moisture had no effect on Cl^- concentrations, while SO_4^{2-} concentrations were higher in the wet treatments on day 3 ($P < 0.05$) and in the dry treatments on days 15 and 29 ($P < 0.01$). In all the urine treatments SO_4^{2-} was strongly and positively correlated with WSC ($r \geq 0.82$, $P < 0.05$), Cl^- ($r \geq 0.95$, $P < 0.01$) and NH_4^+-N ($r \geq 0.87$, $P < 0.05$), and negatively correlated with NO_3^--N ($r \leq -0.72$).

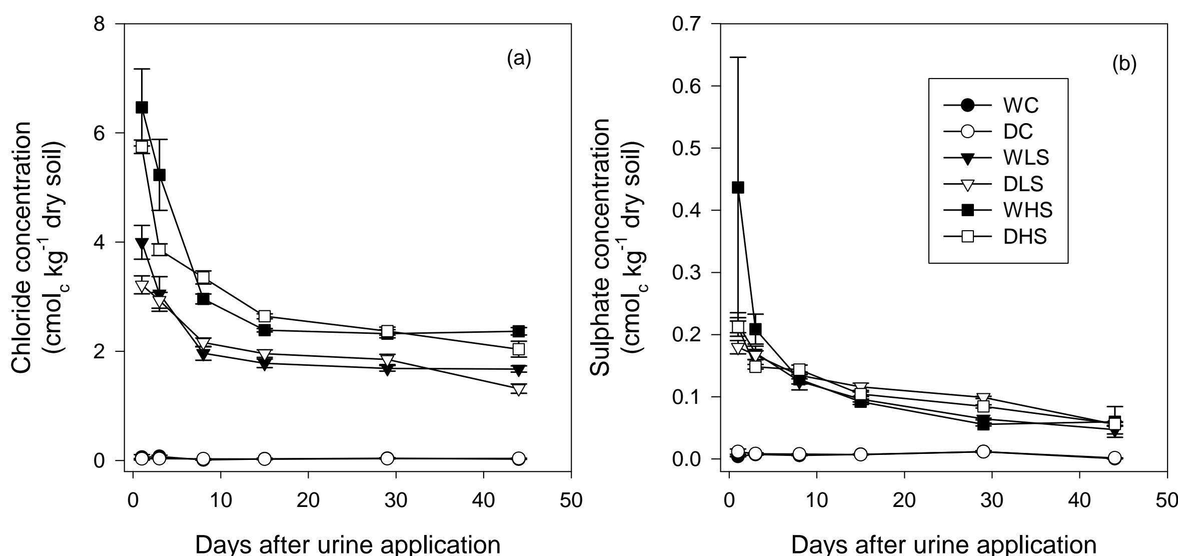


Figure 4.7 Mean concentrations of water-extractable anions; (a) chloride and (b) sulphate ($cmol_c$ kg^{-1} dry soil) measured in the top 20 mm of bare soil cores following urine application (Error bars = SEM; n = 5). NOTE differing scales on y axes.

Phosphate (PO_4^{3-}-P) concentrations rose rapidly in the urine treatments after day 1 and were higher than in the control soils from days 1–15 (Figure 4.8a). Concentrations of PO_4^{3-}-P peaked on day 8 (Figure 4.8b) and had returned to those of the control by day 29. The highest concentration measured was 0.03 $cmol_c$ kg^{-1} (9.2 mg kg^{-1}) in the WHS treatment on day 8. The urinary salt treatments had no effect on PO_4^{3-}-P, while the soil moisture had an effect only on day 44 when the wet treatments had higher PO_4^{3-}-P concentrations than the dry treatments ($P < 0.001$). On day 8, PO_4^{3-}-P concentrations were higher in the urine treatments than in the control treatments ($P < 0.001$), and higher in the WHS treatment than in the DHS treatment ($P < 0.001$) (Figure 4.8b).

There was a negative correlation between PO_4^{3-}-P and EC ($r \leq -0.38$) in all the urine treatments. Excluding day 1 data, the correlation between PO_4^{3-}-P and pH was positive in all the urine

treatments ($r \leq 0.84$). Phosphate-P and NO_2^--N were strongly and positively correlated in the wet urine treatments ($P \geq 0.77$).

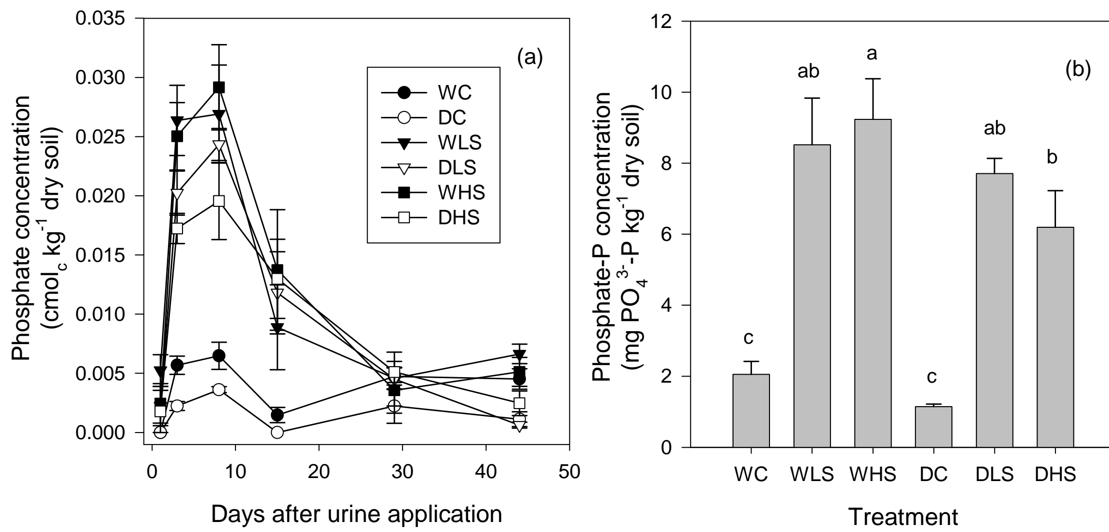


Figure 4.8 Water-extractable phosphate-P concentrations measured in the top 20 mm of bare soil cores: (a) mean concentrations from day 1 to day 44, and (b) mean concentrations at peak on day 8 (Same letter above bars indicates no significant difference; For both graphs, error bars = SEM; n = 5).

4.3.5 Phospholipid fatty acid analyses

Soil PLFA contents were highest on day 3, but had decreased by day 8 (Figures 4.9a–c). The wet soils had a higher total PLFA content than the dry soils on days 3 and 8 ($P < 0.01$), and a higher bacterial PLFA content on days 3–15 ($P < 0.05$), but soil moisture content did not affect the fungal PLFA content. Urine addition resulted in an immediate increase in total and bacterial PLFA content (Figures 4.9a & 4.9b) but caused a reduction in fungal PLFA content after day 3 (Figure 4.9c). The net effect was a significant decrease in the fungal/ bacterial ratio in the urine treatments on days 8 and 29 ($P < 0.01$) (Figure 4.9d). The salt treatments had no effect on this ratio.

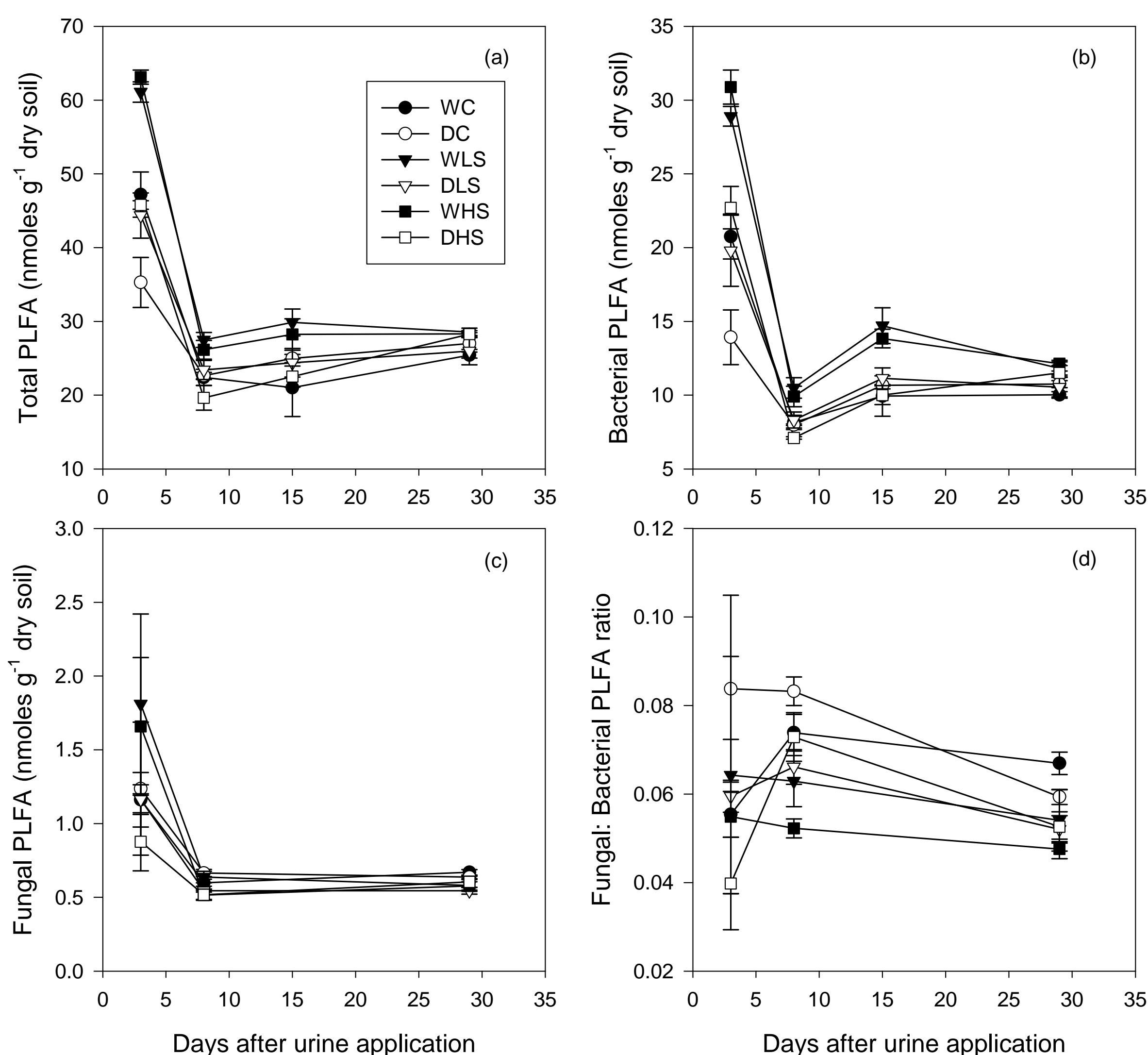


Figure 4.9 Results of PLFA biomass analyses: (a) total of all FAMEs, (b) sum of selected bacterial biomarkers (see Table 3.3), (c) amount of fungal biomarker (18:2ω6), and (d) ratio of fungal: bacterial biomarkers in the top 20 mm of bare soil cores (Error bars = SEM; n = 3). NOTE: differing scales on y axes.

The Gram-positive (G+) bacteria (see Table 3.3 for biomarkers used) showed similar responses to the bacterial PLFA (Figure 4.10a). In the urine-treated soils, PLFA contents were significantly higher in the wet treatments than in the dry treatments from days 3–15. By day 29 no significant differences were seen due to either the moisture treatments or the salt treatments. Gram-negative (G-) bacterial biomarkers (see Table 3.3 for biomarkers used) were significantly higher in the wet urine treatments than in the other treatments on all days except day 15 (Figure 4.10b). Unlike the G+ biomarkers, this effect was still apparent on day 29.

The ratio of G+ to G- biomarkers (G+/G-) (Figure 4.10c) was affected by the soil moisture content only on day 15, when it was higher in the dry treatments ($P < 0.01$). On day 3 the G+/G- ratio was higher in the urine treatments than in the control treatments ($P < 0.01$), but on day 29 the ratio was higher in the control treatments ($P < 0.001$). The G+/G- ratio did not differ between the urine treatments.

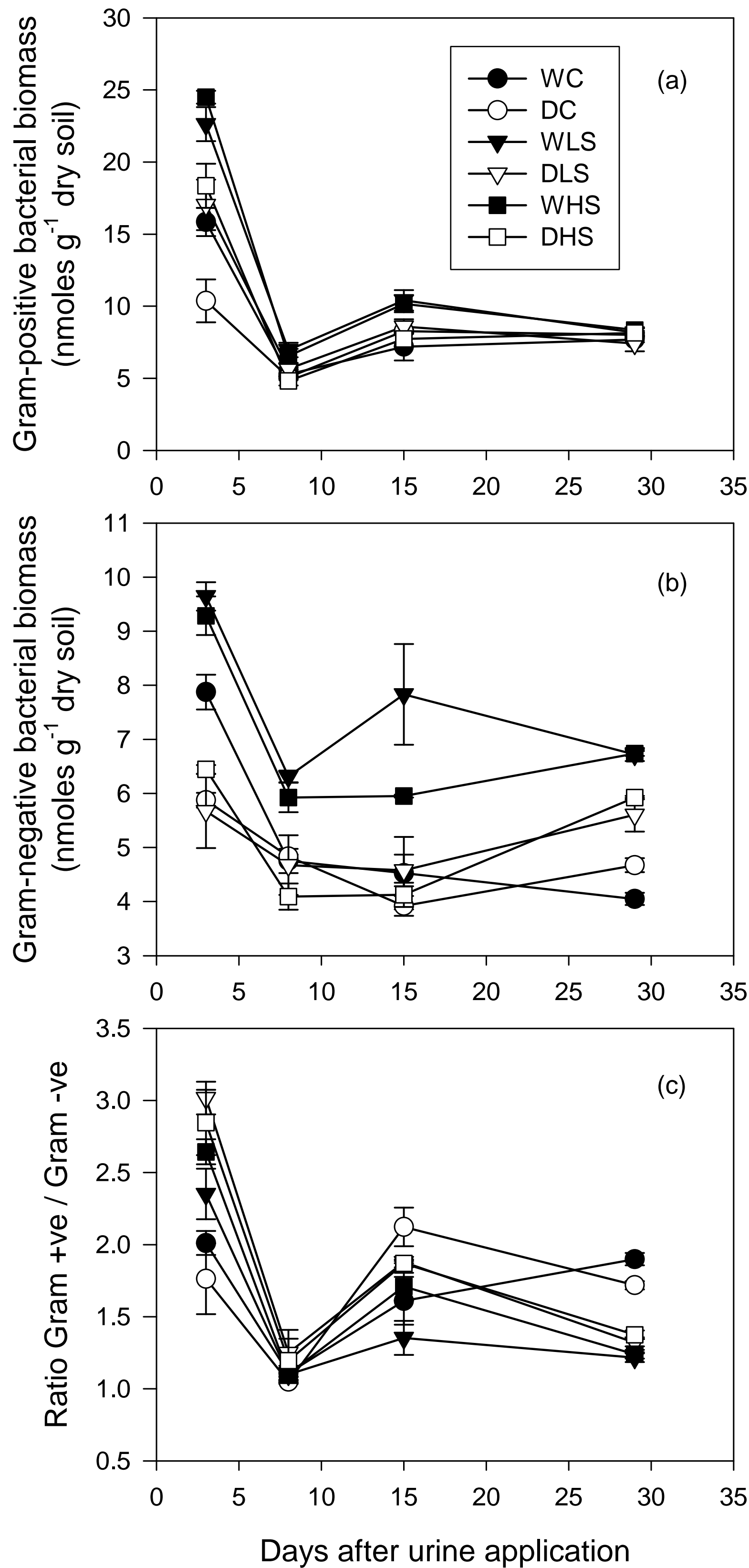


Figure 4.10 Results of PLFA analyses for (a) Gram-positive bacteria and (b) Gram-negative bacteria based on selected biomarkers (see Table 3.3), and (c) Gram-positive/Gram-negative bacterial ratio in the top 20 mm of bare soil cores (Error bars = SEM; n=3). <u>NOTE</u>: differing scales on y axes.

The cy17:0 to 16:1ω7 ratio (cy/ω7), an indicator of microbial stress, was significantly higher in the control treatments than in the urine treatments on day 8. An interaction between the moisture and salt treatments resulted in the WHS treatment having a higher cy/ω7 ratio than any other treatment on day 15, so that the cy/ω7 ratio averaged across moisture treatments, was higher in the high salt treatments than in the low salt treatments on day 15 ($P < 0.01$) (Figure 4.11a).

The microbial stress indicator, the i15:0 to a15:0 ratio (i/a), was not affected by the soil moisture treatments, but the ratio in the urine treatments was lower than that in the control treatments throughout the experiment ($P < 0.001$) (Figure 4.11b). The i/a ratio was not affected by the salt treatments.

The ratio of 18:1ω9t to 18:1ω9c (t/c), another microbial stress indicator, did not differ between treatments on day 3. However, it was strongly affected by both the moisture and urine treatments from day 8 to day 29, when it was higher in the wet soils than in the dry soils ($P < 0.01$) and was higher in the urine treatments than in the control treatments ($P < 0.001$) (Figure 4.11c). On day 29 the t/c ratio was higher in the wet urine treatments than in the other treatments ($P < 0.01$).

In the urine treatments, all PLFA variables except the stress ratios were positively correlated with Cl^- ($r \geq 0.25$). The PLFA stress ratios in the urine treatments were negatively correlated with Cl^- ($r \leq -0.48$), and the i/a and cy/ω7 ratios were negatively correlated with pH ($r \leq -0.32$), while there was no correlation between pH and the t/c ratio. The i/a ratio was strongly and negatively correlated with both SO_4^{2-} concentration ($r \leq -0.79$) and WSC ($r \leq -0.75$).

In all treatments the total, bacterial and fungal PLFAs were positively correlated with each other ($r \geq 0.56$) and with the G+ and G- biomarkers and the G+/G- ratio ($r \geq 0.35$). The PLFA stress indicator ratios (i/a, t/c and cy/ω7) were positively correlated with each other in all treatments ($r \geq 0.12$). The only exceptions were in the DC treatment, where there was a negative correlation between the fungal PLFA content and the G+/G- ratio ($r = -0.32$), and between the i/a and cy/ω7 ratios ($r = -0.40$).

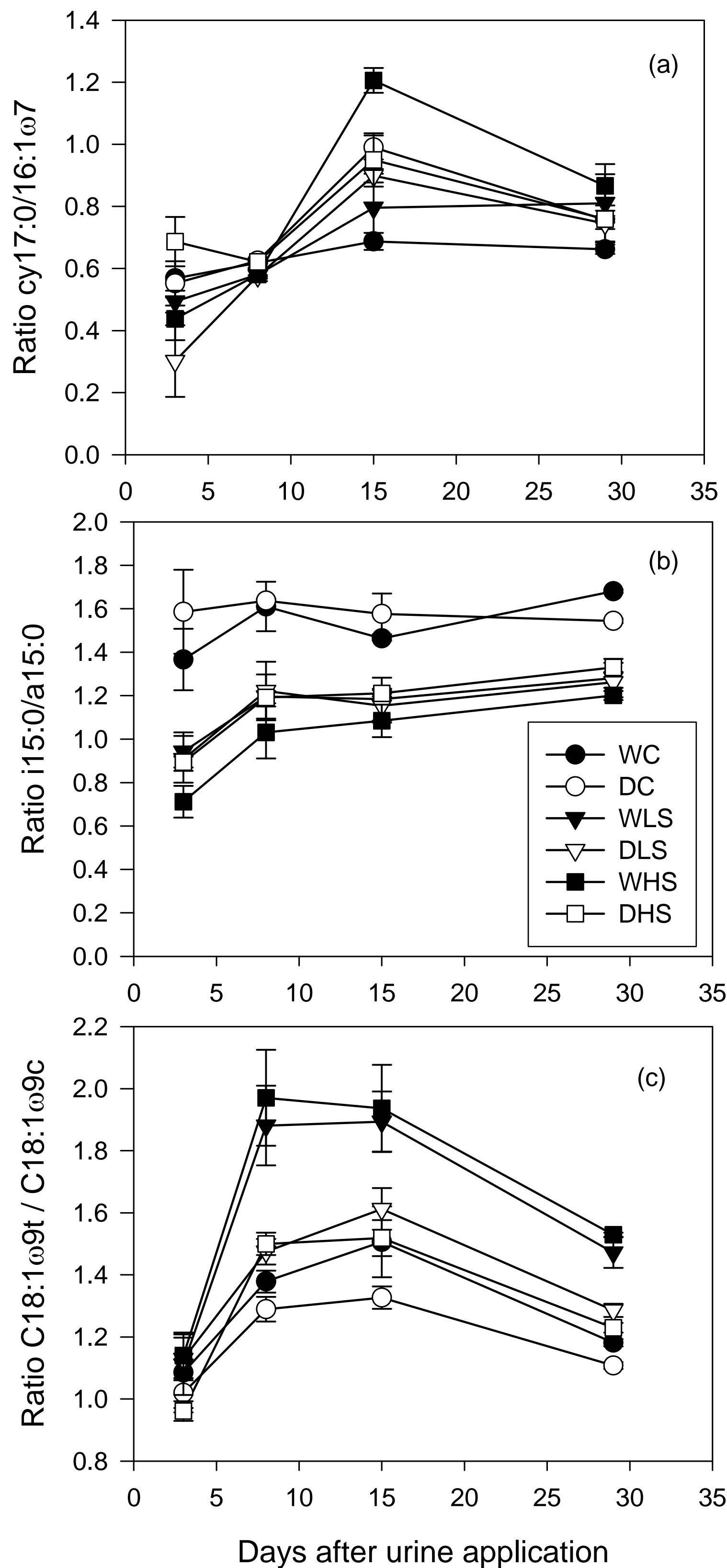


Figure 4.11 Results of PLFA analyses: (a) ratio of cyclopropyl 17:0 to its fatty acid precursor 16:1ω7, (b) ratio of iso 15:0 to anteiso 15:0, and (c) ratio of C18:1ω9 trans to C18:1 ω9 cis in the top 20 mm of bare soil cores (Error bars = SEM; n = 3). NOTE: differing scales on y axes.

Principal component analyses (PCA) were carried out on the mol% data (Section 3.6) of 15 PLFA peaks that were present on every sampling occasion. The PCA results for each individual day indicated that a number of PLFAs, including straight chain, branched, unsaturated, and cyclopropyl fatty acids, were correlated with the principal components on each day. The individual PLFAs with the main influence on principal components 1 and 2 over the four days sampled were 18:1ω9t, 18:1ω9c, 16:0 and a15:0.

The scores for principal components 1 and 2 for days 3–29 were plotted against each other to indicate any differences between the treatments (Figures 4.12a–d). Analyses of variance of the scores indicated that both the moisture and urine treatments affected PC1 on all days ($P < 0.01$), but had little effect on PC2. Urinary salt content had no effect on either of the main components, and no separation was apparent between high and low salt treatments on the score plots (Figures 4.12a–d). There was a trend on all days for the dry treatments to be more negative on PC1 than the wet treatments, and for the control treatments to be more negative on PC1 than the urine treatments. The latter trend was particularly obvious on day 29 (Figure 4.12d), when the main PLFA influences on PC1 were cy17:0, 16:1ω7 and 18:1ω9t with positive loadings and i17:0, 18:2ω6 and 16:0 with negative loadings. Apart from the straight chain PLFAs, the main FAs that influenced PC1 were the branched and unsaturated FAs, which are biomarkers for G+ and G- bacteria. The G+ biomarkers had both positive and negative loadings, while the G- biomarkers had positive loadings. The fungal biomarker had a negative loading on all days. On all the sampling days the WC data were closer on PC1 to the dry treatments than to the wet urine treatments, and the wet urine treatments were consistently situated at the more positive end of the PC1 axis.

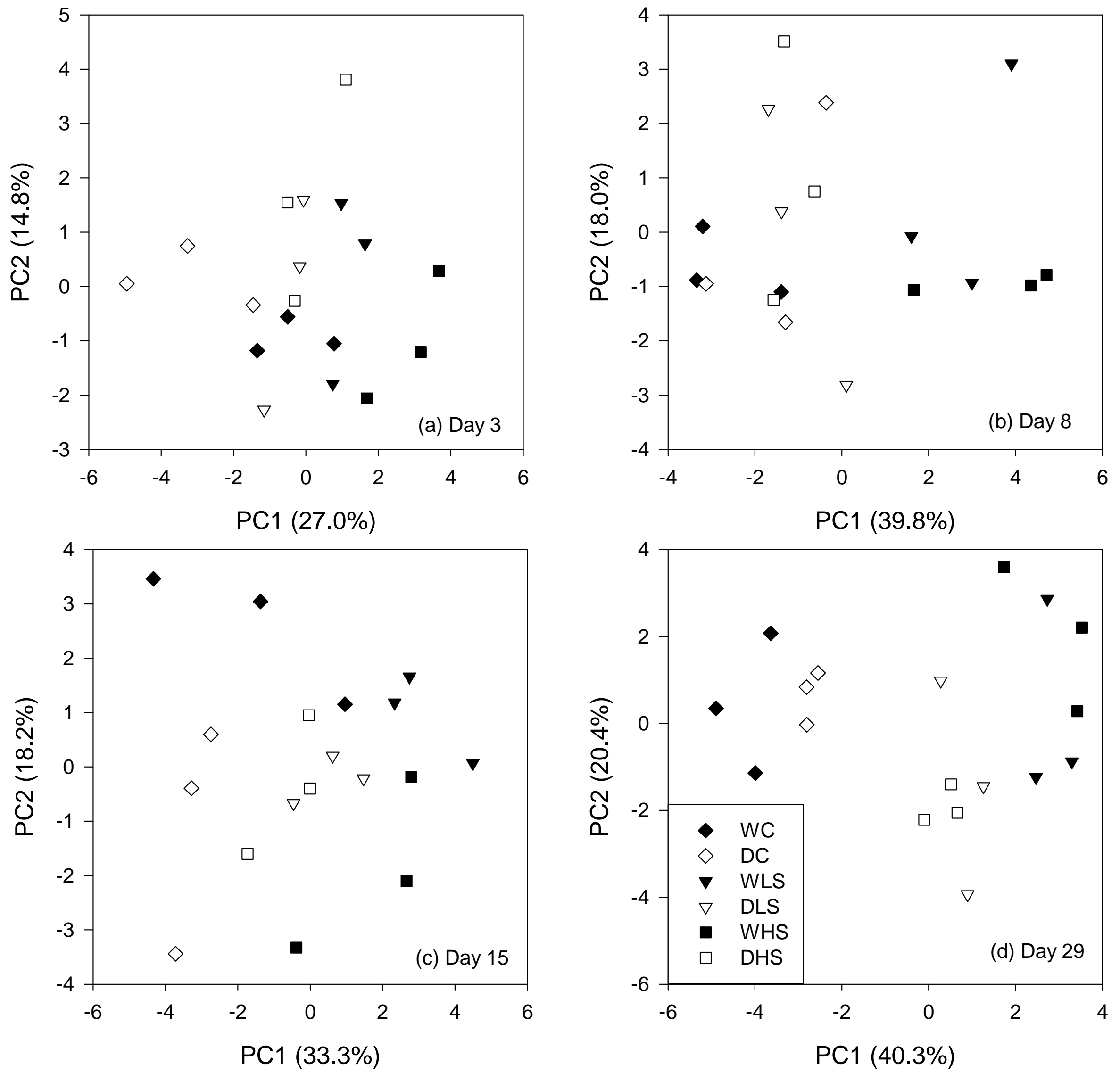


Figure 4.12 Principal component analysis (PCA) results (PC1 vs. PC2) for 15 PLFAs quantified on each sampling day.

When the principal component scores for all days were analysed together, there was a definite separation between the data from different days (Figure 4.13). Sampling time affected both principal components ($P < 0.001$) and was the main factor for PC2, with data from the first two sampling days being more negative on PC2 than data from the latter two days. A day x moisture interaction also influenced both components ($P < 0.05$), and PC1 was affected by urine treatment ($P < 0.05$) and a day x urine interaction ($P < 0.001$) as well. However, although sampling day was the main influence for the complete set of PLFA data, there was no consistent trend over time. The PLFAs that had the main influence on microbial community change over time were 18:1ω9t with a positive loading on PC1 and 18:2ω6 with a negative loading on PC2.

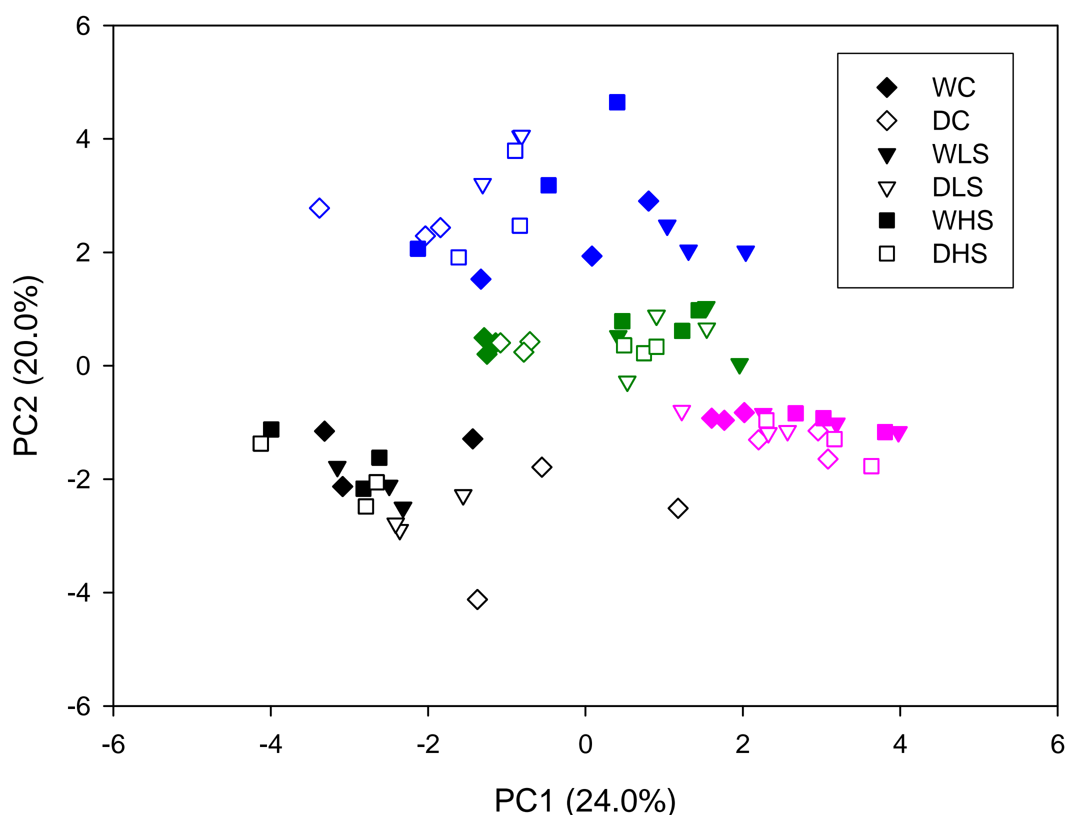


Figure 4.13 Principal component analysis results for all days (PC1 vs. PC2) for 19 extracted PLFA peaks. Black = Day 3, Pink = Day 8, Blue = Day 15, Green = Day 29.

4.3.6 Microbial biomass and activity

Levels of MBC fluctuated over time, particularly in the wet urine treatments (Figure 4.14). On the final two sampling occasions, days 29 and 44, MBC was lower in the wet urine treatments than in the other treatments ($P < 0.001$). Microbial biomass C was higher in the control treatments than the urine treatments on days 8, 29 and 44 ($P < 0.01$), and was lower in the low salt treatments than the high salt treatments on day 15 ($P < 0.05$). On day 8, MBC was lower in the WHS treatment than all other treatments except WLS. Microbial biomass C was not closely related to total PLFAs.

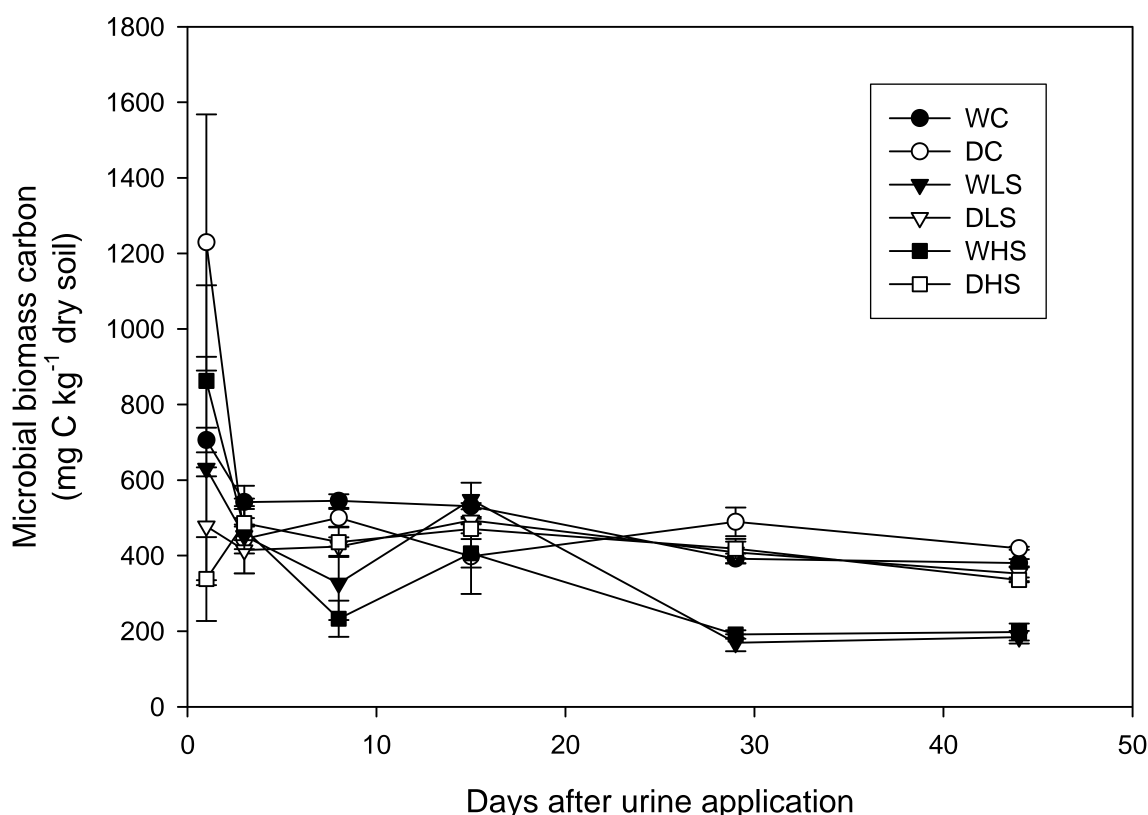


Figure 4.14 Mean microbial biomass carbon measured in the top 20 mm of bare soil cores following urine application (Error bars = SEM; n = 5).

Dehydrogenase activity (DHA) was significantly higher in the urine treatments than in the control treatments until day 15, but was lower by day 44 ($P < 0.05$) (Figure 4.15). The dry treatments had higher levels of DHA than the wet treatments from day 8 onwards ($P < 0.05$), but DHA did not differ between salt treatments.

There was a positive correlation between DHA and WSC in all the urine treatments ($r \geq 0.78$), which was higher in the dry soils ($r \geq 0.88$). There was also a positive correlation between DHA and MBC in the WLS, WHS and DLS treatments ($r \geq 0.80$). This relationship was seen in the

DHS treatment only if data from day 1 (when MBC was low in the DHS treatment) were not included ($r = 0.95$). Dehydrogenase activity was also positively correlated with pH in all treatments ($r \geq 0.41$), and with EC in both the wet urine treatments ($r \geq 0.62$). In all the urine treatments DHA was strongly and positively correlated with SO_4^{2-} ($r \geq 0.88$, $P < 0.05$). There were no consistently strong correlations between DHA and PLFA.

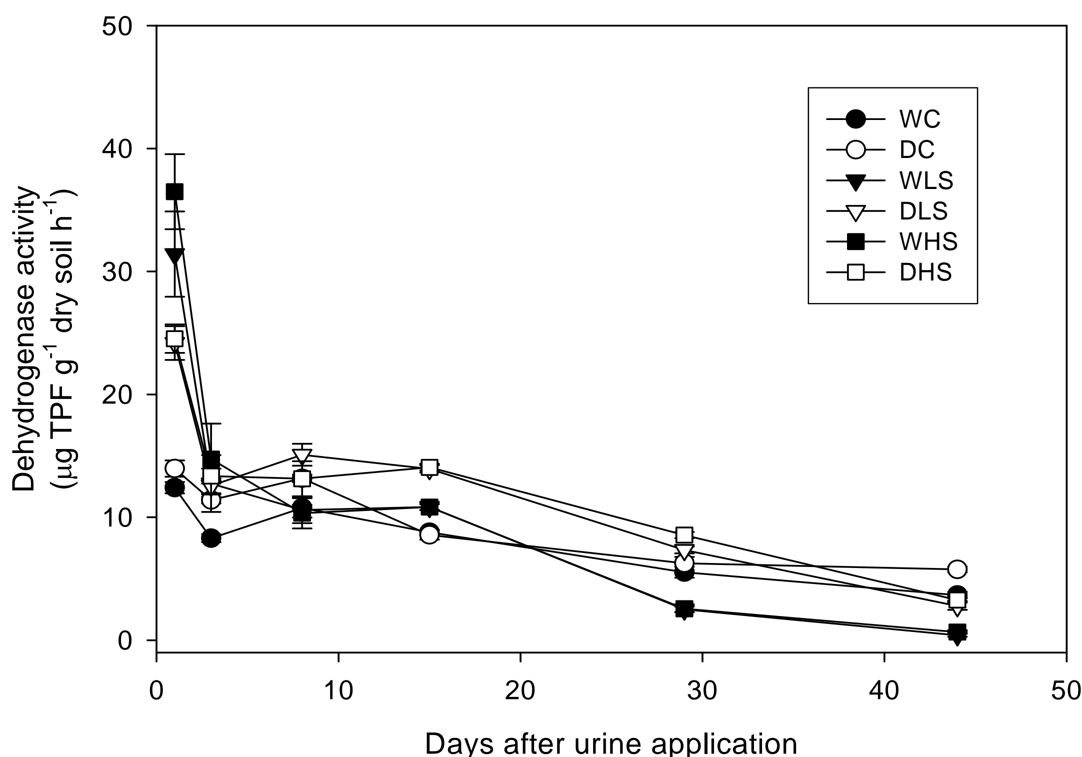


Figure 4.15 Mean dehydrogenase activity (µg triphenyl formazan (TPF) produced) measured in the top 20 mm of bare soil cores following urine application (Error bars = SEM; n=5).

4.3.7 Headspace gas fluxes

In the urine treatments, N_2O-N fluxes were higher from the wet soils than from the dry soils on all sampling occasions ($P < 0.01$) (Figures 4.16a & 4.16b). Fluxes averaged 1669 and 31 µg N_2O-N m^{-2} h^{-1} from the wet and dry urine treatments respectively, with maximum fluxes of 7800 µg N_2O-N m^{-2} h^{-1} in the wet treatments and only 88 µg N_2O-N m^{-2} h^{-1} in the dry treatments. Fluxes of N_2O-N were higher from the urine treatments than from the control treatments for each moisture treatment ($P < 0.001$), with mean maximum fluxes of 160 and 31 µg N_2O-N m^{-2} h^{-1} from the wet and dry control treatments respectively. There was no significant difference on any day between high and low salt treatments when averaged over moisture treatments. Although fluxes from low salt treatments were consistently higher than

fluxes from high salt treatments within each soil moisture treatment, the difference was only significant on day 8 when N_2O-N fluxes were higher from the DLS treatment than from the DHS treatment ($P < 0.01$).

In the urine treatments, N_2O-N was positively correlated with the PLFA stress ratios ($r \geq 0.19$) and negatively correlated with all other measured PLFA variables ($r \leq -0.05$).

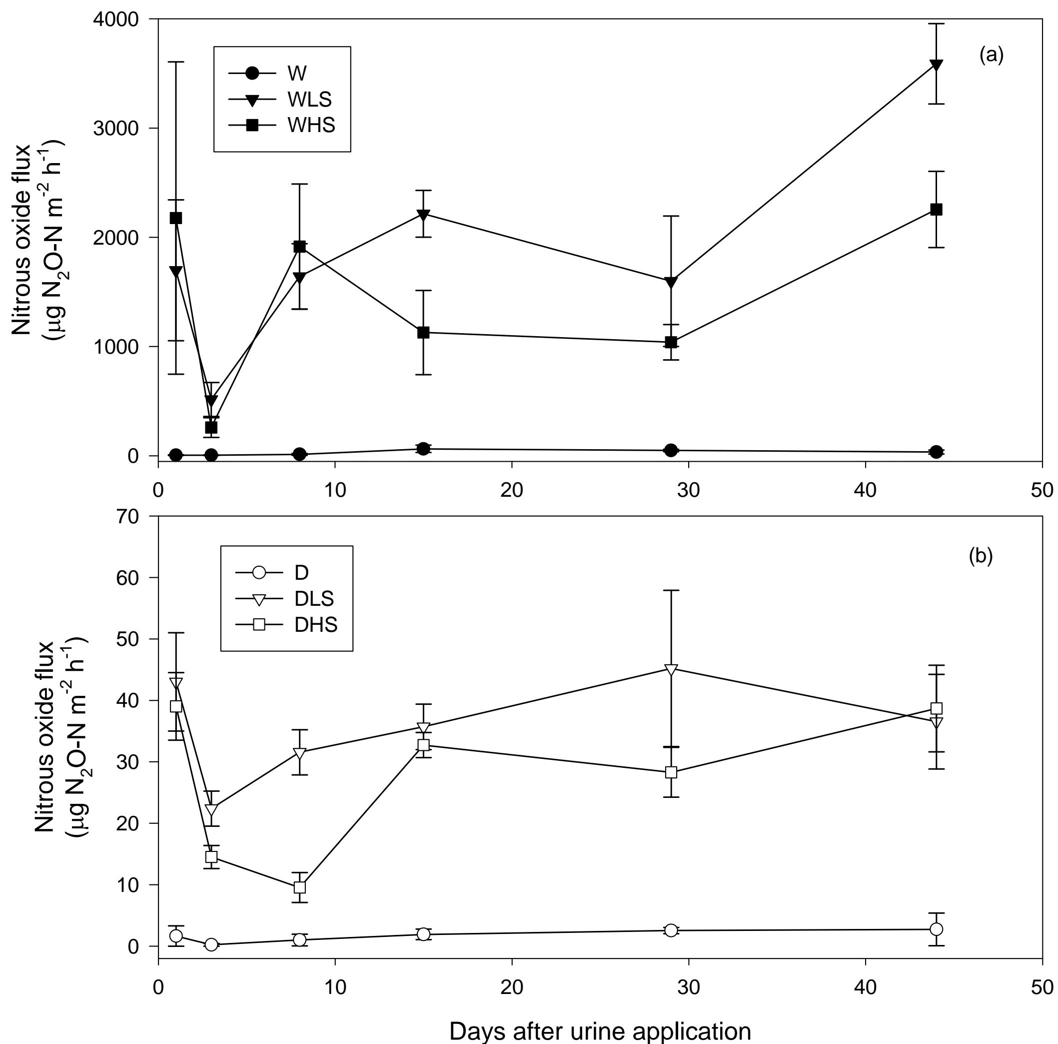


Figure 4.16 Mean nitrous oxide fluxes measured from (a) wet treatments and (b) dry treatments following urine application (Error bars = SEM; n = 5). NOTE differing scales on y axes.

Cumulative N_2O-N fluxes to day 44 were higher from the WLS and WHS treatments than from the other treatments ($P < 0.001$), but did not differ from each other (Table 4.3). Cumulative fluxes from the WC treatment did not differ from fluxes from the two dry urine treatments, while the DC treatment had lower cumulative fluxes than all other treatments ($P < 0.001$). Although there were no differences in N_2O-N fluxes on a day-to-day basis, cumulative fluxes of

N_2O-N from the low salt treatments were higher than from the high salt treatments over the course of the experiment ($P < 0.01$) (Table 4.3). Interaction between moisture and urine influenced cumulative fluxes ($P < 0.001$) but there was no moisture x salt effect.

Cumulative N_2O-N fluxes as a percentage of total N applied were higher from the wet treatments than from the dry treatments ($P < 0.001$), and were also higher from the low salt treatments than from the high salt treatments ($P < 0.01$) (Table 4.3). There was also an interaction effect of moisture x salt ($P < 0.01$).

Table 4.3 Cumulative N_2O-N fluxes over the experimental period. Means and SEMs for total cumulative fluxes (top left), and for cumulative N_2O-N fluxes (top right) as a percentage of the N applied. Below are the *F* ratios and significance levels for each factor as determined by 2-way ANOVA.

Treatment	Cumulative N_2O-N flux (μg)[a]		% total N applied[a]	
	mean	SEM	mean	SEM
WC	96	27	na	na
WLS	4811	470	3.27	0.32
WHS	3270	211	2.22	0.14
DC	5	1	na	na
DLS	90	12	0.061	0.008
DHS	67	5	0.045	0.004
Moisture (M)	635.29***		820.16***	
Urine (U)	479.86***		na	
M x U	16.54***		na	
Salt (S) (high v low)	10.61**		10.97**	
M x S	0.27		8.82**	

[a] Log transformed for analysis na = not applicable
** $P < 0.01$, *** $P < 0.001$

Carbon dioxide fluxes from the urine treatments reached 10 000 mg CO_2-C m^{-2} h^{-1} on day 1 and were still markedly elevated on day 3 (Figure 4.17a). Fluxes from the urine treatments remained higher than from the control treatments until day 15 ($P < 0.01$). Within the urine treatments, CO_2-C fluxes from the wet treatments were higher than from the dry treatments from day 8 onwards ($P < 0.01$) (Figures 4.17a & 4.17b). There was a salt effect only on day 15, when fluxes were higher from the high salt treatments than from the low salt treatments ($P < 0.01$). Carbon dioxide fluxes (days 8–44) were positively correlated with moisture content ($r \geq 0.21$).

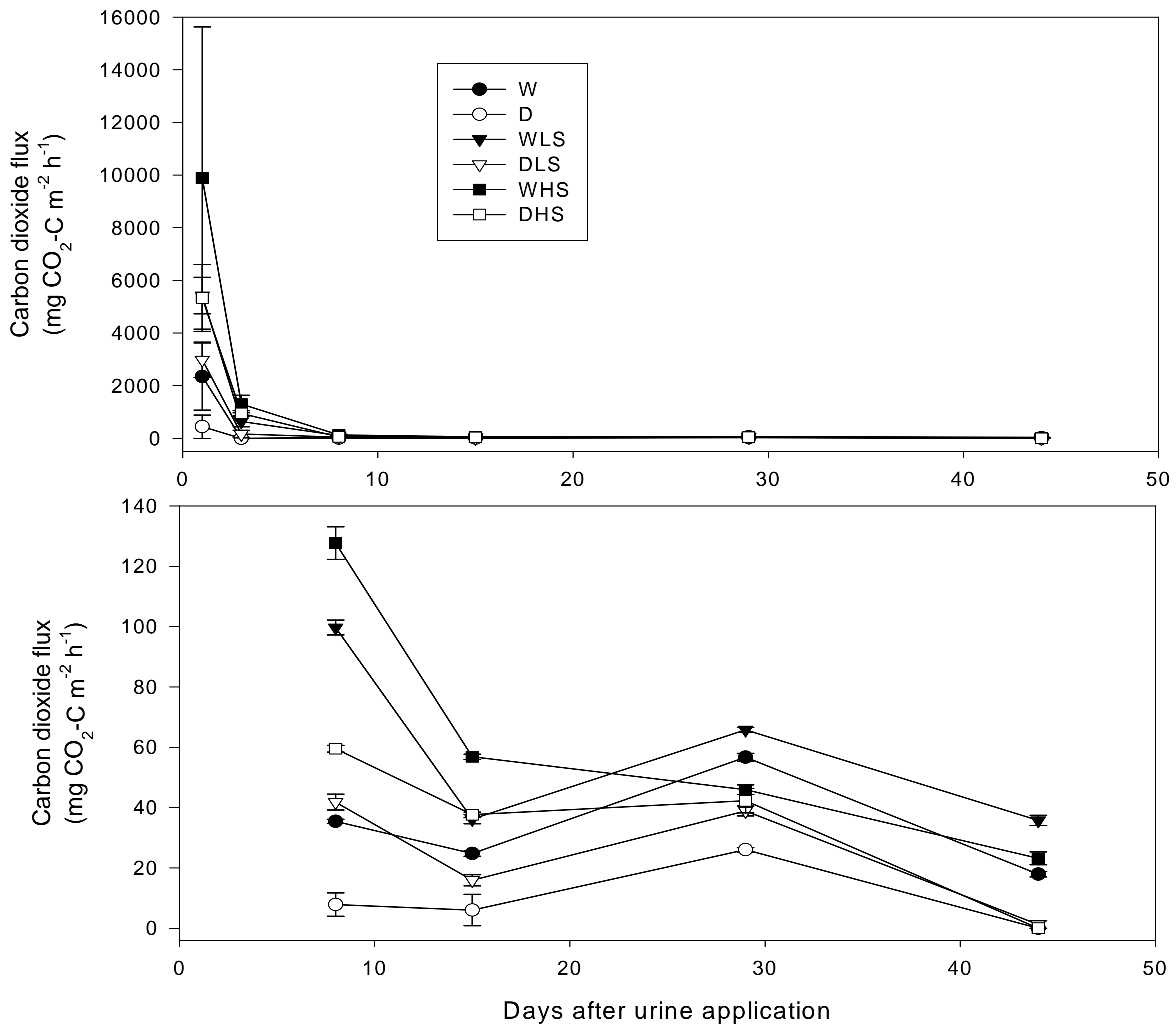


Figure 4.17 Carbon dioxide fluxes for all treatments measured on (a) all sampling days and (b) on days 8-44 only (Error bars = SEM; n=5). NOTE differing scales on y axes.

The metabolic quotient (qCO_2) (Section 3.4.3.1) was calculated for days 8–44, and the results are presented in Table 4.4. On all days the qCO_2 was higher in the wet soils than in the dry soils and higher in the urine treatments than in the control treatments ($P < 0.01$). The qCO_2 was higher in the WHS treatment than all other treatments on day 8, but thereafter was highest in the

WLS treatment. On days 29 and 44 the qCO_2 in the wet urine treatments was significantly higher than in all other treatments.

Table 4.4 Metabolic quotient (qCO_2; µg CO_2-C mg^{-1} MBC h^{-1}) for days 8–44 (n = 5). Treatments with same letter on each day are not significantly different.

	Days after urine application			
Treatment	8	15	29	44
WC	1.56 b	2.40 a	3.12 c	1.08 c
WLS	5.55 b	2.82 a	9.66 a	4.59 a
WHS	16.30 a	2.67 a	5.62 b	2.80 b
DC	0.44 b	1.21 c	1.27 c	0.00 d
DLS	2.39 b	1.81 b	2.24 c	0.07 d
DHS	3.30 b	2.07 ab	2.40 c	0.00 d
significance	***	***	***	***

*** $P < 0.001$

4.3.8 ^{15}N trace analyses

The atom% ^{15}N enrichment of the NH_4^+-N in all urine treatments was highest (≥ 4.9 atom%) on day 3, and had decreased in the wet treatments to 4.5 atom% by day 44 (Figure 4.18a). The NH_4^+-^{15}N enrichment fluctuated in the dry treatments, but was lower than the initial concentration by day 44, while remaining higher than in the wet treatments on days 29 and 44 ($P < 0.001$). The dry treatments were analysed for NO_2^--^{15}N only on day 3, as there was insufficient sample for analyses on other occasions. On day 3, the enrichment in the wet soils was significantly higher than in the dry soils (Figure 4.18b). The mean enrichment in the wet treatments peaked at 5.1 atom% on day 15, and then rapidly declined to < 3.2 atom% on days 29 and 44. The ^{15}N enrichment of the NO_3^--N increased over time (Figure 4.18c), and was higher in the wet treatments from days 3–29 ($P < 0.01$). On day 44, the mean NO_3^--^{15}N enrichment in all treatments was 3.4 atom%. The ^{15}N enrichment of the N_2O-N was higher in the wet treatments than in the dry treatments ($P < 0.001$) on all days, with a peak ^{15}N enrichment on day 15 of > 4.9 atom% in the wet treatments and only 4.1 atom% in the DLS treatment (Figure 4.18d). Throughout the experiment the urinary salt content had no effect on the ^{15}N enrichment of the inorganic-N.

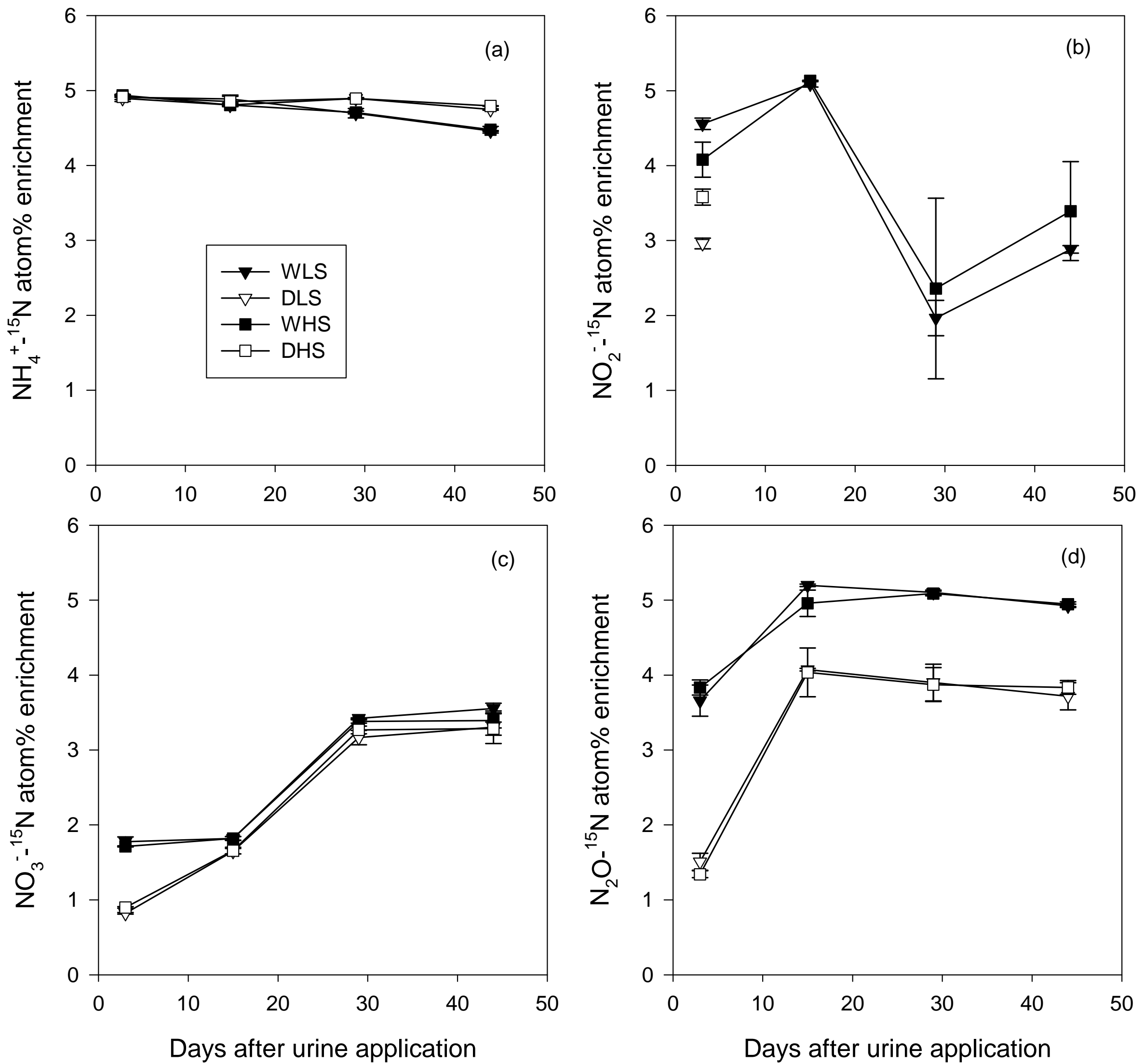


Figure 4.18 ^{15}N atom% enrichment determined for urine treatments on days 3, 15, 29 and 44 for (a) ammonium, (b) nitrite, (c) nitrate, and (d) nitrous oxide (Error bars = SEM; n = 5).

4.4 Discussion

4.4.1 Microbial stress

The three microbial stress ratios used (cy/ω7, i/a, t/c) indicated that the stress imposed by the experimental treatments on the microbial community caused changes in the cellular membranes, but was not of sufficient magnitude to cause cell dormancy. The G+/G- ratio indicated that microbial community composition continued to alter over the 29 days that sampling occurred, probably due to different stresses acting on the microbial population at different times. The addition of anions (Cl^- and SO_4^{2-}) and cations (K^+ and Na^+) in the urine resulted in an immediate

increase in the soil salinity, and thus the EC, which remained elevated because of displacement of Ca^{2+} and Mg^{2+} from soil exchange sites. The extra KCl added to the high salt treatments (to increase the urinary K^+ concentration) resulted in the EC being higher in these soils. These changes in soil salinity could be expected to affect the microbial community.

The higher cy/ω7 ratio (indicating cell dormancy) in the high salt treatments on days 8 and 15 showed that the microbial community was stressed by the higher salt content on these occasions. This was not reflected in the i/a ratio (an indicator of cell membrane rigidity), which did not differ between salt treatments, despite a lower i/a ratio being previously observed under conditions of increased osmotic potential in both bacterial cultures and urine-treated pasture soil (Chihib et al., 2003; Petersen et al., 2004a). In this current study, despite the higher Cl^- concentrations in the high salt treatments and the very strong correlations between the i/a ratio and Cl^- concentrations in the urine treatments, it appears that the difference between high and low salt treatments was not sufficient to affect the i/a ratio. However, the lower i/a ratio indicated increased microbial stress in the urine treatments throughout the experiment, and this was also apparent in the t/c ratio from day 8 onwards (Figure 4.11c). The t/c ratio (an indicator of membrane permeability) has previously been used to examine the effects of osmotic stress on microbes, and was observed to increase in the presence of high salt concentrations (Heipieper et al., 1996). In this current experiment, the t/c ratio, like the i/a ratio, showed no distinction between high and low salt treatments, but was higher in the wet soils than in the dry soils from day 8 onwards, indicating that the microbial community was under increased stress in the wet soils.

As discussed in Section 2.4.2.4, G+ and G- bacteria often respond differently to stress, with G- bacteria believed to be more stress-resistant due to their cell structure (Kaur et al., 2005). Therefore, we would expect to see an increase in G- biomarkers with a corresponding decrease in G+ biomarkers, resulting in a lower G+/G- ratio if the microbial community was under stress. In this experiment, both G+ and G- biomarkers initially increased, suggesting stimulation of the microbial community. In fact, on day 3 the G+/G- ratio was lower in the control treatments, showing that the addition of urine had not caused microbial stress. On day 29, however, the lower G+/G- ratio in the urine treatments indicated a delayed stress response to urine addition, possibly due to a decrease in substrate supply, such as WSC. The significantly higher concentration of G- biomarkers in the wet urine treatments on three days suggests that urine application caused more stress in the wet soil, as also seen in the t/c ratio, but this was reflected in a lower G+/G- ratio only on day 15. This may indicate a soil pH effect, because the pH on day 15 was significantly higher in the wet soils than in the dry soils.

Increased stress due to high soil moisture was also indicated by the qCO_2 (Section 3.4.3.1), which was consistently higher in the wet treatments than in the corresponding dry treatments (Table 4.4). An increase in the qCO_2 may indicate increased microbial stress (Wardle and Ghani, 1995), and shows a reduction in the efficiency of converting substrate-C into cell material (Alexander, 1977) (Section 2.4.2.4). The qCO_2 values seen here were similar to those calculated by Scheu and Parkinson (1994) after rewetting of an air-dried soil. An increase in the qCO_2 has been observed following an increase in salinity (Rietz and Haynes, 2003), and this may be the cause of the higher qCO_2 seen here in the urine treatments.

A number of the PLFAs identified in the PCA analysis as being responsible for changes in the microbial community were stress biomarkers, including 18:1ω9t, 18:1ω9c, cy17:0, and the fungal biomarker 18:2ω6. In addition, the strong positive and negative loadings of the G+ and G- biomarkers would have had a big influence on the separation between the wet and dry soil treatments. Gram-negative bacteria carry out both nitrification and denitrification, and G+ bacteria have genes for denitrification (Sections 2.5.1.3 & 2.5.1.4), so these processes were likely to have been affected by the soil moisture treatments, as was seen in the differences in the net nitrification rates and production of N_2O-N between the two treatments. Other researchers (e.g. Petersen and Klug, 1994; Hamer et al., 2007) also found that a large number of PLFAs, including some of the same biomarkers for G+ and G- bacteria and for stress, were involved in microbial community responses to changed conditions. This suggests that these biomarkers are useful indicators of disturbance.

The PCA results for individual days (Figure 4.12) show no evidence of microbial community differences due to the salt treatments, despite the differences in the cy/ω7 ratio on days 8 and 15, but in agreement with the i/a and t/c ratio results. However, the separation of the control treatments from the urine treatments in each moisture regime on PC1 on all days indicates that the microbial communities were affected by urine application. Within the urine treatments, the separate clustering of the wet soils and the dry soils on all days shows that the soil moisture content also influenced microbial community structure, reflecting the t/c ratio results. These PCA results suggest that the i/a and t/c ratios are the best monitors of microbial community changes caused by urine-induced stress.

The separation of data on the graph of PCA data from all days (Figure 4.13), demonstrates that the microbial community structure changed over time, and similar clustering was seen by Petersen and Klug (1994) after the imposition of stress caused by high soil incubation temperatures and soil sieving.

4.4.2 Microbial biomass and phosphate-P

The response of total PLFAs to urine addition suggests an initial stimulatory effect (Figure 4.9a). This reflects the response of the G+ and G- bacteria because many of the G+ and G- biomarkers are also used in the calculation of total PLFAs. This stimulatory increase in biomass has been seen in previous studies where urine was applied to soil under pasture (Petersen et al., 2004a) or where urea solutions were applied to repacked soil cores (Petersen et al., 2004b). In both of those experiments the period of stimulation was longer than was seen here, despite the higher rates of N applied in this study. The high N application rate used here (15 g N L^{-1}) resulted in higher pH values and NH_4^+-N concentrations than those measured in Petersen et al. (2004a; 2004b), where the highest rate of N application was 10 g N L^{-1}. This would probably have led to higher soil concentrations of NH_3-N, which can be toxic to both fungi and bacteria (Chang and Chang, 1999; Green et al., 2006a), and may have overridden any stimulatory effects of urine application. Alternatively, the absence of plants and subsequent lack of C inputs, in contrast to the study of Petersen et al. (2004a), may have reduced the duration of the period of stimulation. The stimulatory effect of urine may also have involved microbial assimilation of the anions and cations supplied in the applied urine (Section 2.2.2.2), thus contributing to the initial reduction of the ions.

The lack of any detectable increase in microbial biomass by the chloroform fumigation extraction method (Figure 4.14), could have been due to the release of large amounts of CO_2-C following urea hydrolysis (Figure 4.17a), which may have influenced the initial CFE results.

It is possible that where the MBC data corresponded with the PLFA data, in showing a decrease in microbial biomass in the urine treatments between days 3 and 8, that lysis of microbial cells due to osmotic stress contributed to this decline (Kieft et al., 1987). This suggestion is supported by the increase in PO_4^{3-}-P concentrations (Figure 4.8a) at the same time as the biomass decrease, as cell lysis releases P-containing intracellular constituents such as phospholipids (Turner et al., 2003). In addition to cell lysis, PO_4^{3-}-P may have been released by SOM degradation caused by the high soil pH or have been displaced from soil exchange sites. The increased PO_4^{3-}-P could not be directly attributed to the added urine, because ruminant urine contains only trace amounts of P (Betteridge et al., 1986) and because the increase was delayed. The peak concentrations of water-extractable PO_4^{3-}-P in the urine treatments on day 8, ranging between 6.2 and 9.2 mg kg^{-1} (Figure 4.8b), were substantially higher than the concentrations of 0.1–1 mg kg^{-1} generally found in soil (Plante, 2007). This P would be rapidly assimilated by surviving organisms, since P is a limiting nutrient in the soil (Sharpley, 2000), thus explaining the subsequent decline in PO_4^{3-}-P. In a pasture system, the P would be taken up by plants, as

well as being assimilated by microbes, if the soil solution was originally low in P (Section 2.3.1). The positive correlation seen here between PO_4^{3-}-P and pH in the urine treatments, reflects the findings of Hartikainen and Yli-Halla (1996), with the highest concentrations of PO_4^{3-}-P measured when the pH was highest.

After the minor recovery of the urine-treated microbial populations by day 15, the subsequent decrease in MBC in the wet soils may have been because of the rapid depletion of resources due to the higher initial microbial activity in these soils, as seen in the DHA results (Figure 4.15). Comparable with this experiment, Rooney et al. (2006) measured a reduction in MBC in soil 50 days after urine application, although they saw no change 10 days after application.

4.4.3 Microbial activity

The total PLFA increase in this study corresponded with an immediate rise in DHA (Figure 4.15), however, DHA was more closely correlated with MBC than total PLFAs. The relationship between microbial biomass and activity has been seen in other studies (e.g. Rietz and Haynes, 2003), although Rooney et al. (2006) detected a rise in DHA before MBC increased. In this current experiment, the strong and positive correlation between DHA and CO_2-C in the WHS, DLS and DHS treatments from days 8–44, supports the use of CO_2-C respiration as a measure of microbial activity (Section 2.4.2.2).

The positive correlation between DHA and pH has been seen in other studies (e.g. Bardgett and Leemans, 1995; Liu et al., 2008). DHA is usually very low in soils with a pH < 5 (Alef, 1995), and soil bacteria are known to be inhibited by very acid or very alkaline conditions (Alexander, 1977). Since, in this experiment, microbial activity was highest when the pH was highest, the pH measured here was apparently not high enough to induce inhibition. This was supported by the lack of a clear relationship between pH and the PLFA stress ratios. Both the i/a and cy/ω7 ratios were negatively correlated with pH, although this corresponded to increasing stress with pH for the i/a ratio and decreasing stress with pH for the cy/ω7 ratio.

The correlation observed between EC and DHA in the wet urine treatments is comparable with the results of Rooney et al. (2006), who also saw an increase in DHA in urine-treated soils. Dehydrogenase is more sensitive to salinity than urease is (Frankenberger and Bingham, 1982), and the higher pH in the low salt treatments on day 0 suggests that there was inhibition of urea hydrolysis in the high salt treatments. Therefore, it is reasonable to assume that DHA inhibition would also have occurred in the high salt treatment, but may not have been detected because DHA was not measured on day 0. The extracellular urease enzymes that catalyse urea

hydrolysis (Section 2.5.1.1) could possibly have been affected by the extracellular osmotic concentrations. However, the highest EC reading in this current experiment was 1.8 dS m^{-1} (day 1, WHS) which is not quite as high as the ostensible critical level for urease inhibition of 2 dS m^{-1} calculated by Inubushi et al. (1999). It is, however, possible that the EC was above this critical level on day 0, when the lower pH in the high salt treatments was observed. Since urea hydrolysis is also an intracellular process (Section 2.5.1.1), it is possible that the high EC may have caused some slight initial inhibition of microbial activity.

The lack of response by DHA to the elevated EC and pH may have been due to other factors, such as the increase in soil nutrients, including WSC, and the positive correlation between DHA and WSC emphasises the dependence of microbes on an available source of C. Concentrations of WSC increased nearly tenfold immediately after the addition of urine (Figure 4.3). It is unlikely that all of this C came from urinary constituents, and it may have also come from the solubilisation of SOM due to the high pH (Monaghan and Barraclough, 1993; Shand et al., 2000) or the addition of salt in the urine (Jones and Willett, 2006). Alternatively, organic solutes may have been released from microbial cells, either as a physiological response (Halverson et al., 2000) or through lysis of microbial cells (Kieft et al., 1987) due to the change in soil water potential after urine deposition. The positive correlation between WSC and PO_4^{3-}-P in the urine treatments may reflect the release of microbial cell components, with input from SOM preventing the correlation from being stronger.

4.4.4 Nitrogen transformations

4.4.4.1 Denitrification and nitrous oxide emissions

The supply of available C may be the rate-determining factor for denitrification when NO_3^--N is readily available (Bremner and Shaw, 1958; Firestone, 1982; Haynes and Sherlock, 1986), and the increase in WSC following urine application in this experiment should certainly have been sufficient to promote denitrification. Although it is not known if this C was microbially available (Petersen et al., 2004b), a strong correlation has previously been seen between organic C and the denitrification capacities of anaerobic soils (Burford and Bremner, 1975). Here, the strong positive correlation between WSC and NH_4^+-N and the strong negative correlation between WSC and NO_3^--N in the urine treatments show a close link between C availability and denitrification.

As expected, denitrification occurred to a greater extent in the wet urine treatments, with N_2O-N fluxes approximately 50 times greater than from the dry urine treatments (Figures 4.16a & 4.16b), and cumulative emissions significantly higher (Table 4.3). Reinforcing the effect of

moisture on N_2O-N emissions, cumulative N_2O-N fluxes from the WC soil were 20 times higher than from the DC soil. Bateman and Baggs (2005) also found that N_2O-N fluxes were higher from soil at 70% WFPS than at 35% WFPS, and the maximum fluxes in their study (5.7 mg N_2O-N m^{-2} d^{-1}) were similar to those measured here (3.6 mg N_2O-N m^{-2} d^{-1} (Figure 4.16a)). Using isotopic labelling, Bateman and Baggs (2005) determined that the high fluxes from the wet soil were completely attributable to denitrification, whereas the low fluxes from the dry soil were largely from nitrification. However, nitrification was entirely inhibited in the wet treatment in that experiment, which was not the case in this current experiment, so it is probable that some of the N_2O-N measured here was produced by nitrification. The very high rates of N_2O-N production from denitrification in the wet soils would have been supported by the supply of NO_3^--N produced by nitrification in these soils (Figure 2.1), with N_2O-N also being produced during nitrification.

In this experiment, the N_2O-N from the wet soils had higher ^{15}N enrichment than either the NO_2^--N or NO_3^--N pools, but was similar to the original labelled urea. This suggests that the KCl extraction included soil zones that were not a source of the N_2O-N measured, an effect also seen by Carter (2007). The lower N_2O-^{15}N enrichment in the dry soils was closer to the enrichment seen in the NO_3^--N pool, indicating that the N_2O-N produced in the dry soils was more closely related to the KCl-extracted NO_3^--N pool and was also affected by inputs of ^{14}N from other sources.

The inhibitory effect of the high salt treatment on cumulative N_2O-N fluxes (Table 4.3) shows that the salt content of the urine affected the soil microorganisms involved in N transformations, despite the PLFA stress indicators only showing increased stress in the high salt treatments by a higher cy/ω7 ratio on day 8. A decline in N_2O-N emissions was also observed by Adviento-Borbe et al. (2006) as the EC increased from 0.5–2.0 dS m^{-1} in a soil at 60% WFPS, and this was attributed to an inhibitory effect of Cl^- ions on nitrifying bacteria. However, denitrifier activity was observed to decline when an agricultural grassland soil was treated with saline water (1.3 g Cl^- L^{-1}) (Antheunisse et al., 2007). In this current experiment, the magnitude of the difference between N_2O-N fluxes from the high and low salt treatments was similar in both soil moisture treatments, suggesting that both nitrifiers and denitrifiers were similarly affected by the salinity.

An early flush of N_2O-N, seen here on day 1, has been observed in other studies (e.g. Sherlock and Goh, 1983; Williams et al., 1999b) and has previously been ascribed to the CO_2 produced by urea hydrolysis creating anaerobic soil sites in which native soil NO_3^--N could be denitrified.

This would be expected to lead to larger fluxes of N_2O-N from the wet soils, as was seen, because a larger proportion of the soil volume would be anaerobic and thus conducive to denitrification. This initial denitrification would have been supported by the high initial abundance of WSC after urine application (Figure 4.3). The early N_2O-N flux accumulated, probably until synthesis of *de novo* N_2O-N reductase began 16–33 h after the onset of anaerobic conditions (Firestone and Tiedje, 1979; Dendooven and Anderson, 1994), after which N_2O-N concentrations declined. Nitrous oxide reductase reduces N_2O-N to N_2-N, so the N_2-N/N_2O-N ratio would increase once synthesis of this enzyme began. However, the increasing NO_3^--N concentration and declining WSC concentration and pH as the experiment progressed, would increasingly favour N_2O-N production (Focht, 1974; Firestone, 1982; Weier et al., 1993).

4.4.4.2 Nitrification and soil moisture content

Soil moisture treatments were chosen for this experiment based on the supposition that the dry treatment (35% WFPS with urine added) would favour nitrification while the wet treatment (70% WFPS with urine added) would favour denitrification, based on numerous studies (e.g. Linn and Doran, 1984; Franzluebbers, 1999) in which the highest nitrification rates occurred at 35–60% WFPS. Linn and Doran (1984) postulated that nitrification was water limited below 60% WFPS and aeration limited above 60% WFPS. In this current study, the higher rates of net NO_3^--N production from days 0–3 and higher concentrations of NO_2^--N on day 1 in the dry urine treatments supported the assumption that nitrification rates would be higher in the dry soils. From days 3–29, however, the net rates of NO_3^--N production and NH_4^+-N disappearance indicated that nitrification was higher in the wet urine treatments. This contrasts with the results of Bateman and Baggs (2005), also using a silt loam soil, who found that nitrification was severely inhibited at 70% WFPS, and suggests that the soil cores in the current study contained sufficient aerobic micro-sites to support nitrification. However, other studies have found optimal nitrification rates at soil moisture contents of $\geq$ 80% WFPS (e.g. Stanford and Epstein, 1974; Schjønning et al., 2003).

The elevated NO_2^--N concentrations in the dry soils on day 1 had declined by day 3, showing that nitrification was not being retarded. However, the continued elevation of NO_2^--N concentrations in the wet soils until day 15 indicates that NO_2^--N reduction during nitrification or denitrification was being inhibited. Accumulation of NO_2^--N can indicate inhibition of nitrification by NH_3 (Whitehead, 1995c), and occurs because NO_2^--N oxidisers are more inhibited by high NH_3 concentrations than NH_4^+-N oxidisers are (Aleem and Alexander, 1960; Alexander, 1977). High concentrations of NH_3 in solution arise when there is a high concentration of NH_4^+-N in conjunction with a high soil pH (Alexander, 1977), and, in this

experiment, the NH_4^+-N concentration and the pH were both higher in the wet soil when NO_2^--N concentrations were highest. The strong correlation between NO_2^--N and PO_4^{3-}-P in the wet urine treatments may indicate that the accumulation of NO_2^--N was due to higher numbers of microbial nitrifier cells lysing or releasing cell constituents in response to the greater osmotic pressure in the higher soil moisture.

High salinity can also inhibit nitrification, and the 12–20-fold increase in the EC following urine deposition may have contributed to osmotic stress of microbial cells, potentially resulting in the NO_2^--N accumulation (Jin et al., 2007) and the reduction in N_2O-N fluxes (Adviento-Borbe et al., 2006) observed here.

Monaghan and Barraclough (1992) also observed accumulation of NO_2^--N, which peaked 7–29 days after urine application. In their experiment the peak NO_2^--N concentration was five times higher than that measured here at the corresponding N application rate, possibly due to higher pHs, which were not reported. Morrill and Dawson (1967) found that higher pH values corresponded to an increasing lag in the generation time of NO_2^--N oxidisers and thus greater NO_2^--N accumulation.

The accumulation of NO_2^--N in the wet soils could also indicate that nitrification rates were higher in the wet urine treatments than in the dry urine treatments after day 3, which may have led to an accumulation of NO_2^--N before the denitrifying community could respond. The enzymes involved in denitrification, including NO_2^--N reductase, are inducible and there can be a lag between the appearance of the substrate and its consumption (Robertson, 2000). It is, however, unlikely that this lag could account for the 8 d accumulation of NO_2^--N seen in the wet soils. Studies by Smith and Tiedje (1979) and Dendooven and Anderson (1994) found that synthesis of *de novo* NO_2^--N reductase enzymes started 3–6 h after the onset of anaerobic conditions. Coupled with persistent NO_3^--N reductase activity this led to an early accumulation of NO_2^--N, similar to that observed in this current study. Thus the lag of several hours until synthesis of NO_2^--N reductase might explain the high NO_2^--N concentrations in all the urine treatments on day 1, but does not account for its increase in the wet soils after this time. It is therefore more likely that inhibition of nitrification is responsible for the enhanced NO_2^--N concentrations measured in the wet soils after day 1.

The relatively high ^{15}N enrichment of the NH_4^+-N showed that the majority of this pool was sourced from the urinary urea in both soil moisture treatments. The declining enrichment in the wet soils indicates that this pool was being diluted by NH_4^+-^{14}N during the course of the study. In all treatments, the NO_3^--N pool was diluted by NO_3^--^{14}N throughout the experiment, and

never reached the same level of ^{15}N enrichment as the NH_4^+-N pool. The ^{14}N may have come from soil NO_3^--N, which averaged 25 mg NO_3^--N kg^{-1} in the control treatments during the experiment, or from organic N solubilised by the urine and subsequently mineralised. Since concentrations of WSC increased following urine application, it is probable that the DON content of the soil also increased (Shand et al., 2000; Shand et al., 2002).

On day 15, ^{15}N enrichment of NO_2^--N in the wet soils was similar to that of the NH_4^+-N, indicating that, at this stage, the NH_4^+-N pool was the main source of NO_2^--N (Figure 2.1). The decrease in the NO_2^--^{15}N enrichment by day 29 showed that another source of N was contributing to the NO_2^--N pool, and it is likely that this source was denitrification of the less enriched NO_3^--N (Figure 2.1). This addition to the NO_2^--N pool through both nitrification and denitrification has been seen in other studies (Russow et al., 2000; Müller et al., 2006). As with the NO_3^--N, ^{15}N enrichment of the NO_2^--N was higher in the wet soils than in the dry soils on day 3, the only day the dry soils were analysed.

4.5 Discussion summary

- The wet soil treatments increased microbial stress, as demonstrated by the higher t/c ratio and qCO_2, and also inhibited nitrite oxidation, probably via high NH_3 concentrations.

- The high soil moisture content stimulated N transformations, resulting in higher concentrations of NH_4^+-N and NO_3^--N and higher emissions of N_2O-N.

- Urine application increased microbial stress, as shown by the higher t/c ratio and lower i/a and G+/G- ratios in the urine treatments.

- Urine addition also resulted in an increase in PO_4^{3-}-P, possibly as a result of cell lysis caused by changes in osmotic pressure.

- The high salt treatment caused increased microbial stress, shown by a higher cy/ω7 ratio, and also reduced cumulative N_2O-N emissions.

4.6 Conclusions

Hypothesis 1: That the addition of urine with a higher salt content would stress the soil microbial community.

There was some support for this hypothesis, with higher microbial stress in the high salt treatments being indicated by the cy/ω7 ratio being higher than in the low salt treatments. This

occurred on only one sampling occasion, however, and urinary salt content did not affect the other PLFA stress ratios and was not apparent in the PCA. Although there was evidence of an inhibitory effect of urinary salt on urease activity, indicated by the lower pH in the high salt treatments on day 0, no inhibition of DHA was evident. In general, differences between control and urine treatments were greater than differences between high and low salt treatments. Separation of control and urine treatments by PCA showed that urine application had affected the soil microbial community. Application of urine also increased microbial stress, as shown by an increased t/c ratio and reduced i/a ratio. A reduced G+/G- ratio after urine application was seen on day 29, and may have been caused by a reduction in soil substrates (e.g. WSC) that had increased after urine addition.

Hypothesis 2: That altering the soil moisture content would cause measurable changes in the soil microbial community.

This hypothesis was supported, with higher total PLFAs measured in the wet soils on days 3–8 and higher bacterial PLFAs on days 3–15. There was also evidence of increased microbial stress in the wet soils indicated by the higher concentrations of G- biomarkers and higher t/c ratio compared with the dry soils. This was also shown by the higher qCO_2 in the wet treatments. The separation of the wet and dry treatments in the PCA showed that the soil moisture treatments had an impact on the soil microbial community, particularly affecting the G+ and G- bacteria, which are involved in nitrification and denitrification (Sections 2.5.1.3 & 2.5.1.4). On days 29 and 44 DHA and MBC were significantly lower in the wet soils than in the dry soils.

Hypothesis 3: That any changes in microbial stress or microbial community structure would be reflected in changes in nutrient dynamics.

There was also support for this hypothesis, with changes in nutrient dynamics resulting from both urinary salt treatments and soil moisture treatments. It is probable that both nitrification and denitrification were inhibited by the high salt content, as cumulative N_2O-N emissions were lower in the high salt treatments at both moisture levels. The influence of the soil moisture content on the microbial community was shown by N transformation rates being higher in the wet soil treatments, resulting in significantly large increases in N_2O-N production, which were apparent even in the control treatments. When urine was added to the soil, the wet treatments produced 50 times more N_2O-N than the dry treatments. This demonstrated that, as expected, denitrification was higher in the wet treatments. However, nitrification also appeared to be higher in the wet soil after day 3, as shown by the faster rates of NO_3^--N appearance. There was some inhibition of NO_2^--N oxidation in the wet treatments, resulting in accumulation of NO_2^--N,

which peaked on day 8, and was probably caused by the high pH in conjunction with high concentrations of NH_4^+-N creating high levels of NH_3. Increased concentrations of PO_4^{3-}-P in the urine treatments may have come from microbial cells that lysed because of changes in osmotic pressure (due to urinary salt or added moisture) in the soil solution after urine application. The timing of this increase corresponded with the decrease in microbial biomass seen in both MBC and PLFA measurements.

Future research

- Field studies on the effects of urine application on nutrient addition and release in the presence of plants
- Monitoring of a greater range of trace elements in the urine patch
- Effects of urine application on microbial stress and community dynamics in the presence of plants
- Experiments using urine with a greater variation in salt concentrations than was possible here

Chapter 5
Nutrient dynamics in urine patches in soil under pasture

5.1 Introduction

Experiment 2 was planned to contrast with the 'bare soil' laboratory experiment (Chapter 4) and aimed to measure changes in soil variables after *in situ* urine application to pasture. Under suitable growing conditions, the presence of plants would be expected to reduce the accumulation of soil nutrients, both those sourced from the urine and those potentially released by soil constituents due to urine addition (Sections 2.2.2 & 2.3.2.4). The increase in PO_4^{3-}-P measured in experiment 1 may also have been due to the absence of plants, so one aspect of this study was P concentrations in the soil compared with plant P and total PLFAs. However, plants may also add nutrients to the soil via root exudation and through root decomposition; the latter can occur as a result of damage due to urine application (Section 2.3.1). Thus differences in the soil pH, DHA, and concentrations of inorganic N, WSC, anions and cations between this experiment and experiment 1 could elucidate the impact of plants on soil nutrient changes after urine addition.

Previous studies on nutrient dynamics in urine patches have not included trace element measurements, despite the potential for urine deposition to cause their release from the soil via subsequent dissolution of the SOM (Shand et al., 2000) or as a result of urine constituents displacing them from soil exchange sites. Release of trace elements and other metals has implications for microbes, which require trace elements for enzyme synthesis, while other metals can be toxic at high concentrations; therefore selected metals were monitored for several days after urine application.

The soil microbial community in a field soil could be expected to respond differently *in situ* compared to soil in a laboratory experiment because of differences in nutrient dynamics and environmental conditions (e.g. temperature and moisture), and due to the presence or absence of plants. Release of toxic trace elements and a lack of nutrients through competition from plants could result in microbial stress. The fluctuating moisture conditions in the field (in this case 23–60% WFPS), compared with the constant WFPS of 35 or 70% in experiment 1 could also alter

microbial dynamics, in accordance with previous studies on the wetting and drying of soils (e.g. Kieft et al., 1987; Gordon et al., 2008).

Using templates to contain the urine patches, it was possible to demarcate the urine patch area, so that measurements from soil in the urine patch could be compared with measurements from soil just outside the urine patch, as well as in control plots. Thus any influence of urine immediately outside the urine patch could be assessed, as lateral movement of urine through the soil is believed to be minimal (Williams and Haynes, 1994; Somda et al., 1997; Monaghan et al., 1999), but plants outside the urine patch may be influenced (Section 2.2.1). However, few studies have examined this, possibly because it is often difficult to delineate the exact area of a urine patch.

Therefore this experiment focused on *in situ* nutrient dynamics and potential microbial stress following urine deposition, with the hypotheses being:

1) that the presence of plants would reduce the accumulation of nutrients including N and P,

2) that soil microbes would be stressed by urine application and by competition with plants for nutrients,

3) that the soil microbial community would be stimulated by plants via rhizodeposition and by the release of trace elements following urine application, and

4) that urine effects in soil adjacent to the urine patch would be minimal.

5.2 Materials and Methods

5.2.1 Field site selection and preparation

The field site was located in the Horticultural Research Area at Lincoln University (43°38.8'S, 172°27.3'E), and the soil was a Wakanui silt loam (Mottled Immature Pallic; New Zealand Soil Classification (Hewitt, 1998)). The field had most recently been used for sheep grazing, and the trial area (approximately 16 m x 16 m) was surrounded by an electric fence 5 months prior to the start of the experiment to exclude grazing animals. Site preparation began 4 months before the experiment began, when the site was mowed and sprayed with herbicide to kill the existing vegetation. The soil was then rotovated and large pieces of vegetation were removed, after which the site was levelled with a roller. Two weeks after spraying, the area was resown with perennial ryegrass (*Lolium perenne* var. Meridian Plus AR1) at a rate of 10 kg ha^{-1}, in rows 7.5 cm apart. The site was fertilised twice, at 9 and 6 weeks before the experiment began, at a rate

of 25 kg N ha^{-1} on both occasions. Herbicide was applied eight weeks (Pulsar; 5.0 L ha^{-1}) and six weeks (Versatile; 0.7 L ha^{-1}) before the experiment began. Hand weeding was carried out one week prior to urine application, and the ryegrass was mown to 5 cm height one week before the experiment began (Plate 5.1). The experiment ran from February to April (summer/autumn).

5.2.2 Experimental design and treatments

The experiment was a randomised block design with 5 blocks. Each block contained 6 urine patch plots and 6 control plots. The plots were 2.00 m^2 in area, and the urine patch, which was centred in the plot, covered 0.36 m^2, which is within the range of cow urine patch areas measured (Section 2.2.1). This left a 0.40 m edge around each urine patch within the plot, and the plots were further separated by a 0.10 m buffer strip, so that the urine patches were at least 0.90 m apart.

Plate 5.1 The field site immediately prior to urine application.

5.2.3 Urine collection and application

Urine was collected from Friesian dairy cows at the Lincoln University Dairy Farm 18 h before application, and was stored overnight in a covered opaque PVC tank in a cool location to minimise temperature fluctuations and prevent ammonia volatilisation. The measured urinary N concentration was 8.51 g N L^{-1}. For each patch, 2 L of urine was decanted into a measuring

cylinder and slowly poured onto the plot at a rate of 473 kg ha^{-1}. The location and size of the urine patch was demarcated by an aluminium template (Plate 5.2), which prevented the spread of urine outside the designated area. Urine was evenly spread over the soil within the template, which was left in place until the urine had soaked into the soil.

Plate 5.2 Urine patch template (0.6 m x 0.6 m). (Marker pen for scale.)

5.2.4 Soil and plant sampling

The soil was sampled on 8 occasions (days 3, 6, 9, 13, 21, 30, 43 and 69) after urine application. Two soil cores of 5.0 cm diameter x 5.0 cm depth were taken from inside the urine patch (Ip), from 5.0 cm outside the urine patch (Op), or from the control plot (C). The cores were immediately placed into labelled resealable plastic bags. All the plots in each block were sampled at least once during the experimental period, and two of the plots were reused for the two final samplings. In the laboratory the soil was sieved to < 4 mm and stones and organic matter were removed. The soil was mixed well and a small amount (5–10 g) of each sieved soil sample was frozen at -80°C for later PLFA analysis (Section 3.3.1). Subsamples of the field moist soil were analysed for gravimetric moisture content, inorganic N, water soluble carbon (WSC), anions, and dehydrogenase activity (DHA) (Sections 3.2.1, 3.2.3, 3.2.5, 3.2.6 & 3.3.2). The remainder of the soil was dried and subsamples were analysed for pH and DRP (Sections 3.2.2 & 3.2.4).

Samples of ryegrass herbage were collected on days 3, 6, 9, 13, 21 and 30, by cutting plants at the soil surface. The plant samples were collected adjacent to the soil collection site on each occasion, and were immediately placed in labelled paper bags, which were dried in a 60°C incubator for 4 d in the laboratory. These dried samples were commercially analysed for P (NZLabs, Hamilton, NZ) using ICP-OES after digestion with nitric acid and perchloric acid.

5.2.5 Short-term nutrient and trace element dynamics

Adjacent to the location of the main field trial, further urine patches of 0.25 m^2 were created using cow urine amended with urea to give 10 g N L^{-1} and applied at a rate equivalent to 865 kg N ha^{-1}. The rate of N application was higher in this experiment to create a higher concentration of NH_4^+-N and a higher pH to enhance the potential release of trace elements and other metals from soil exchange sites and SOM (Section 2.3.2.4). Soil samples (0–5 cm) from the centre of the urine patches (Ip) were removed immediately after urine application (day 0) and at the same time on subsequent days (days 1–4), as were samples from control plots (C). The water extractable nutrient fraction was analysed by ICP-OES (Section 3.2.9) for cations (Ca^{2+}, K^+, Mg^{2+} and Na^+) and metals (Al, Cu, Fe and Mo). The metals measured were selected from a suite of results produced by the ICP-OES, because Cu, Fe and Mo are required for enzyme synthesis, and Al can be toxic to microbes at high concentrations.

5.3 Results

5.3.1 Soil pH

The soil pH had increased to 6.90 in the Ip soil 3 days after urine application (Figure 5.1). The pH in the Ip soil was higher than in the Op and C soils on days 3 and 6 ($P < 0.01$), but by day 21 had declined to be lower than in the Op and C soils ($P < 0.001$). The pH in the Op and C soils did not differ from each other throughout the experiment, and averaged 6.30. Over the experimental period, the mean pH in the Ip soil (6.33) was higher than that in the Op soil (6.25) ($P < 0.05$), with the mean pH in the C soil (6.28) of intermediate value and not differing significantly from either of the other treatments. Averaged over all treatments, the soil pH was highest on days 3 and 6 and lowest on day 21, because of the influence of the Ip soil. There was a time x treatment interaction, with the pH in the Ip soil declining and then increasing, while the pH in the C and Op soils increased slightly over time.

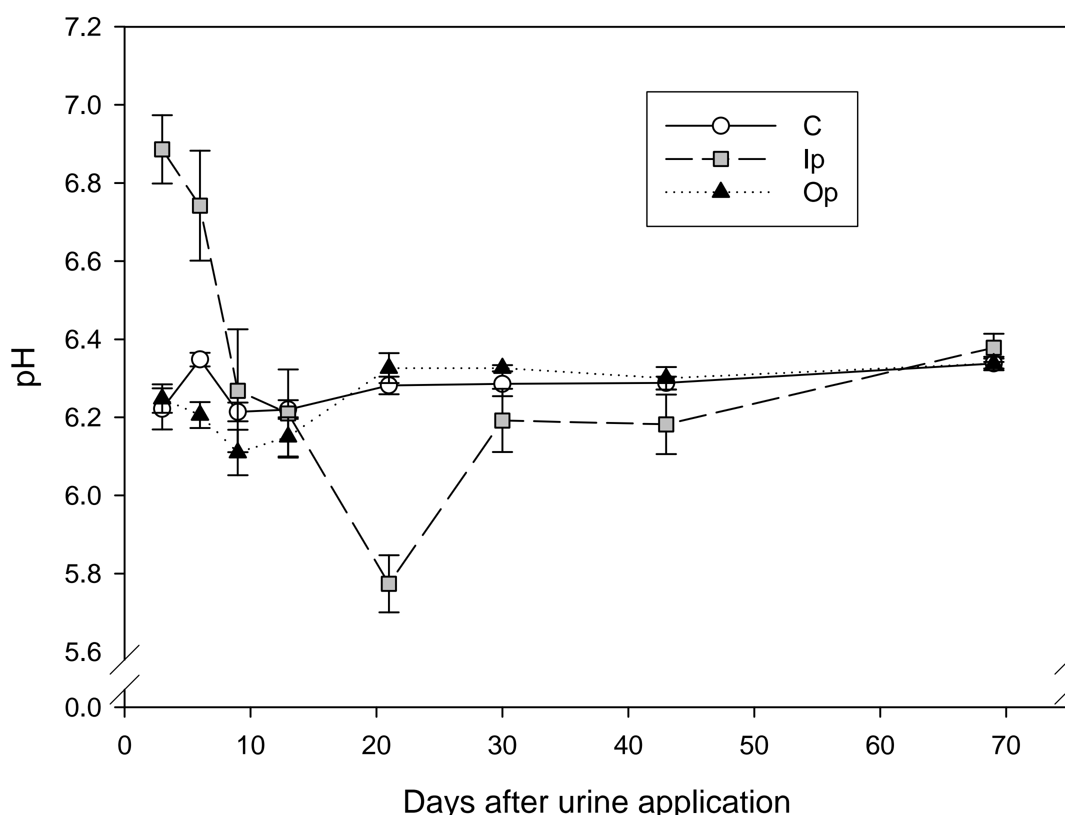


Figure 5.1 Soil pH measured over time, following urine application on day 0, where C = control, Ip = inside urine patch and Op = 5 cm outside urine patch (Error bars = SEM, n = 5).

5.3.2 Inorganic N

Soil NH_4^+-N concentrations had increased to 351 mg N kg^{-1} in the Ip soil by day 3, and they were higher than in the Op and C soils on days 3–21 ($P < 0.01$) (Figure 5.2a). Concentrations of NH_4^+-N did not differ between the Op and C soils at any sampling time, despite being elevated in the Op soil until day 13. However, averaged over the experimental period, NH_4^+-N concentrations were higher in the Op soil than in the C soil ($P < 0.001$), being 22 and 2 mg N kg^{-1} respectively, and both were lower than concentrations in the Ip soil ($P < 0.001$), which averaged 147 mg kg^{-1}. Sampling time had a significant influence on NH_4^+-N ($P < 0.001$), and average concentrations decreased with time to day 13 and did not differ thereafter. There was a strong time x treatment effect ($P < 0.001$), with NH_4^+-N concentrations decreasing over time in the Ip and Op soils and showing little change in the C soil.

Soil NO_2^--N concentrations were significantly higher in the Ip soil than in the Op and C soils on days 3, 6, 21 and 30 ($P < 0.05$), peaking on day 6 at 0.16 mg kg^{-1} and again on day 21 at 0.12 mg kg^{-1} (Figure 5.2b). Concentrations of NO_2^--N in the C and Op soils did not differ on any sampling occasion or over the experimental period, and averaged 0.02 and 0.03 mg kg^{-1} respectively. Overall the mean NO_2^--N concentrations were higher in the Ip soil than in the Op or C soils ($P < 0.001$). Sampling time also had a significant effect, with higher NO_2^--N concentrations on days 3–6, followed by days 13–21, and not differing thereafter ($P < 0.001$). There was a time x treatment interaction effect on NO_2^--N, with concentrations generally being higher in the first half of the experimental period and in the Ip soil.

Soil NO_3^--N concentrations peaked at 129 mg kg^{-1} in the Ip soil on day 21, and were higher in this soil than in the Op and C soils on days 3–30 ($P < 0.01$) (Figure 5.2c). Concentrations of NO_3^--N did not differ between the soils on days 43 and 69. There was also no significant difference between NO_3^--N concentrations in the Op and C soils at any sampling time, although concentrations peaked in the Op soil at < 13 mg kg^{-1} (day 13) and reached only 2.5 mg kg^{-1} in the C soil (day 30). Averaged over the experimental period, the concentration of NO_3^--N was higher in the Ip soil than in the Op and C soils, and did not differ between Op and C soils ($P < 0.001$). The changing concentrations in the Ip soil meant that sampling time had a significant effect on NO_3^--N concentration over the experimental period ($P < 0.001$), and also meant that there was a time x treatment effect ($P < 0.001$). Soil NO_3^--N was negatively correlated with pH in both the Ip and Op soils ($r \leq -0.65$). Correlations between NO_3^--N and NH_4^+-N were not strong, and were not consistent in the different treatments.

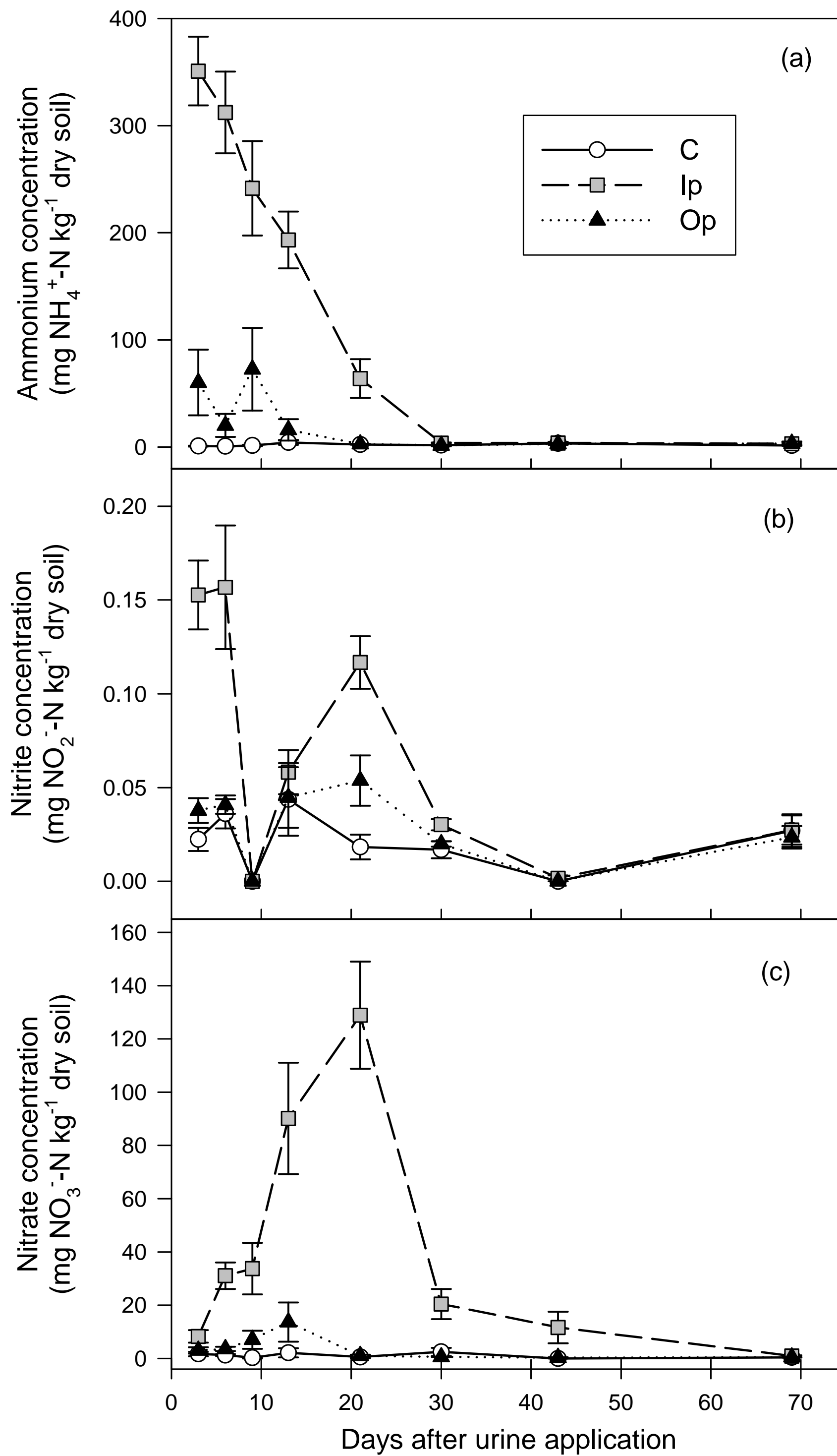


Figure 5.2 Soil inorganic nitrogen concentrations measured over time: (a) ammonium-N, (b) nitrite-N, and (c) nitrate-N, where C = control, Ip = inside urine patch and Op = 5 cm outside urine patch (Error bars = SEM, n = 5). NOTE differing scales on y axes.

5.3.3 Water soluble C and soil moisture content

Water soluble C was highest in all treatments on day 6, with the highest concentration of 326 mg kg^{-1} measured in the Ip soil (Figure 5.3). On days 3 and 6, WSC concentrations were higher in the Ip soil than in the Op and C soils ($P < 0.05$), and on day 69 concentrations in the C and Ip soils were higher than in the Op soil ($P < 0.01$). Sampling time had a significant effect on WSC, with concentrations being higher on day 6 than on all other sampling occasions ($P < 0.001$). There was also a significant treatment effect, with mean WSC concentrations in the Ip soil being higher than those in the Op and C soils over the experimental period ($P < 0.05$). There was a positive correlation between WSC and pH in the Ip soil ($r = 0.74$).

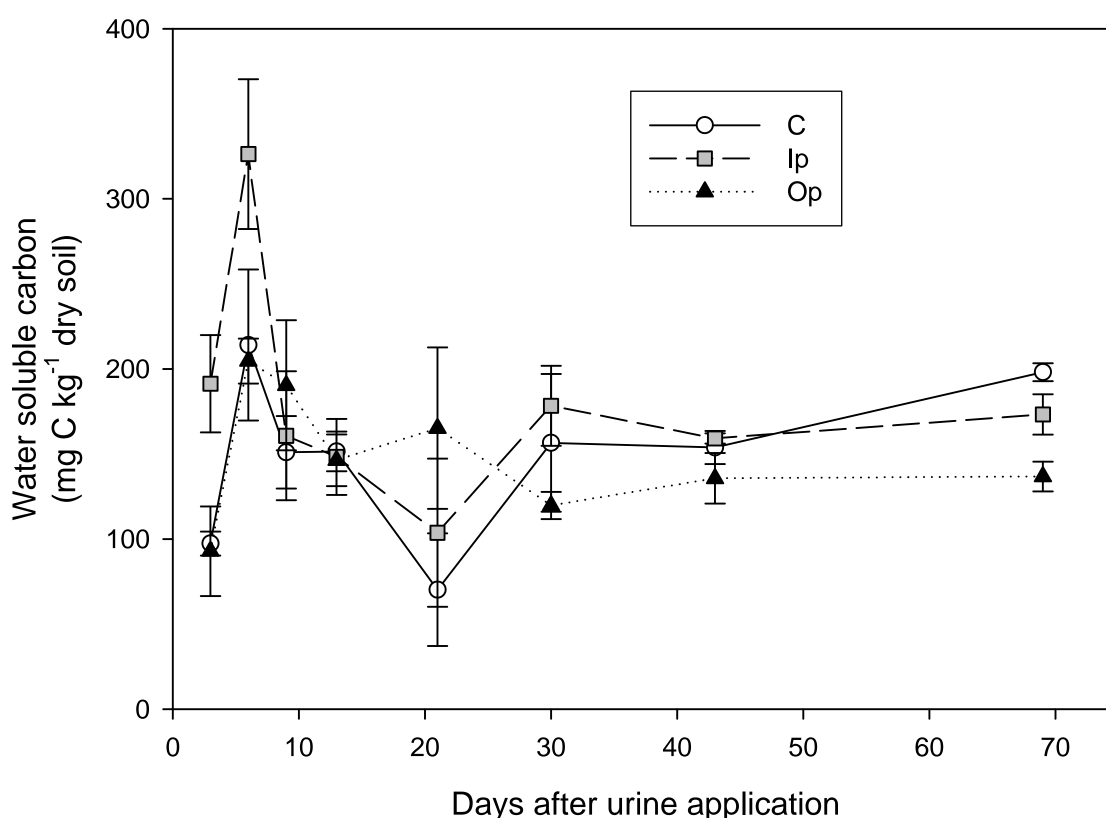


Figure 5.3 Water soluble carbon concentration measured in soil over time following urine application on day 0, where C = control, Ip = inside urine patch and Op = 5 cm outside urine patch (Error bars = SEM, n = 5).

The gravimetric soil moisture content (Figure 5.4a) fluctuated between 0.11 and 0.27 g g^{-1} (23.2 and 59.7% WFPS) over the experimental period due to irrigation and rainfall (Figure 5.4b), and was highest on day 21. The soil moisture content was higher in the Ip soil than in the Op or C soil on day 3, because of the added urine ($P < 0.001$). On days 30 and 43, soil moisture was higher in the C and Op soils than in the Ip soil ($P < 0.05$), and on day 69 the soil moisture

content was higher in the C soil than in both the Ip and Op soils ($P < 0.05$). There was a significant effect of sampling time on the soil moisture content due to the added water ($P < 0.001$), which also created a time x treatment interaction effect ($P < 0.001$).

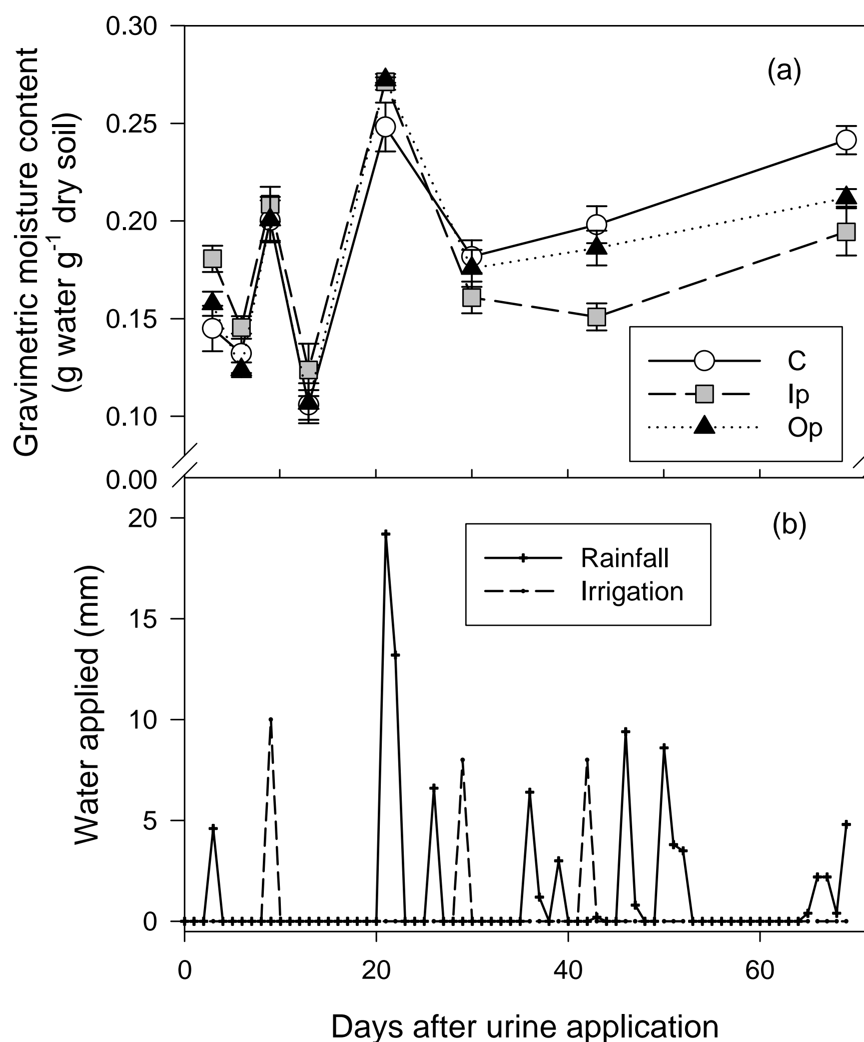


Figure 5.4 (a) Gravimetric soil moisture content measured in soil over time, where C = control, Ip = inside urine patch and Op = 5 cm outside urine patch (Error bars = SEM, n = 5), and (b) water applied.

5.3.4 Ionic concentrations

The Cl^- concentration had increased to 0.90 $cmol_c\ kg^{-1}$ in the Ip soil 6 d after urine application (Figure 5.5a), while averaging 0.04 and 0.10 $cmol_c\ kg^{-1}$ in the C and Op soils respectively, throughout the experiment. On every sampling occasion, the Cl^- concentrations were higher in the Ip soil than in the Op and C soils ($P < 0.001$). Averaged over all sampling occasions, the mean concentration of Cl^- was higher in the Ip soil than in the Op soil and higher in the Op soil than in the C soil ($P < 0.001$). Time of sampling had a significant effect on Cl^- concentration,

which was higher on days 6–13 than on days 21–30 ($P < 0.001$). There was also a time x treatment interaction effect, with Cl^- concentrations declining over time in the Ip and Op soils ($P < 0.001$). Chloride concentration was positively correlated with both NH_4^+-N and SO_4^{2-}-S in the Ip and Op soils ($r \geq 0.95$), and negatively correlated with pH in the Op soil ($r = -0.94$).

Concentrations of SO_4^{2-}-S were elevated following urine application, with the highest concentration of 0.103 $cmol_c\ kg^{-1}$ recorded in the Ip soil on day 6 (Figure 5.5b). The SO_4^{2-}-S concentration was higher on all sampling occasions in the Ip soil than in the Op or C soils ($P < 0.001$). There were no significant differences between SO_4^{2-}-S concentrations in the Op and C soils on any sampling occasion or averaged across the experimental period. Sulphate-S concentrations in the Op and C soils averaged 0.018 and 0.012 $cmol_c\ kg^{-1}$ respectively during the experiment. Sampling time significantly affected SO_4^{2-}-S concentration, which was higher on days 6–13 than on day 21, and day 30 was lower than on all previous sampling occasions ($P < 0.001$). There was also a time x treatment interaction effect, due to the relatively larger decrease in SO_4^{2-}-S concentrations in the Ip soil over time ($P < 0.001$). Sulphate-S was positively correlated with NH_4^+-N in the Ip and Op soils ($r \geq 0.89$), and negatively correlated with pH in the Op soil ($r = -0.96$).

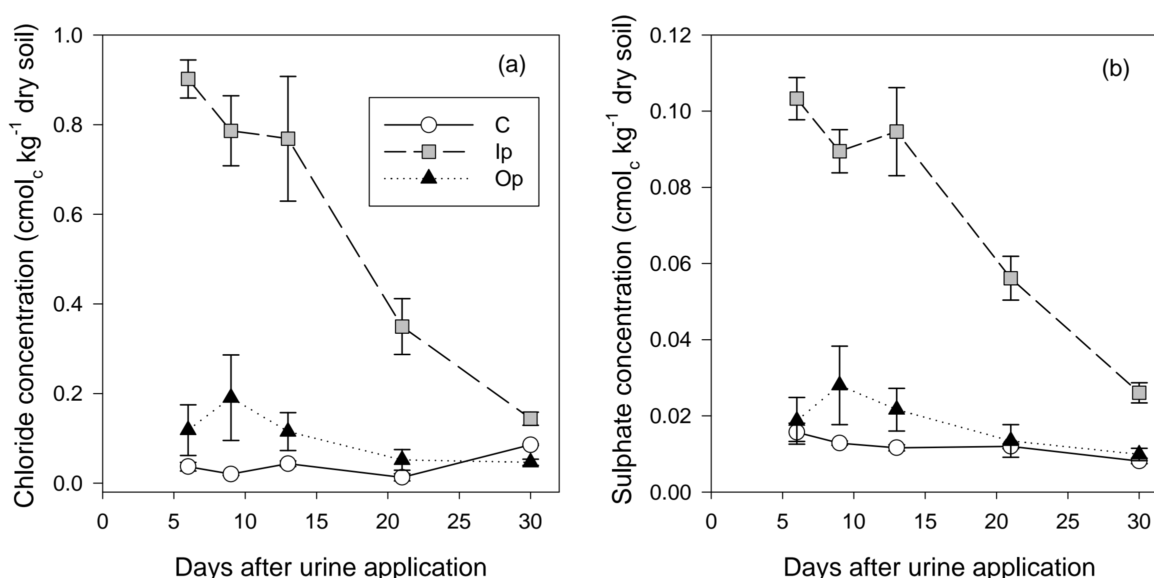


Figure 5.5 Concentrations of water extractable (a) chloride and (b) sulphate-S measured over time following urine application to soil on day 0, where C = control, Ip = inside urine patch and Op = 5 cm outside urine patch (Error bars = SEM, n = 5). NOTE: differing scales on y axes.

Concentrations of K^+ were higher in the Ip soil than in the C soil on all sampling occasions ($P < 0.05$), while Na^+ concentrations were significantly higher in the Ip soil only on days 0 and 4 ($P < 0.01$) (Figures 5.6a & 5.6b). Averaged over the 5 d sampled, concentrations of the nutrients

were higher in the Ip soil than in the C soil ($P < 0.001$). Concentrations of Na^+ and K^+ initially decreased, but then increased between days 2 and 4, to be higher at the end of the experiment than at the start ($P < 0.001$). On day 4, concentrations of K^+ and Na^+ were 0.34 and 0.10 $cmol_c$ kg^{-1} respectively. There was a significant time x treatment interaction for K^+ ($P < 0.01$) due to the changes in concentrations over time.

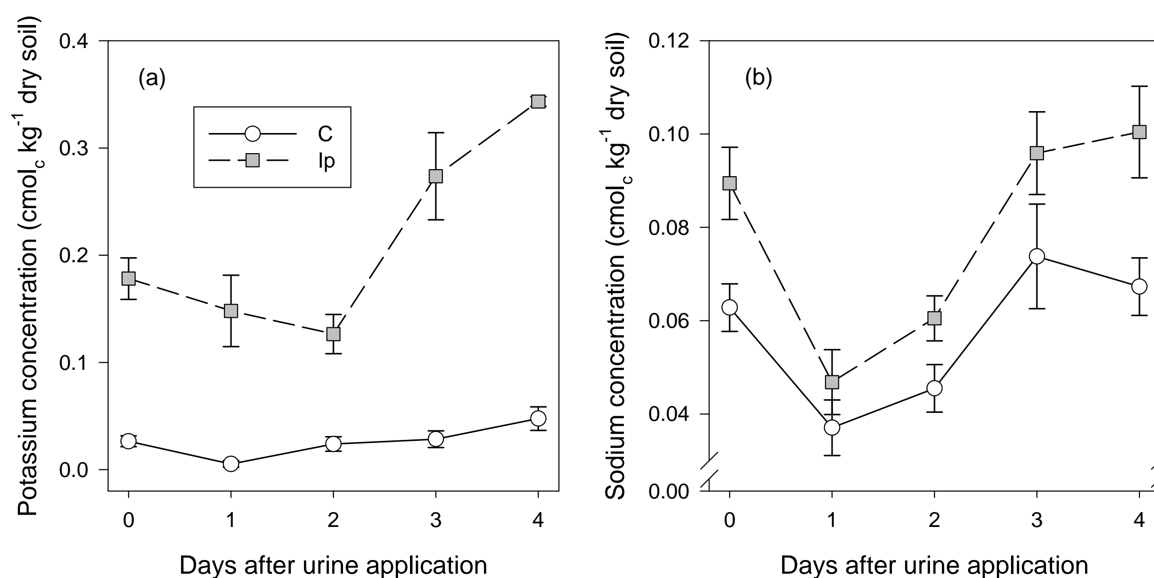


Figure 5.6 Concentrations of water extractable (a) potassium and (b) sodium measured over time following urine application to soil on day 0, where C = control, Ip = inside urine patch and Op = 5 cm outside urine patch. NOTE: differing scales on y axes.

Concentrations of Ca^{2+} were initially low in the Ip soil, at 0.05 $cmol_c$ kg^{-1}, but had increased by day 3, and were highest on day 4 at 0.37 $cmol_c$ kg^{-1} (Figure 5.7a). Calcium concentrations were higher in the Ip soil than in the C soil on all days except day 1 ($P < 0.05$), and were higher in the Ip soil when averaged over the sampling period ($P < 0.001$). The increase in Ca^{2+} over time meant that the concentration on day 4 was higher than the concentration on day 3, and days 0–2 had lower concentrations than the last 2 d sampled ($P < 0.001$). There was also a time x treatment effect ($P < 0.001$), because of the increase in Ca^{2+} in the Ip soil. Concentrations of Mg^{2+} in the Ip soil were higher than expected initially, at 0.051 $cmol_c$ kg^{-1}, but declined to 0.017 $cmol_c$ kg^{-1} by day 2 and then increased to 0.082 $cmol_c$ kg^{-1} by day 4 (Figure 5.7b). Magnesium concentrations were higher in the Ip soil than in the C soil on all sampling occasions ($P < 0.05$) and overall ($P < 0.001$). Sampling time had a significant effect on Mg^{2+} concentration ($P < 0.001$), with concentrations on days 0 and 3 being higher than on days 1 and 2 but lower than on day 4. There was a significant time x treatment effect ($P < 0.01$), due to the changes in Mg^{2+} concentration in the Ip soil over the sampling period.

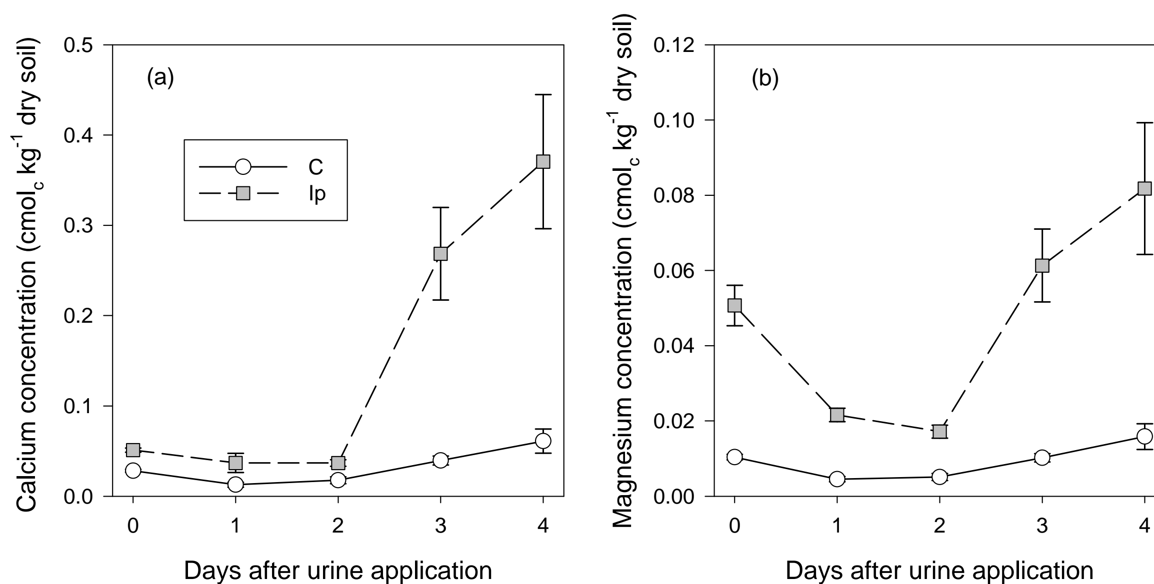


Figure 5.7 Concentrations of water extractable (a) calcium and (b) magnesium measured over time following urine application to soil on day 0, where C = control, Ip = inside urine patch and Op = 5 cm outside urine patch. (Error bars = SEM, n = 4). NOTE: differing scales on y axes.

5.3.5 Trace elements and other metals

The trace elements essential for enzyme synthesis were all elevated by urine addition, with the highest concentrations of 0.077, 19.7 and 0.022 mg kg^{-1} for copper (Cu), iron (Fe), and molybdenum (Mo), respectively (Figures 5.8a–c), measured 1 h after urine application on day 0. The concentration of Mo was higher in the Ip soil on days 0 and 1, but higher in the C soil on days 3 and 4 ($P < 0.05$). Molybdenum concentrations were higher on day 0 than on days 3 and 4 ($P < 0.05$). Averaged over the period sampled, concentrations of Mo were higher in the Ip soil than in the C soil ($P < 0.01$), and there was a time x treatment effect ($P < 0.001$), due to the reduction of Mo over time in the Ip treatment. Iron concentrations were higher in the Ip soil than in the C soil on days 0–2 ($P < 0.01$), and higher in the Ip soil over the period sampled ($P < 0.001$). Concentrations on days 1 and 2 were lower than on day 0, but higher than on days 3 and 4 ($P < 0.001$). There was a time x treatment effect, with the Fe concentration in the Ip soil reducing over time ($P < 0.001$). Concentrations of Cu fluctuated, but were higher in the Ip soil on day 0 and higher in the C soil on day 4 ($P < 0.05$). There was no difference between Cu concentrations in the two treatments when averaged over the sampling period. The concentration of Cu was higher on days 0–2 than on day 3 ($P < 0.01$), and there was a time x treatment effect, with an overall reduction in Cu concentration over time ($P < 0.05$).

The Al concentration was highest on day 0 at 45.5 mg kg^{-1} (Figure 5.8d). Aluminium concentrations were higher in the Ip soil than in the C soil on days 0–2 ($P < 0.05$) and did not differ thereafter. Over the sampling period concentrations of Al were higher in the Ip soil ($P < 0.001$), and sampling time had a significant effect, with concentrations on days 1 and 2 higher than on days 3 and 4 but lower than on day 0 ($P < 0.001$). The decline in Al concentration with time meant that there was a significant time x treatment effect ($P < 0.001$).

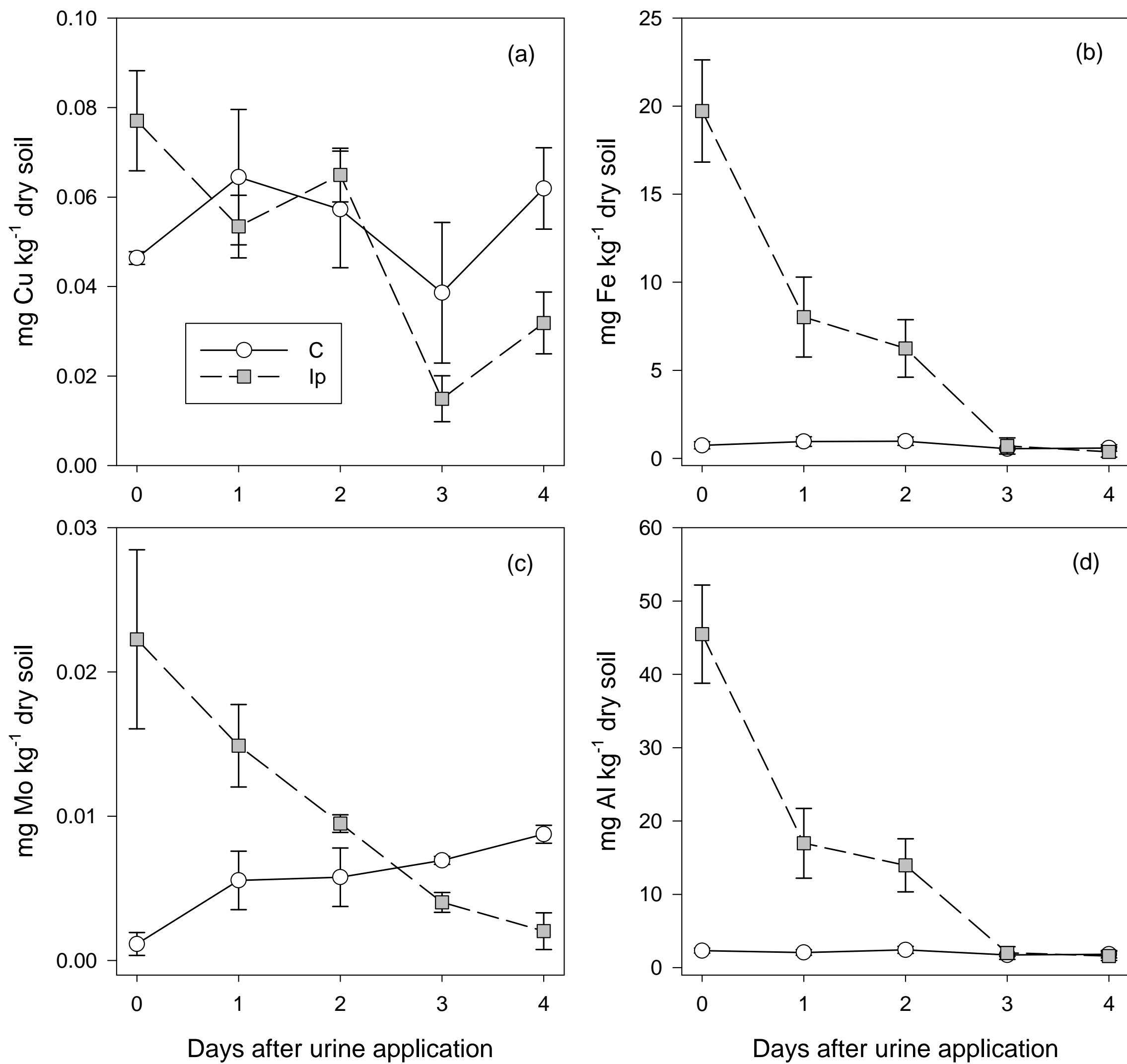


Figure 5.8 Concentrations of water extractable trace elements and other metals, measured following day 0 urine application to soil: (a) copper, (b) iron, (c) molybdenum, and (d) aluminium (Error bars = SEM, n = 5). NOTE: differing scales on y axes.

5.3.6 P analyses

Dissolved reactive P (DRP) concentrations in the C soil were higher than in the Ip soil on days 3–13 and higher than in the Op soil on days 6–13 ($P < 0.05$) (Figure 5.9). The DRP was higher in the Op soil than in the Ip soil on day 6 ($P < 0.01$), but they did not differ on any other occasion. By day 21 there were no differences between DRP concentrations in the different treatments. Over the course of the experiment, mean DRP concentrations were higher in the C soil than in the Op soil, and higher in the Op soil than in the Ip soil ($P < 0.001$). Dissolved reactive P concentrations were positively correlated with soil moisture content in the Ip and Op soils ($r \geq 0.74$).

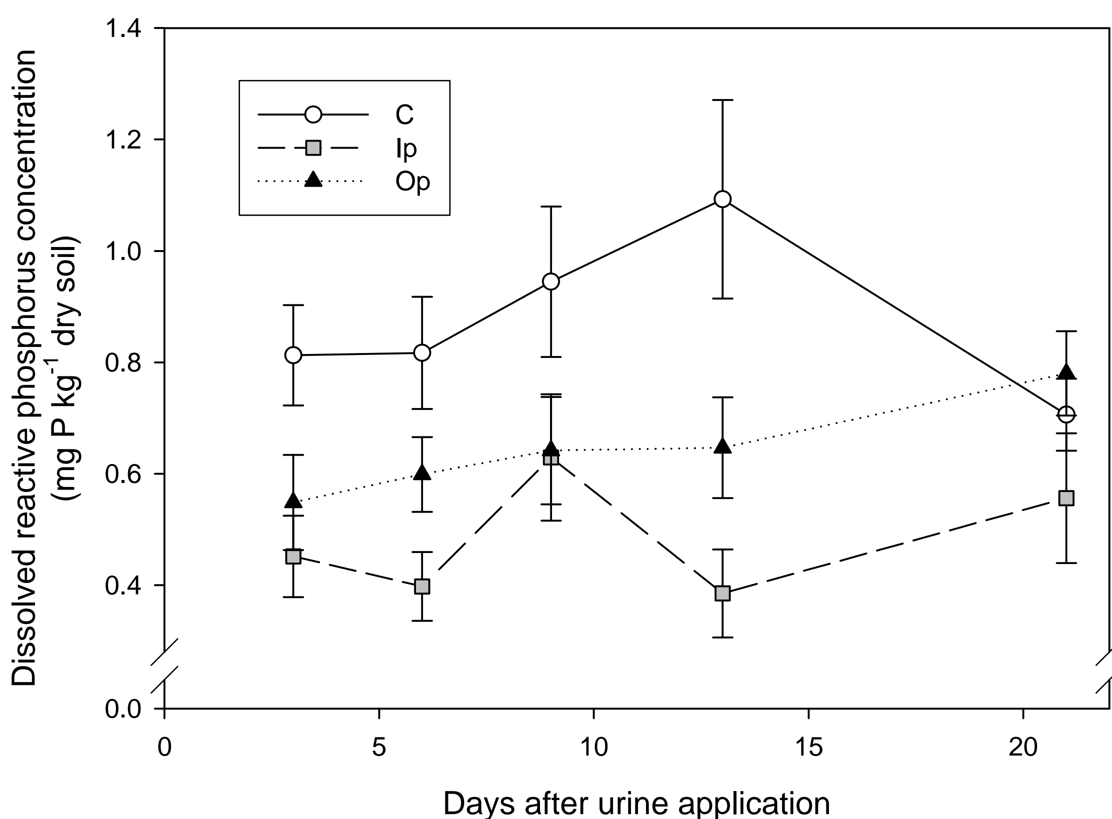


Figure 5.9 Concentrations of dissolved reactive phosphorus (DRP) in the soil, measured over time following day 0 urine application (Error bars = SEM, n = 5).

The measured concentrations of P in the dried plant material were 2300 to 4800 mg kg^{-1} (0.23–0.48%) (Figure 5.10). Averaged over the sampling period, mean plant P concentrations were lower in the Ip treatment than in the C and Op treatments ($P < 0.001$). Plant P was significantly lower in the Ip treatment compared with the C and Op treatments on days 3 and 13 ($P < 0.05$), and was lower in the Ip and Op treatments than in the C treatment on day 30 ($P < 0.01$). On day 6 the difference between the Ip treatment and the other two treatments was only significant at the $P < 0.10$ level. The plant P concentration was higher on day 6 than on all other sampling occasions ($P < 0.001$). Plant P and DRP were positively correlated ($r = 0.25$, $P < 0.05$).

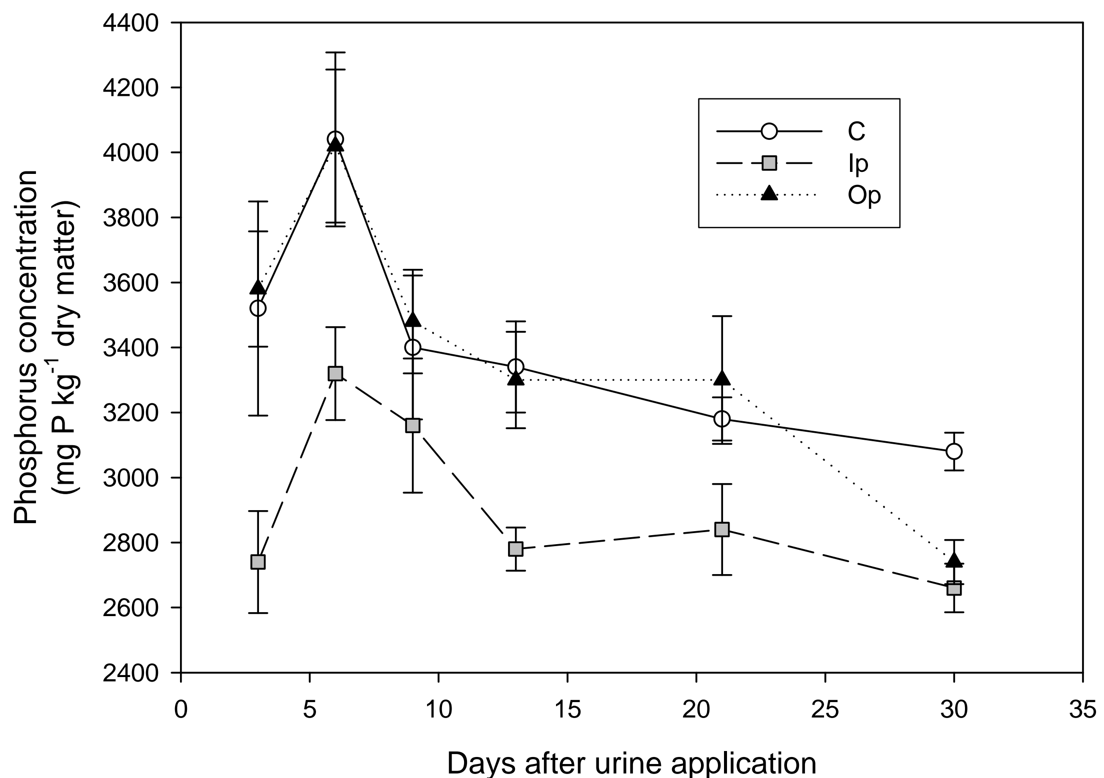


Figure 5.10 Concentrations of phosphorus measured in air-dried plant herbage over time (Error bars = SEM, n = 5).

5.3.7 Phospholipid fatty acid analyses

The concentration of total PLFA biomarkers was significantly higher on day 30 than on day 9 ($P < 0.001$), averaging 12.3 and 27.4 nmoles g^{-1} of soil on days 9 and 30 respectively (Figure 5.11a). Total PLFAs were significantly higher in the Ip soil than in the Op and C soils on day 30 ($P < 0.05$).

Bacterial PLFA biomarkers, G+ bacterial biomarkers and G- bacterial biomarkers were highest in the Ip soils on both days, but the difference was only significant for the G+ biomarkers on day 30 ($P < 0.05$). The G+/G- ratios were higher in all the soils on day 9 than on day 30 (Figure 5.11b). This reflected a mean increase in G- biomarkers of 152.3% between the two days, while the mean increase of G+ biomarkers was only 37.1%. On day 9 the G+/G- ratio was higher in the Ip and Op soils than in the C soil, although not significantly, while on day 30 the ratio in the C soil was significantly higher than in the Op soil ($P < 0.05$), but the ratio in the C and Op soils did not differ from the ratio in the Ip soil.

Concentrations of the fungal biomarker (18:2ω6) were higher in the Ip soil than in the Op and C soils on both days and this difference was significant on day 30 ($P < 0.01$). This was reflected in the ratio of fungal to bacterial biomarkers (F/B ratio), which was significantly higher in the Ip soil than in the C soil on day 30 ($P < 0.05$) (Figure 5.11c). The concentration of fungal biomarker and the F/B ratio were higher on day 30 than on day 9 ($P < 0.001$).

Two of the three microbial stress ratios used (cy/ω7 and t/c) did not differ significantly between any of the treatments on either sampling occasion. However, the i/a ratio showed significant treatment differences on both days (Figure 5.11d). On day 9, the i/a ratio was lower in the Ip soil than in the C soil ($P < 0.05$), and on day 30 the ratio was lower in the Ip soil than in both the C and Op soils ($P < 0.001$), with a lower ratio indicating increased stress.

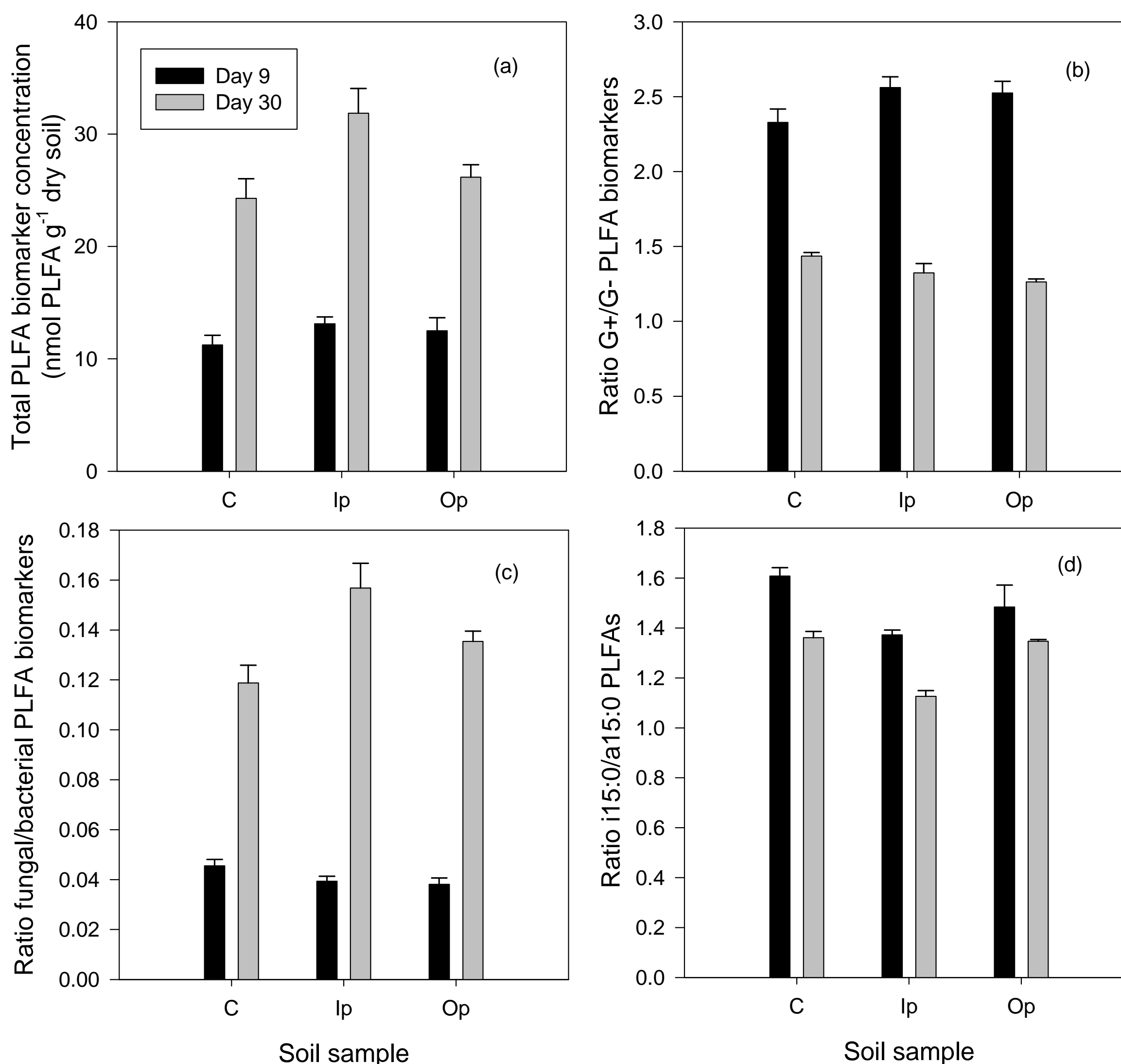


Figure 5.11 Results of phospholipid fatty acid analyses for days 9 and 30: (a) Total PLFA biomarker concentration, (b) Gram positive/Gram negative biomarker ratio, (c) fungal/bacterial biomarker ratio, and (d) iso 15:0/anteiso 15:0 ratio.

Total PLFAs were negatively correlated with plant P (r = -0.92, $P < 0.01$) (Figure 5.12a) and with DRP (based on day 9 data) (r = - 0.47, $P > 0.10$) (Figure 5.12b).

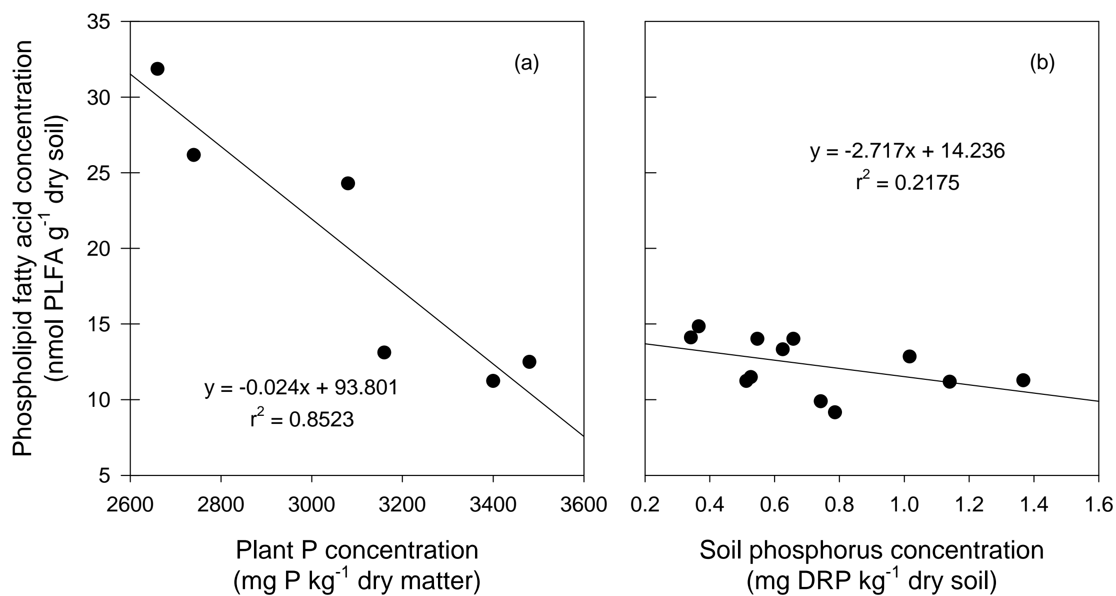


Figure 5.12 Relationships between (a) total PLFAs and plant P and (b) total PLFAs and soil DRP.

A plot of PC1 vs. PC2 principal component scores for day 9 indicated that there were few differences between treatments (Figure 5.13a), and this was supported by the ANOVA results for this data, which showed that neither PC1 nor PC2 were affected by soil treatment. In contrast, the day 30 PCA data showed that the Ip soil differed significantly from the Op and C soil on PC1 ($P < 0.001$), while the Op soil differed from the Ip and C soil on PC2 ($P < 0.001$) (Figure 5.13b). On day 30, the main influences on the PC1 axis were the PLFAs 18:1ω9t, 18:1ω9c and 18:2ω6 with positive loadings, and i15:0 and 16:0 with negative loadings. The PLFAs with the biggest influence on PC2 on day 30 were cy19:0 with a positive loading and a15:0 with a negative loading. These PLFAs include stress indicators, G+ and G- biomarkers, and the fungal biomarker. When graphed on the same axes (data not shown), the PCA scores for the two days could not be differentiated.

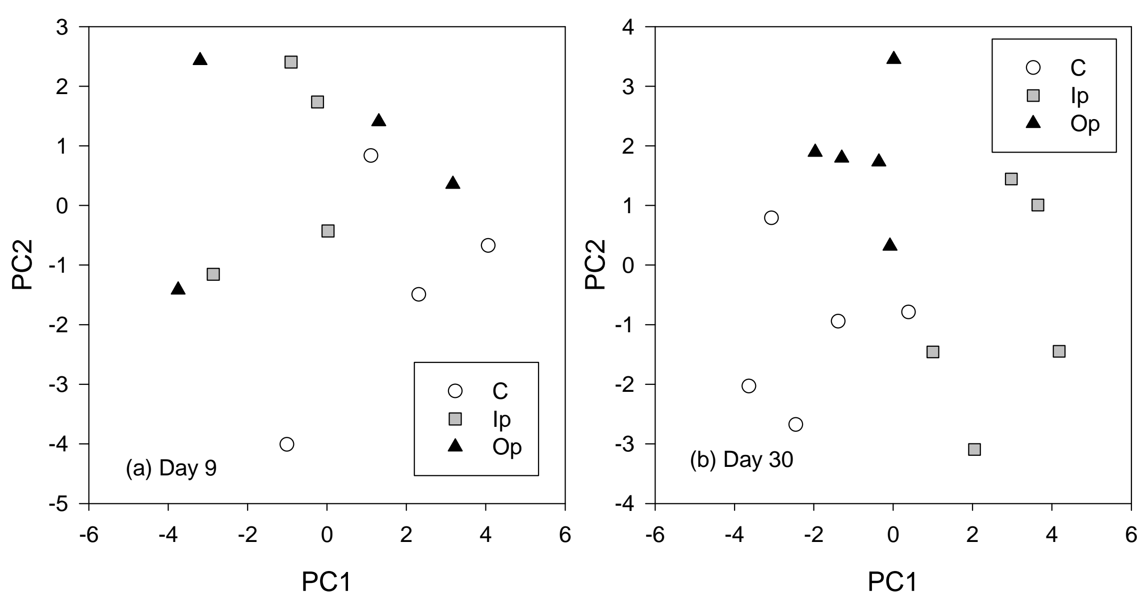


Figure 5.13 Principal component analyses of PLFAs as mol% values for (a) day 9 and (b) day 30.

5.3.8 Microbial activity

Dehydrogenase activity was higher in the Ip soil than in the Op and C soils on days 3 and 6 ($P < 0.01$), but the reverse was true on days 30 and 43 ($P < 0.05$) (Figure 5.14). The highest DHA measured in the Ip soil was 8.0 μg TPF g^{-1} soil h^{-1} on day 6, but the highest activity measured overall was 8.5 μg TPF g^{-1} soil h^{-1} in the C soil on day 69. There was no difference between DHA in the Op and C soils throughout the experiment. Over the experimental period, sampling time had a significant effect on DHA, with higher activity on day 69 and lower activity on days

13 and 43 than on all other occasions ($P < 0.001$). Dehydrogenase activity was positively correlated with soil moisture content in the C and Op soils ($r \geq 0.74$) and with pH in the Ip and Op soils ($r \geq 0.64$). There were also positive correlations between DHA and DRP in the Op soil ($r = 0.80$) and between DHA and Cl^- in the Ip soil ($r = 0.67$). Dehydrogenase activity and WSC were positively correlated in the Ip soil ($r = 0.62$).

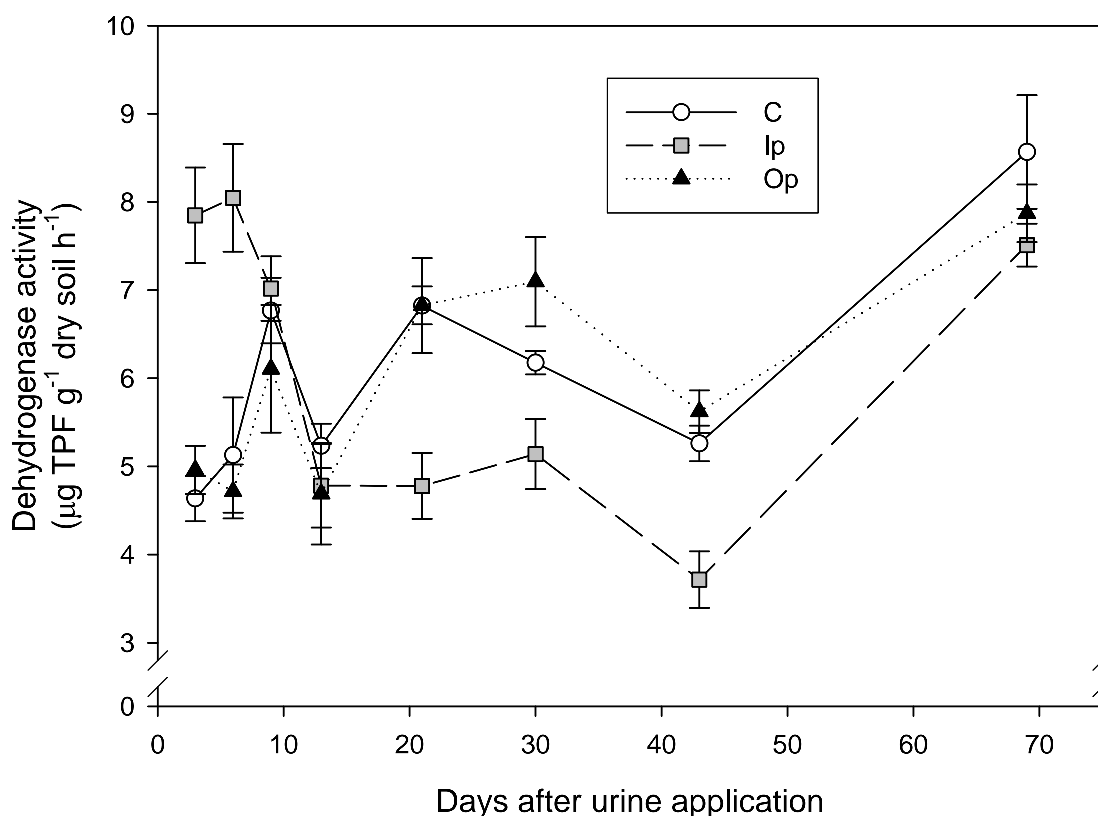


Figure 5.14 Dehydrogenase activity measured over time following urine application on day 0 (Error bars = SEM, n = 5).

5.4 Discussion

5.4.1 Nutrient dynamics and plant effects

As in experiment 1, the soil pH and NH_4^+-N concentration increased after urine application and declined with time, while the concentration of NO_3^--N was initially low and increased as the NH_4^+-N decreased. In contrast to experiment 1, a laboratory study, the maximum soil pH in this field trial was only 6.9 (c.f. > 8.5), with both values measured 3 d after urine application. The pH had returned to control values by day 9 in this study, whereas in experiment 1 the pH remained elevated until after day 15. The same trend was seen with NH_4^+-N, with a concentration of 350 mg kg^{-1} measured in the Ip soil on day 3 in this study, compared with a

mean concentration of 1440 mg kg^{-1} on day 3 in experiment 1 (Chapter 3). Similarly, concentrations of NO_3^--N in the Ip soil in this current experiment peaked at ca. 130 mg kg^{-1}, but were > 500 mg kg^{-1} in experiment 1.

The main reason for these differences between the two experiments was the lower rate of N application in this experiment (473 kg ha^{-1}) compared with experiment 1 (750 kg ha^{-1}). This would have reduced the amount of NH_4^+-N in the soil and thus the amount of NO_3^--N produced, an effect also seen by Clough et al. (2003b) at N application rates between 0 and 500 kg ha^{-1}. It is likely, however, that the presence of plants and the probability of higher diffusion of NH_3-N from the soil also had an impact. The absence of plants in experiment 1 meant that NH_4^+-N and NO_3^--N accumulation would occur, whereas the growing plants in this experiment could have removed substantial quantities of both of these ions, e.g. Barraclough et al. (1985) measured annual plant uptake of 76% of the N applied. Meanwhile, the relatively confined headspace and semi-permeable evaporative barrier on the soil cores together with a lack of wind meant that NH_3-N could not disperse from the soil to the same extent as it would in the field, thus reducing volatilisation rates, so that NH_4^+-N concentrations remained elevated in experiment 1. This could have resulted in a large reservoir of NH_4^+-N available for nitrification in the soil cores, so that NO_3^--N concentrations were still at or near their peak on day 44 in experiment 1, while in this current experiment the NO_3^--N concentration was highest in the Ip soil on day 21 and had returned to control values by day 43.

Concentrations of Cl^- and SO_4^{2-}-S were elevated in the Ip soil, as in experiment 1, because they are constituents of ruminant urine (Section 2.2.2.2). The decline over time may have been due to uptake by plants, assimilation by microbes, leaching, and/or adsorption on SOM (Broyer et al., 1954; Williams and Haynes, 1992, 1993a, 1993b; Öberg and Sandén, 2005). Since additional Cl^- was added to the urine used in experiment 1, the concentrations in the two experiments cannot be compared. Concentrations of SO_4^{2-}-S, however, were comparable in these experiments, with the slightly lower concentrations in this experiment suggesting possible plant utilisation and/or microbial uptake (Williams and Haynes, 1992, 1993b).

The cations K^+ and Na^+ are also deposited in urine, with some adsorbed onto cation exchange sites (Section 2.3.2.4). The delayed increases in K^+ and Na^+ concentrations, as measured in this experiment (Figures 5.6a & 5.6b), were due to displacement of the cations from soil exchange sites by H^+ and NH_4^+ (Section 2.3.2.4). The much higher concentrations of K^+ and Na^+ on day 3 in experiment 1 (even when only the unamended low K^+ urine was considered) could have been due to greater displacement caused by the higher concentrations of NH_4^+. This same mechanism

results in increased concentrations of soil Ca^{2+} and Mg^{2+} which are present in only low concentrations in urine (Section 2.2.2.2). Concentrations of Ca^{2+} were similar on day 3 in both experiments, while concentrations of Mg^{2+} were slightly higher on day 3 in experiment 2, but this may have been due to variation in the applied cow urine. It is plausible that the higher total cation concentration in experiment 1 was responsible for the greater degree of microbial inhibition and stress measured in that experiment. However, as discussed in Section 5.4.2 below, the i/a stress ratio seemed to indicate salt induced stress in both experiments following urine application.

Ruminant urine generally contains only low concentrations of P (Section 2.2.2.2). However, after measuring an increase in PO_4^{3-}-P after urine addition in experiment 1 (Figure 4.8), it was anticipated that there might also be an increase in soil DRP concentrations in this field experiment. Increases in total dissolved P (TDP) were measured by Shand et al. (2000; 2002) after the application of synthetic sheep urine to soil. In this current experiment, in contrast to experiment 1, soil P concentrations were lower in the Ip and Op soils than in the C soil at most sampling times. At the pH values of 6–7 measured during this experiment, much of the soil P would be in the form of Al or Ca phosphates, with fixation of phosphate expected to be slightly reduced at the elevated pH in the Ip soil on days 3–6 (Sauchelli, 1965). Furthermore, if carbonic acid (H_2CO_3) formed as a result of CO_2 production following urea hydrolysis and from microbial activity, it could have dissolved some of the Ca phosphates (Sauchelli, 1965), so that the amount of available P would be expected to be higher in the Ip soil than in the other treatments. Since the reverse was seen, rapid uptake of available P was indicated.

However, plant P concentrations showed the same trend as soil DRP concentrations, with the lowest P values in the plant herbage being measured in the Ip treatment. This differed from some previous experiments, where plant P concentrations (% DM) were either enhanced or unaffected by the application of synthetic sheep urine or urea to the soil, while total plant P uptake (g P m^{-2}) increased (Reid et al., 1966; Williams et al., 1999a; Shand et al., 2002). However, these current results are in agreement with other studies that showed a decline in plant P with increasing N uptake rates following urine or fertiliser application to soil (Dijkshoorn, 1958; Riley and Barber, 1971; Joblin and Keogh, 1979). Thus, in this current study, the enhanced concentrations of soil N in the Ip soil, which were already apparent by day 3, may have inhibited plant P uptake.

It is plausible that the lower P concentration in the Ip plants in this current study resulted from greater dilution of the plant P due to the greater plant biomass in the Ip treatment, but since the

effect was present from day 3, this is unlikely. Since only the plant herbage was analysed, it is possible that there were higher concentrations of P in the plant roots in the Ip treatment. Relocation of plant P from older herbage to roots can occur when P is limiting (Schachtman et al., 1998). This may have been the case in this experiment, as soil DRP was certainly depleted in the Ip soil compared with the C and Op soils, although in other studies, addition of urine or urea to soil increased water-soluble P concentrations (Hartikainen and Yli-Halla, 1996; Williams et al., 1999a; Shand et al., 2000). The relationship between soil DRP and PLFA concentrations is discussed in Section 5.4.3 below.

Addition of urine resulted in transient increases in concentrations of Al, Cu, Fe and Mo. The water soluble fraction that was analysed consists of free ions and ions complexed with organic and inorganic ligands (Shuman, 1991; Stevenson and Cole, 1999). Since this fraction was significantly lower in the C soils, the increased concentrations in the Ip soils must have come either from the urine or from disruption of the more stable forms of the nutrients within the soil. Concentrations of urinary Al, Cu, Fe and Mo are generally negligible (Dick, 1956; Safley et al., 1986; Barrow, 1987; Crowe et al., 1990), so it is unlikely that the elevated concentrations measured in the soil (particularly the Al and Fe) were directly attributable to the added urine. It is improbable that the increase in pH directly released metals from soil complexes, as the solubilities of Al, Cu and Fe all decrease with increasing pH (Driscoll and Postek, 1996; Stevenson and Cole, 1999). Furthermore, although Mo becomes more soluble with increasing pH (Stevenson and Cole, 1999), its increase on days 0–1 and ensuing decline followed the same trend as that of Al and Fe, which would not be expected simply from a change in the soil solution pH.

The increase in soil pH, following urine application, may have enhanced SOM degradation (Barrow, 1960; Williams and Haynes, 1994; Shand et al., 2000; Shand et al., 2002), so that trace elements bound in organo-metallic complexes were released (McBride and Blasiak, 1979; Stevenson and Cole, 1999). Alternatively, the addition of cations in the urine (Section 2.2.2.2) could have displaced some metal ions from soil cation exchange sites (Stevenson and Cole, 1999) so that they were temporarily solubilised before being readsorbed onto exchange sites or taken up by plants or microbes (Kochian, 1991; Shuman, 1991; Stevenson and Cole, 1999). Since Cu is more tightly bound to organic matter than the other elements measured (Shuman, 1991), the weaker response of Cu to urine application, compared with Al, Fe and Mo, suggests that the metals released were from the organically bound fraction.

5.4.2 Microbial stress

Aluminium can be toxic to both bacteria and fungi, although this generally occurs when the soil pH is low (e.g. Firestone et al., 1983; Guida et al., 1991). The enhanced concentrations of Al measured in the Ip soil on days 0–2 in this experiment were potentially high enough to induce inhibition of soil microbes (Guida et al., 1991). However, in this current experiment Al toxicity would be difficult to distinguish from inhibition of microbes due to high pH, and because of the extremely short-term nature of the enhanced concentrations, it would be hard to attribute urine-induced microbial inhibition to Al toxicity with any certainty. Furthermore, complexation with SO_4^{2-} reduces Al toxicity (Robert, 1995), so the high initial SO_4^{2-}-S concentrations in this experiment would have moderated the potential effects of Al on the soil microbial community.

The higher G+/G- ratios on day 30 compared with day 9 reflected the greater magnitude of the increase in G- bacteria, and indicated that the stress on the microbial communities had increased, with the more resilient G- bacteria predominating (Section 2.4.2.4). A similar change over time was seen in experiment 1. The decrease in the i/a ratio in all the treatments between days 9 and 30 support this conclusion.

The i/a ratio was also the only one of the three PLFA stress ratios monitored that indicated increased microbial stress in the urine patch, with a lower i/a ratio in the Ip soil than in the C soil on both sampling occasions. Thus, as in experiment 1, the i/a ratio seemed to be the most suitable for indicating urine-induced stress in the soil microbial community. A reduction in the i/a ratio was also measured by Petersen et al. (2004a) following application of urea (5.6 and 9.5 g N L^{-1}) to soil in a field trial. The lower i/a ratio in the Ip soil showed that, although the microbial biomass had increased, at least some section of the microbial community was under increased stress in the urine affected soil. The higher stress in the urine treatments, also measured in experiment 1, was attributed to increased salt concentrations, which the i/a ratio has been used to indicate in other studies (Section 2.4.2.4). Thus, despite the intact soil profile and the inclusion of plants, the soil microbial community in this experiment still showed the same urine-related stress response as in the previous bare soil study, experiment 1.

Although the presence of plants in this experiment was expected to lead to increased microbial stress due to competition for nutrients, including P, there was in fact a greater increase of PLFA biomass in this experiment, between days 9 and 30, than was measured in experiment 1, where plants were absent. This may have been because rhizodeposition by the pasture plants supplied substrates to the microbes, which were lacking in experiment 1. The accumulation of PO_4^{3-}-P in

experiment 1, which may have been sourced from the microbial biomass (Section 2.3.2.3), was only possible because plants were absent.

Inhibition of nitrite oxidising bacteria (NOB) was indicated by the early accumulation of NO_2^--N in both experiments 1 and 2, and was probably caused by high concentrations of NH_3-N (Section 2.5.1.3), although it is perhaps possible that Al toxicity contributed. The lower concentration of NO_2^--N in this current experiment could have been due to the lower pH and lower NH_4^+-N concentration. Accumulation of NO_2^--N on day 21, however, may have been caused by inhibition of NOB by the high concentrations of NO_3^--N, since *Nitrobacter* are inhibited by NO_3^--N, and the inhibition increases as the pH decreases (Hunik et al., 1993). Alternatively, the measured NO_2^--N may have been caused by a lag in the synthesis of NO_2^--N reductase (Robertson, 2000), coupled with persistent NO_3^--N reductase activity (Dendooven and Anderson, 1994). Denitrification was probably occurring after day 21 since the soil moisture content was between 35 and 60% WFPS on days 21–30 and there was a rapid decline in NO_3^--N after day 21.

5.4.3 Microbial stimulation

The release of Cu, Fe and Mo could possibly stimulate microbial function since, unlike Al, these elements are required by microbes for the synthesis and functioning of the enzymes involved in many microbial processes, including nitrification and denitrification (Starkey, 1955; Berks et al., 1995; Hooper et al., 1997; Zumft, 1997). Thus their increased availability in urine patches could potentially influence the magnitude and time of onset of N transformations. Although Carter et al. (2006) did not detect any change in denitrifying enzyme activity (DEA) following urine application, nitrifying enzyme activity (NEA) was enhanced by the added urine. However, this cannot be definitely attributed to trace element availability. Copper, Fe and Mo are also required by plants for a variety of purposes (Römheld and Marschner, 1991). Thus there might be competition between plants and microbes for the trace elements released by urine addition. However, there do not appear to be any previous studies into trace element dynamics in urine patches.

While both soil DRP and plant P were reduced in the Ip treatment, there was an increase in total PLFAs. The negative correlation of total PLFAs with soil DRP, in conjunction with the greater increase in total PLFAs in the Ip soil than in the C soil between days 9 and 30, indicated that soil microbes were utilising the available soil P to produce biomass (Plante, 2007). A reduction in soil inorganic P in conjunction with an increase in microbial P has been observed previously, following the addition of a source of C and N to soil (Thien and Myers, 1992). Although the

calculated difference between total PLFA-P in the C and Ip soils on both sampling occasions (0.03–0.12 mg P kg^{-1} soil) was somewhat less than the difference between the soil DRP across the sampling period (mean 0.39 mg kg^{-1} soil), this pool of P does not represent the entire mass of P contained in microbial cells. Therefore it is possible that the majority of the P depletion in the Ip soil was due to assimilation into the soil microbial biomass. If there was competition for P between soil microbes and plants in this current experiment, which is possible since pasture growth was strongly stimulated by urine application, it would seem that the microbes were outcompeting the plants. Plant uptake of soil DRP is discussed in Section 5.4.1 above. Microbes have a role as suppliers of P for plants (e.g. Richardson et al., 2001), although their contribution is hard to quantify (Richardson et al., 2009), and no studies specifically on competition between plants and microbes for soil P could be found.

Dehydrogenase activity was stimulated by the addition of urine, but the DHA in this field soil was low compared with that seen in experiment 1. After urine application the DHA increased to ca. 8 μg TPF g^{-1} soil h^{-1} compared with activity of > 24 μg TPF g^{-1} soil h^{-1} after urine application in experiment 1. This may have been due to the soil used in experiment 1 coming from the top 2 cm, while in this experiment soil from the top 5 cm was used, potentially diluting the DHA. The correlation between DHA and WSC in the Ip soil reflects the increase in both after urine application, and may indicate the importance of available C for DHA, a relationship that was also seen in experiment 1. However, the different trends after day 21 showed that WSC was not the main influence on DHA. In fact, apart from the short-term stimulatory effect of urine application, soil moisture was the major influence on DHA, as was apparent from the close correlations between these two variables in the Op and C soils. The importance of soil moisture to DHA (Ross and Roberts, 1970; Tate and Terry, 1980) and to soil microbial activity in general (Barros et al., 1995) has been observed in other studies. Its effect on DHA is probably due to the greater mobility of soil microorganisms and substrates in higher soil moisture conditions, but may also reflect higher anaerobic activity, as a continued increase in DHA has been measured in flooded soils (Tate and Terry, 1980).

The higher concentrations in the Ip soil of total PLFA and G+ biomarkers on day 30 showed that urine application had stimulated the soil bacterial community, as was previously observed in experiment 1. In contrast to experiment 1, however, the concentration of fungal biomarker was higher in the urine affected soil in this experiment on day 30. If there was an inhibitory effect of urine addition prior to day 9, the soil may have been rapidly recolonised by fungi from outside the urine affected soil, which could not have occurred in the soil cores used in experiment 1. In contrast to the results of this current experiment, Petersen et al. (2004a)

measured a reduction in the fungal PLFA biomarker 14 days after urea application in a field study, while Williams et al. (2000) saw no difference in the number of culturable fungi following urine application. The increase in the F/B ratio between day 9 and day 30 shows that the fungal biomass had increased to a greater extent than the bacterial biomass in the wetter soil conditions, possibly because fungi could better access recalcitrant soil substrates (Schnürer et al., 1986). Fungi dominate the microbial biomass in many temperature grassland and pasture soils (Ruzicka et al., 2000). The higher fungal biomarker in the Ip soil than in the C or Op soils on day 30 was probably a reflection of the greater amounts of nutrients still present in the urine affected soil (e.g. NO_3^--N).

Rhizodeposition by plants may have influenced the PLFA content, although the concentration of WSC generally remained between 100 and 200 mg kg^{-1} in all the treatments, similar to the values measured in the control soils throughout experiment 1, where plants were absent. The unvarying concentration of this WSC suggests that this fraction of the soil C was unavailable to microbes (Boyer and Groffman, 1996; Petersen et al., 2004b), showing that WSC is not an appropriate measure of microbially-available C. This was in contrast to the fraction that appeared after urine application. Possibly any nutrients supplied by plants were so rapidly utilised or at such low concentrations that they were not detected. Generally rhizodeposition is reduced when nutrients are readily available (e.g. Hoffland et al., 1992; Paterson and Sim, 1999), so urine application would not be expected to result in increased plant nutrient inputs, unless the roots were damaged by the urine (Richards and Wolton, 1975), and there was no indication of this.

The increase in total PLFAs between days 9 and 30 in all treatments was unexpected, since total PLFAs decreased with time in experiment 1. Since it was apparent in all the soils, the increase in PLFAs in this current experiment was obviously not caused solely by the addition of urine. It is possible that the relatively high soil moisture on days 21–30 was responsible for this increase, as wetting of a dry soil can result in a greatly increased microbial biomass (Lund and Goksoyr, 1980; Schnürer et al., 1986).

5.4.4 Lateral movement of urine

There were few significant differences between the measured soil properties in the Op and C soils, indicating that there had been minimal lateral movement of urine either on or under the soil surface, as was also seen in other studies (Williams and Haynes, 1994; Somda et al., 1997; Monaghan et al., 1999). Key properties, including soil pH and DHA did not differ on any sampling occasion. Inorganic N was largely unaffected outside the urine patch, with only mean

soil NH_4^+-N concentrations differing between the Op and C soils, and this did not result in differences in mean NO_3^--N concentrations. Concentrations of Cl^- were higher in the Op soil, while DRP was higher in the C soil. Chloride is considered an ideal tracer of water movement (Liu et al., 1995), so its presence in the Op soil suggests that there was some lateral movement of urine. The slightly elevated Cl^- and NH_4^+-N in the Op soil early in the experiment may have reduced over time due to leaching and/or plant uptake. These results show that there was only a minor influence of urine application outside the urine patch, some of which may have been due to the influence of plants with roots inside the urine patch (Section 2.2.1).

5.5 Discussion Summary

- Increased concentrations of trace elements after urine application indicated release from the soil, possibly due to dissolution of SOM in the high pH.

- Lower soil DRP in the Ip soil correlated with higher total PLFAs, indicating utilisation of soil P for microbial biomass.

- Plant P was also reduced in the Ip soil, which may have been due to inhibition of P uptake as a result of high N concentrations in soil, relocation of P to roots and/or competition from microbes.

- Total PLFAs increased between day 9 and day 30 in all treatments, possibly because of higher soil moisture at this time, which also resulted in higher DHA.

- Fungal PLFAs also increased from day 9 to day 30, possibly via recolonisation from outside the urine patch.

- Microbial stress may have been caused by enhanced salt concentrations after urine application, as indicated by the i/a PLFA stress ratio.

- There was little evidence of lateral movement of urine through the soil.

5.6 Conclusions

Hypothesis 1: That the presence of plants would reduce the accumulation of nutrients including N and P.

There was some support for this hypothesis, although other factors would also have been influential in these reductions. Inorganic N and pH dynamics were similar to those in experiment 1, which was laboratory-based, but the magnitude of changes was smaller. This was

because the N concentration of the urine was lower, but may also have been influenced by plant uptake of NH_4^+-N and NO_3^--N, and higher NH_3-N volatilisation. However, the reduced plant P in conjunction with lower DRP in the Ip soil did not support this hypothesis. The reduced plant P may have been due to high N concentrations inhibiting P uptake or to relocation of herbage P to roots, which were not analysed.

Hypothesis 2: That soil microbes would be stressed by competition with plants for nutrients.

This hypothesis was not supported by the P results. The decrease in soil DRP with a corresponding increase in total PLFAs in the Ip treatment, suggested that the soil P was being used by the microbes to make biomass, and it is possible that microbes were outcompeting plants for the soil DRP, since the plant P concentrations were also reduced in the Ip treatment. The high Al concentrations could have caused microbial inhibition, but it was not possible to differentiate any toxic effects due to Al from inhibition due to other sources. However, inhibition of microbes was detected in the changes in PLFA stress ratios and biomarkers following urine application. This may have been due to salt stress, since it was also seen in experiment 1, but stress could not be attributed to plants being present. The lower DHA in this field trial than in the laboratory study (experiment 1) could have been due to competition for substrates by plants. However, the main influence on DHA in the *in situ* field soil was the soil moisture content.

Hypothesis 3: That the soil microbial community would be stimulated by plants via rhizodeposition and by the release of essential trace elements following urine application

There was support for this hypothesis, since total PLFAs increased in this experiment, in contrast with experiment 1, where plants were absent. The greatest impact was on the fungal biomarker, which increased in this experiment but declined over time in experiment 1. The increase in PLFAs may have been facilitated by plant rhizodeposits, and enhanced by the increased soil moisture content. Stimulation of the microbial community could also have occurred through the release of the trace elements, Cu, Fe and Mo, as their concentrations increased after urine application.

Hypothesis 4: That urine effects in soil adjacent to the urine patch would be minimal.

There was also support for this hypothesis, with little evidence seen of lateral movement of urine. Of the variables that were elevated in the Op soil compared to the C soil, the differences were only seen early in the experiment.

Future research

- Response of soil fungal communities to urine application, including effects of different rates of urine application, species of fungi affected, and urinary compounds responsible for inhibition.

- Analysis of trace elements and other metals in urine patches, including measurements of these elements in cow urine and their fate.

- Phosphorus dynamics in urine patches with and without plants including sources of soil P after urine application and determination of P concentrations in cow urine.

- Longer-term monitoring (≥ 1 year) of microbial dynamics and nutrient fluxes in urine patches under field conditions.

Chapter 6
Hippuric acid and benzoic acid impacts on cow urine-derived N_2O-N emissions and soil microbial communities

6.1 Introduction

During the course of this thesis study, two papers were published that showed a > 50% reduction in N_2O-N emissions from synthetic ruminant urine when either the hippuric acid or benzoic acid content of the urine was increased (Kool et al., 2006b; van Groenigen et al., 2006). Hippuric acid is a naturally occurring minor urine constituent (Section 2.2.2.1), and benzoic acid is a product of its hydrolysis. Benzoic acid has antimicrobial properties (Marwan and Nagel, 1986) and is a known denitrification inhibitor (Her and Huang, 1995), and van Groenigen et al. (2006) found that it had the same inhibitory effect as hippuric acid on N_2O-N emissions from synthetic urine applied to soil.

In view of this highly relevant development, Experiment 3 was instigated to investigate, for the first time, the effects of hippuric acid and benzoic acid on N_2O-N emissions from real cow urine applied to soil. This was a significant next step, because it is recognised that results from experiments using synthetic urine may differ from those using real urine (Kool et al., 2006a). If a similar inhibitory effect on N_2O-N fluxes was seen with real urine as with synthetic urine, this study might lead to effective strategies for reducing N_2O-N fluxes from urine patches by altering the hippuric acid content of urine via modifications in ruminant diet and/or pasture management (Section 2.2.2.1). This would also be the first study examining the effects of urinary hippuric acid and benzoic acid concentrations on soil microbes.

Since earlier studies found a stimulatory effect on NH_3-N emissions of increasing hippuric acid concentrations (Section 2.2.2.1), it is conceivable that any decrease in emissions of N_2O-N could be the result of increased N losses via NH_3-N emissions. Therefore emissions of NH_3-N were also monitored.

An additional aim of the experiment was to link any changes in N_2O-N fluxes, which occurred as a result of elevated hippuric acid or benzoic acid concentrations, to microbial responses by simultaneously measuring MBC, DHA and PLFA variables.

The hypotheses tested were:

1) that the addition of hippuric acid or benzoic acid to real cow urine would reduce emissions of N_2O-N over the course of the experiment,

2) that any reduction in N_2O-N would result in an increase in NH_3-N volatilisation, and

3) that these changes would be reflected by measurable changes in the microbial community.

6.2 Materials and Methods

6.2.1 Soil collection and soil core preparation

Topsoil (0–10 cm) from a Wakanui silt loam (Mottled Immature Pallic; New Zealand Soil Classification (Hewitt, 1998)) was collected from a pasture site with > 20 years grazing history at Lincoln, New Zealand (43°38.7′S, 172°28.3′E) and sieved to 5.6 mm, with stones and large aggregates removed. The sieved soil was packed to a depth of 60 mm in poly-vinyl chloride (PVC) tubes (70 mm high x 50 mm ID), at a bulk density of 1.0 g cm^{-3}. Soil loss was prevented by means of nylon mesh fabric attached to the base of the tubes. The soil moisture content of each core was adjusted so that the subsequent addition of 15 mL of a treatment solution would bring the water-filled pore space (WFPS) to 70%. The cores were covered with pierced clear plastic to limit moisture loss while allowing gas diffusion, and they were incubated at 18°C for several days prior to treatment application.

6.2.2 Urine treatments and experimental design

Cow urine was collected from Friesian dairy cows 24 h before application, and stored at 4°C while its constituents were analysed (Section 3.1). The measured N concentration of the urine was 4.3 g L^{-1} and the hippuric acid concentration was 8.2 g L^{-1} (46 mmol L^{-1}), comprising 15.2% of the total urinary N.

The final urine compositions for the different treatments are summarised in Table 6.1. The four experimental treatments consisted of: 1) urine + ^{15}N-urea (U), 2) urine + ^{15}N-urea + hippuric acid (UHA), 3) urine + ^{15}N-urea + benzoic acid (UBA), and 4) DI water control (C). The ^{15}N-enriched urea was added to the urine immediately prior to its application, to take the urine concentration to 10 g N L^{-1}. The UHA treatment was formulated by adding 4.5 g of hippuric acid to 500 mL of the urea-amended urine to increase the concentration by 50 mmol L^{-1} to 96 mmol L^{-1} of hippuric acid, which is within the range of natural variation (Kreula et al., 1978). The additional ^{14}N added in the hippuric acid meant that the ^{15}N enrichment was lower in the

UHA treatment than in the other urine treatments (Table 6.1). Another 500 mL of the urea-amended urine received 3.1 g of benzoic acid to take the concentration to 50 mmol L^{-1} (treatment UBA), congruent with the hippuric acid addition.

Each urine-treated core had 15 mL of urine pipetted onto the soil surface at an application rate equivalent to 750 kg N ha^{-1}, while the C treatment cores received 15 mL of DI water. The cores were arranged in four randomised blocks and were incubated at 18°C throughout the experiment. The pierced plastic covers were retained on the cores, and only removed during gas sampling, to minimise water loss, and the soil moisture content was controlled by adding DI water twice weekly to maintain each core at the desired moisture level. There were a total of 112 cores, comprising 4 replicates of each treatment for 7 destructive soil sampling times.

Table 6.1 Composition of the urine treatments: U (urine + ^{15}N-urea), UHA (urine + ^{15}N-urea + hippuric acid) and UBA (urine + ^{15}N-urea + benzoic acid).

Treatment	Total N content (g L^{-1})	^{15}N atom% enrichment	HA concentration			BA concentration (mM)
			(mM)	g N L^{-1}	% total N	
U	10.0	5.9	46	0.64	6.4	0
UHA	10.7	5.6	96	1.35	12.6	0
UBA	10.0	5.9	46	0.64	6.4	50

6.2.3 Gas and soil sampling

One set of 16 soil cores (4 replicates x 4 treatments) was used for headspace sampling of N_2O-N and CO_2-C throughout the experiment (Section 3.4). Acid traps (Section 3.4.4) were used to measure NH_3-N volatilisation until day 17, by which time NH_3-N emissions in the urine treatments were not significantly different from the C treatment. A further headspace gas sample was collected for ^{15}N analysis of N_2O-N (Section 3.5.2).

A subset of the soil cores was destructively sampled at 1, 3, 6, 10, 20, 35 and 55 days after treatment applications, after measurement of the soil surface pH (Section 3.2.3). The top 20 mm of soil from each core was removed and thoroughly mixed in a resealable plastic bag, before being sub-sampled for subsequent analyses. The soil chemical analyses carried out included; 2 mol L^{-1} KCl extractions for inorganic-N, water extractions for WSC, and isotope tracer analyses for soil NH_4^+-^{15}N and NO_3^--^{15}N (Sections 3.2.3, 3.2.5, 3.5.2 & 3.5.3). Microbial analyses performed included; CFE for MBC, DHA determinations of microbial activity, and PLFA analyses for assessment of the microbial community structure (Section 3.3).

6.3 Results

6.3.1 Soil pH

The mean surface pH of the C treatment over the course of the study was 5.8 (Figure 6.1). One day after urine application the soil pH of the urine-treated soil had increased to ca. 8.6 and remained constant for 6 days. The pH in the urine treatments then declined, and had returned to control values in the U treatment by day 20 and in the UHA and UBA treatments by day 35 (Figure 6.1). By the end of the experiment the soil pH in all the urine-treatments (mean pH = 4.11) was significantly lower than in the C treatment (mean pH = 5.15) ($P < 0.001$).

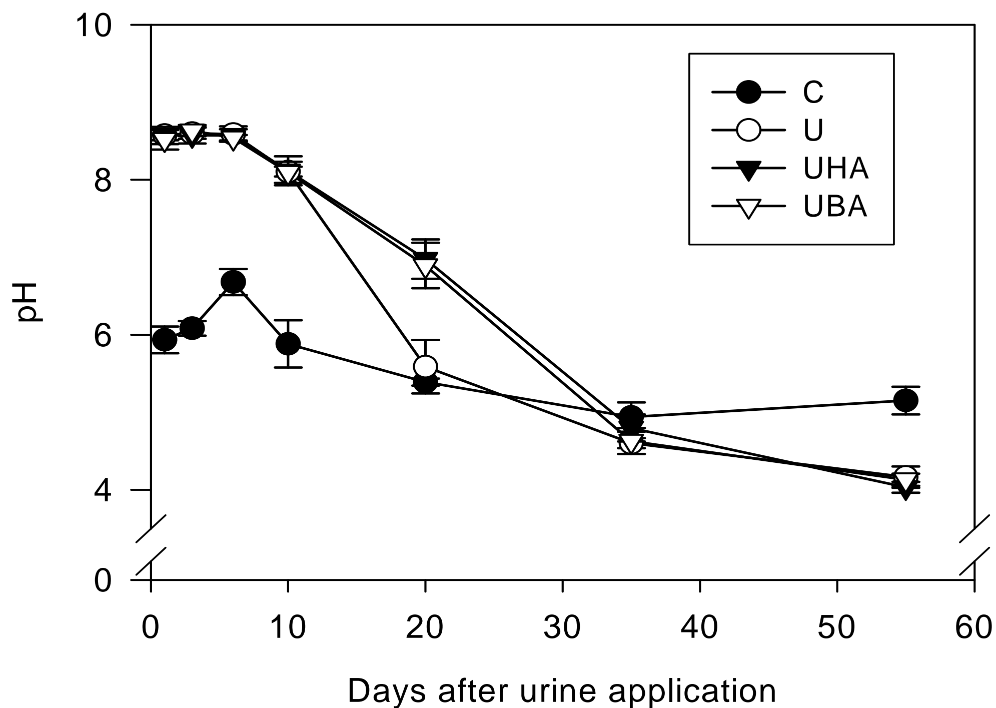


Figure 6.1 Soil surface pH determined for all treatments following urine application on day 0 (equivalent to 750 kg N ha^{-1}) (Error bars = SEM, n = 4).

6.3.2 Inorganic N

Soil NH_4^+-N concentrations peaked on day 1 in all the urine treatments at ca. 1700 mg NH_4^+-N kg^{-1} dry soil (Figure 6.2a), and then decreased steadily, although remaining higher than in the C treatment throughout the study ($P < 0.001$). The NH_4^+-N concentration in the U treatment was lower than in the UHA and UBA treatments only on day 20 ($P < 0.001$), due to a faster rate of decline in the NH_4^+-N concentration in the U treatment (Table 6.2). There was no difference between the NH_4^+-N concentrations in the UHA and UBA treatments. Soil surface pH and NH_4^+-N concentrations in the urine treatments were strongly correlated ($r > 0.97$; $P < 0.01$), particularly between days 10 and 55 ($r > 0.99$; $P < 0.01$), when NH_3-N volatilisation had diminished and nitrification was occurring.

Soil NO_2^--N concentrations in the C treatment remained low (<0.10 mg NO_2^--N kg^{-1} dry soil) and were consistently lower than concentrations in the urine treatments, although the difference was only significant on day 1 ($P < 0.001$). Concentrations peaked on day 10 in the U treatment (9 mg NO_2^--N kg^{-1} dry soil) and on day 20 in the UHA and UBA treatments (12 and 27 mg NO_2^--N kg^{-1} dry soil, respectively) (Figure 6.2b). Concentrations were higher in the U treatment than in the UHA and UBA treatments on days 6 and 10 and again on day 55 ($P < 0.01$). Despite the large NO_2^--N concentrations in the UHA and UBA treatments on day 20, this difference was only significant between the U and UBA treatments at the $P < 0.10$ level. There was no difference in NO_2^--N concentrations between the acid treatments throughout the experiment.

Nitrate concentrations in the C treatment remained low during the experiment at < 35 mg NO_3^--N kg^{-1} dry soil (Figure 6.2c). Before day 10 the NO_3^--N concentrations in all the urine treatments were significantly lower than in the C treatment ($P < 0.01$), whereas after day 10 they were higher ($P < 0.01$). The NO_3^--N concentration was significantly higher in the U treatment than in the UHA and UBA treatments from day 6 to day 20, but there was no difference between the UHA and UBA treatments. By day 55, NO_3^--N concentrations had declined to the same level in all the urine treatments.

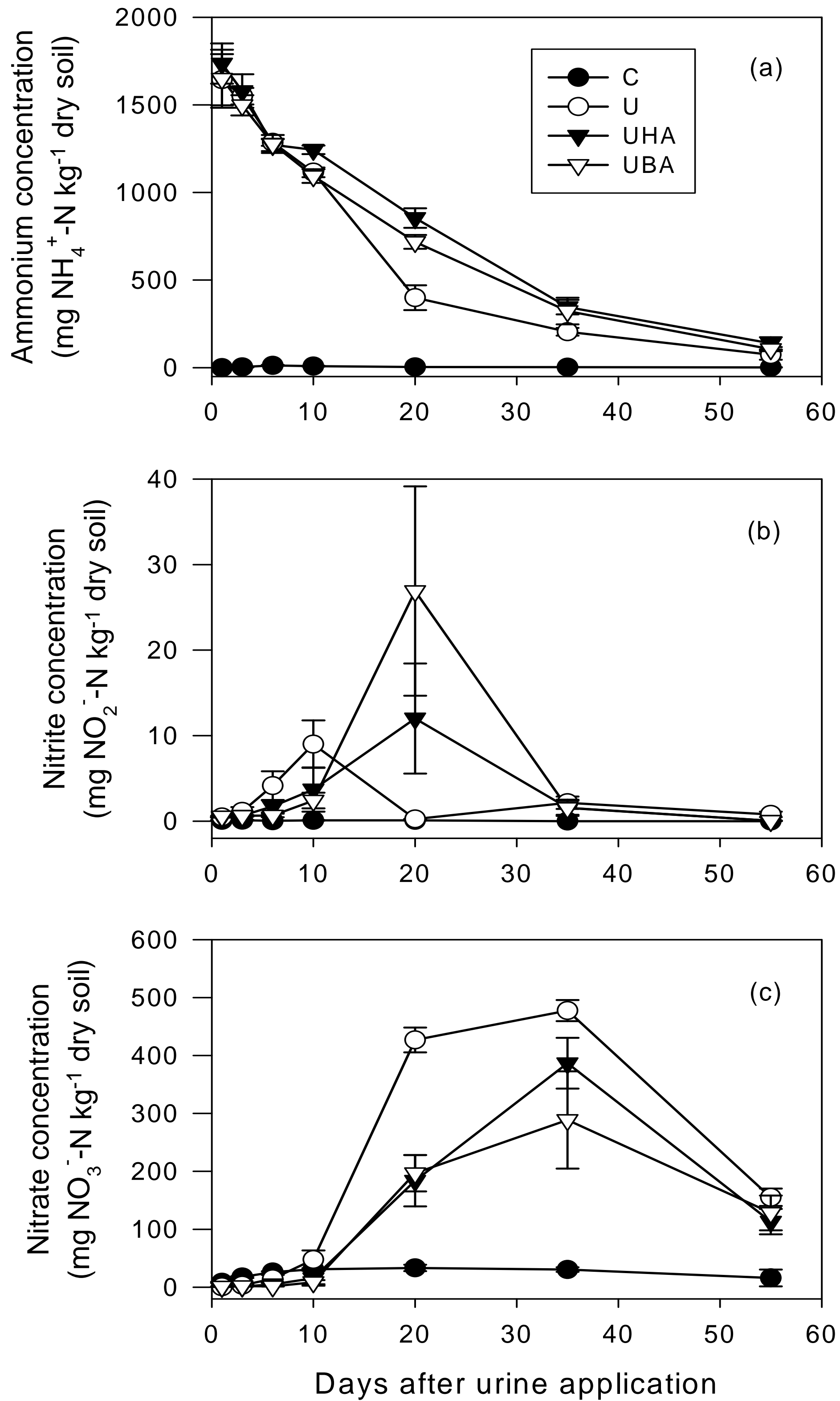


Figure 6.2 Mean concentrations of (a) ammonium, (b) nitrite, and (c) nitrate (mg N kg^{-1} dry soil) determined for all treatments (Error bars = SEM, n = 4). NOTE differing scales on y axes.

Net rates of NH_4^+-N oxidation (Table 6.2) did not differ between the urine treatments until days 6-10, when the rate of decline in soil NH_4^+-N concentrations was lower in the UHA treatment than in the U or UBA treatments ($P < 0.05$). Differences also occurred on days 10–20 when NH_4^+-N in the U treatment declined faster than in the UHA and UBA treatments. On days 20–35 the reverse was seen, with UHA and UBA treatments showing a faster net decline in NH_4^+-N than the U treatment. Net NO_3^--N production rates differed only on days 10–20 when NO_3^--N production in the U treatment was more than double that of the UHA and UBA treatments (Table 6.2).

Table 6.2 Net rates of change in the soil NH_4^+-N and NO_3^--N concentrations (mg N kg^{-1} dry soil d^{-1}). Negative rates indicate declining concentrations.

	Time (days)						
	0-1	1-3	3-6	6-10	10-20	20-35	35-55
	NH_4^+-N (mg N kg^{-1} dry soil d^{-1})						
Treatment							
U	1643	-47	-88	-42	-72	-13	-7
UHA	1736	-78	-102	-7	-39	-34	-10
UBA	1649	-76	-74	-46	-38	-26	-11
significance	NS	NS	NS	*	**	**	NS
LSD (0.05)				27	19	12	
df = 11, n = 4							
	NO_3^--N (mg N kg^{-1} dry soil d^{-1})						
Treatment							
U	0.83	1.29	3.69	8.35	37.91	3.38	-16.12
UHA	0.84	0.32	1.18	2.62	16.84	13.52	-13.67
UBA	1.18	0.43	-0.14	1.80	18.80	6.14	-8.03
significance	NS	NS	NS	NS	**	NS	NS
LSD (0.05)					9.53		
df = 11, n = 4							

* $P < 0.05$, ** $P < 0.01$, NS = not significant

6.3.3 Water soluble C

Water soluble carbon increased immediately after urine application in the urine-treated soils and concentrations remained significantly higher in the urine treatments than in the C treatment until day 35 (Figure 6.3). The U treatment had lower WSC concentrations than the acid treated soils on days 6 and 20 ($P < 0.05$). The only difference between concentrations of WSC in the two acid treatments was on day 55, when the UHA treatment was higher. In the urine treatments there were strong positive correlations between WSC and NH_4^+-N concentrations ($r > 0.97$; $P < 0.01$) and between WSC and pH ($r > 0.90$; $P < 0.05$).

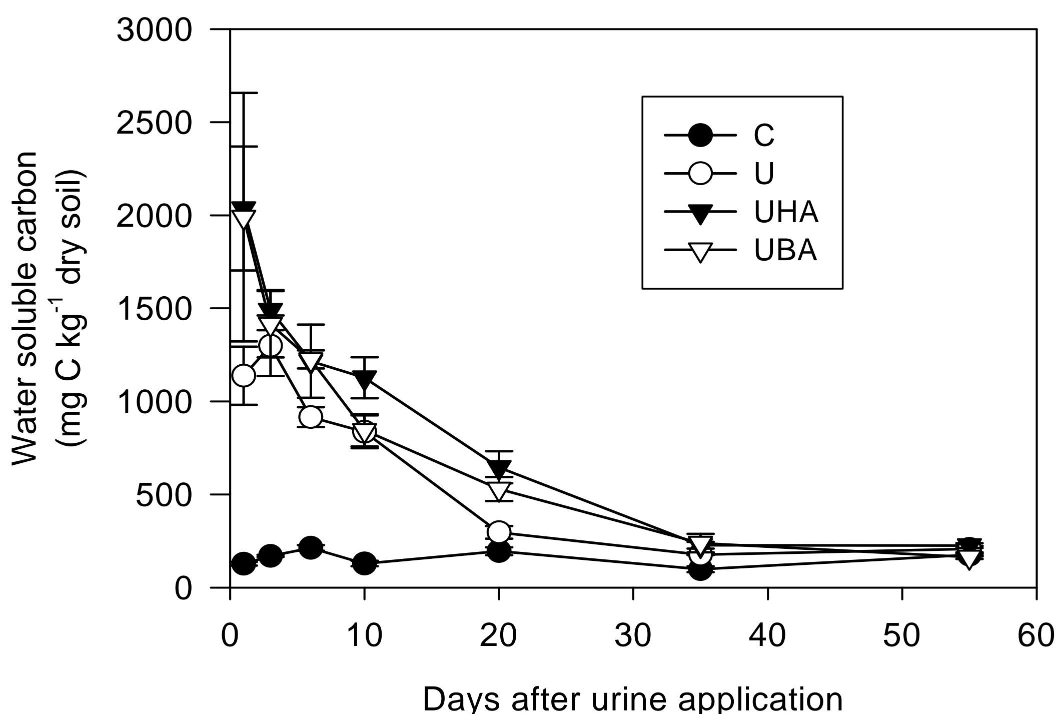


Figure 6.3 Mean water soluble carbon concentrations (mg C kg^{-1} dry soil) determined for all treatments (Error bars = SEM, n = 4).

6.3.4 Phospholipid fatty acid analyses

A stimulatory effect was seen on the microbial biomass due to urine addition, with total PLFAs being higher in the urine treatments than in the C treatment on days 3, 10 and 20 ($P < 0.001$). Phospholipid fatty acid analyses (Section 3.3.1) showed a sharp decline in total PLFA biomarkers between days 3 and 10 in the urine treatments (Figure 6.4a). On days 3 and 10, total PLFAs were lower in the U treatment than in the UHA treatment ($P < 0.001$). Bacterial PLFAs were lower in the C treatment than in the urine treatments on all days except day 55 ($P < 0.01$),

and they were also lower in the U treatment than in the acid amended treatments on day 3 (P < 0.001) (Figure 6.4b). Fungal PLFA biomarkers were higher in the C treatment than in the urine treatments on all days, and this was significant on days 10 and 55 (P < 0.05) (Figure 6.4c). Consequently the PLFA fungal/bacterial ratio (F/B) was significantly lower in the urine treatments than in the C treatment throughout the experiment (P < 0.05) (Figure 6.4d).

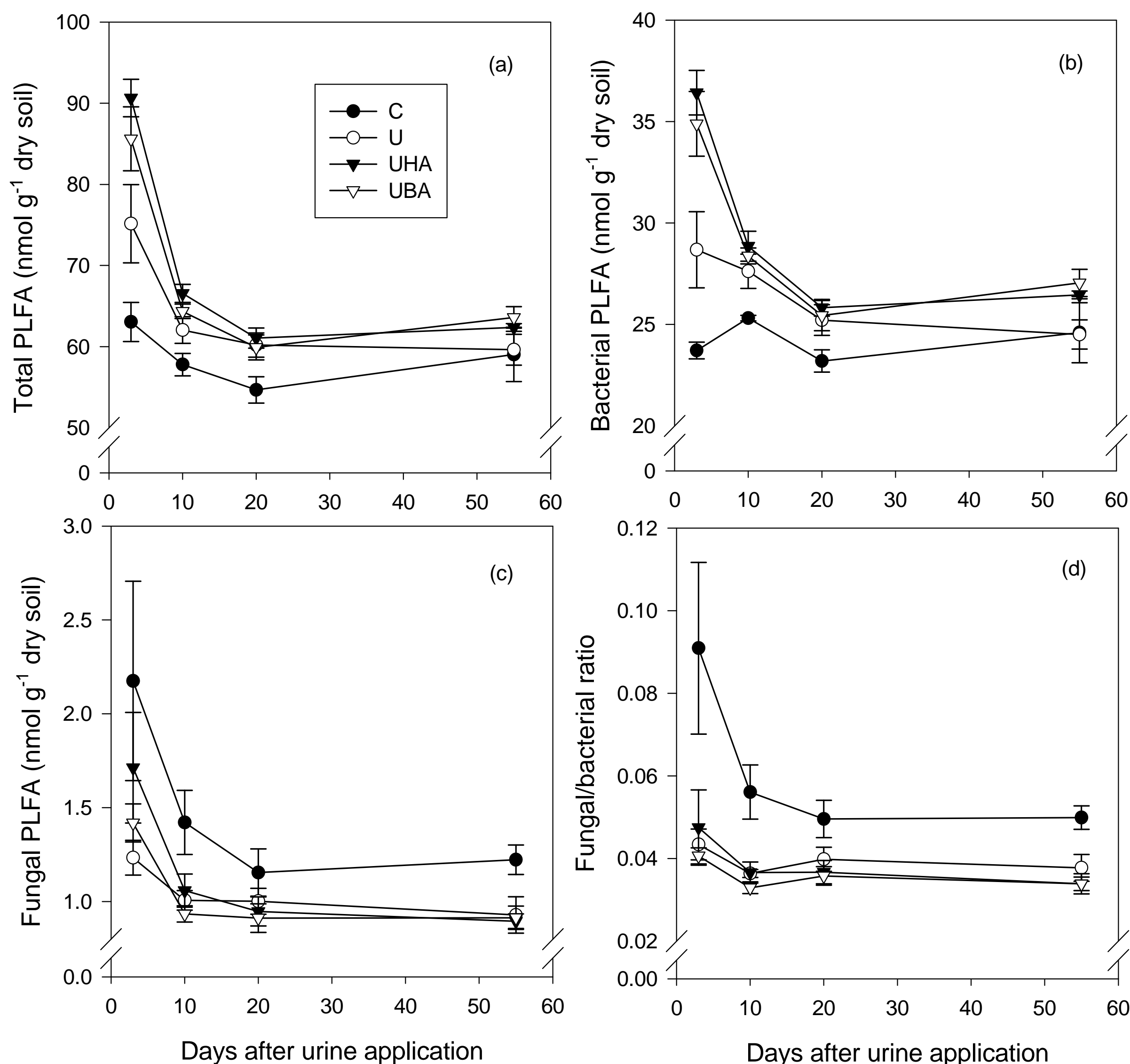


Figure 6.4 Results of PLFA biomass analyses: (a) total of all FAMEs, (b) sum of selected bacterial biomarkers (see Table 3.3), (c) amount of fungal biomarker (18:2ω6), and (d) ratio of fungal: bacterial biomarkers (Error bars = SEM; n = 4).

The G+/G- ratio (Section 2.4.2.4) was lower in the C treatment than in the UHA and UBA treatments on day 3 (P < 0.05) (Figure 6.5a), but on subsequent sampling occasions it was higher in the C treatment than in all the urine treatments (P < 0.05). The cy/ω7 ratio, a stress indicator (Section 2.4.2.4), was significantly higher in the C treatment than in all the urine

treatments on day 3 ($P < 0.001$) and was also higher in the C treatment than the UHA and UBA treatments on day 10 ($P < 0.01$) (Figure 6.5b). The i/a ratio (Section 2.4.2.4) decreased following urine application and, although it then increased between days 3 and 20, the ratio remained significantly higher in the C treatment throughout the experiment ($P < 0.001$) (Figure 6.5c). The t/c ratio was higher in the urine treatments on all sampling days ($P < 0.01$) (Figure 6.5d).

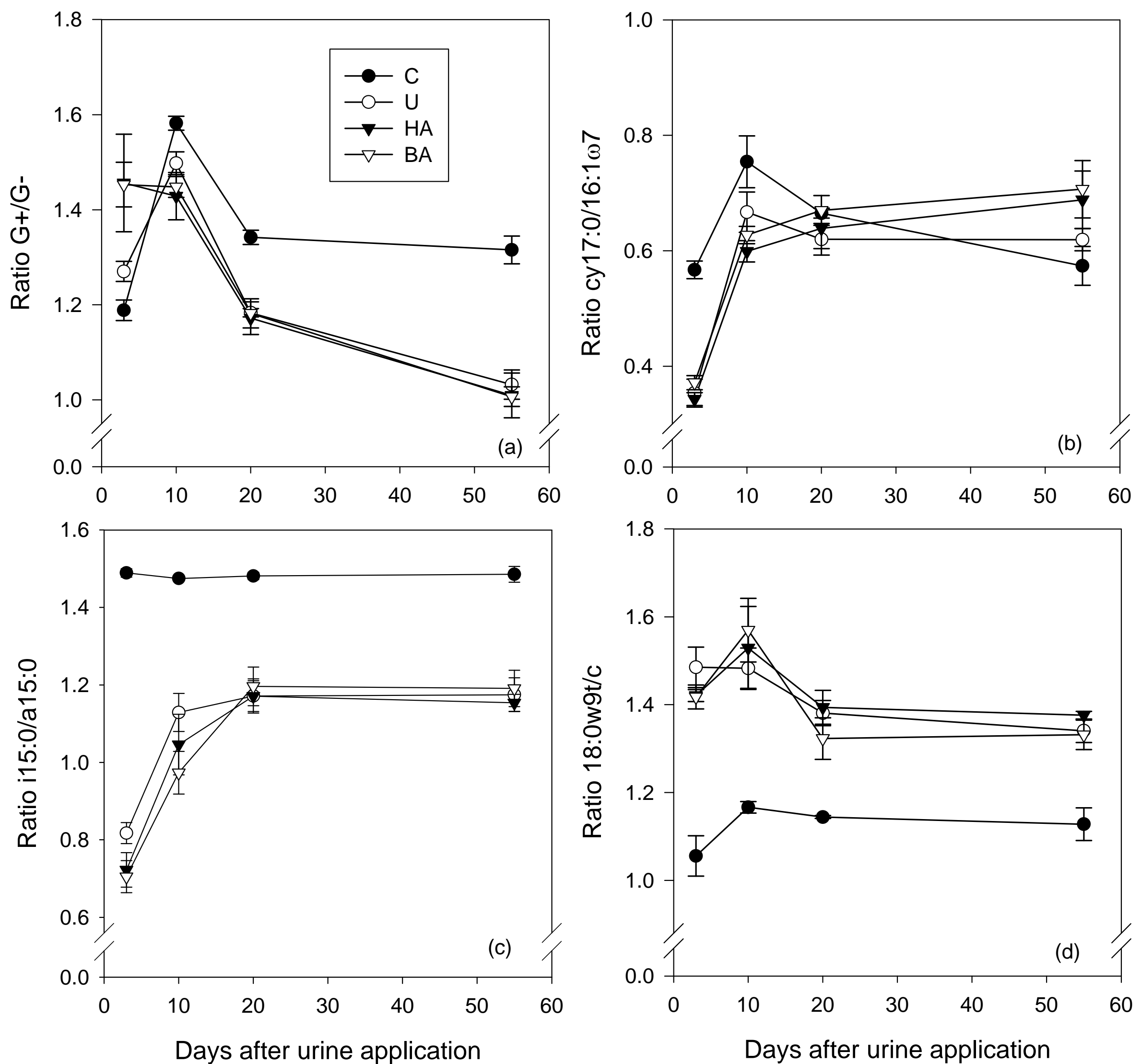


Figure 6.5 PLFA stress indicator ratios: (a) Gram-positive bacteria/Gram-negative bacteria, (b) cy17:0/16ω7, (c) i15:0/a15:0, (d) 18:1ω9t/18:1ω9c (Error bars = SEM, n = 4).

Principal component analyses of PLFAs as mol% (Figures 6.6a-d) showed separation along the PC1 axis of the C treatment from the urine treatments on all days, with the C treatment being more positive on all days except day 20. There was no evidence of separation due to urine treatment along either axis on any sampling day. A number of PLFAs were correlated with the PC1 and PC2 axes, and the major influences were the branched FAs; i15:0, i16:0 and a15:0, the straight chain FAs; 14:0 and 16:0, and the unsaturated FAs; 18:2ω6 and 18:1ω9c. ANOVAs carried out on the PCA scores showed that the PC1 axis was affected by the urine treatments on all sampling days ($P < 0.001$), while the PC2 axis was not affected by the urine treatments.

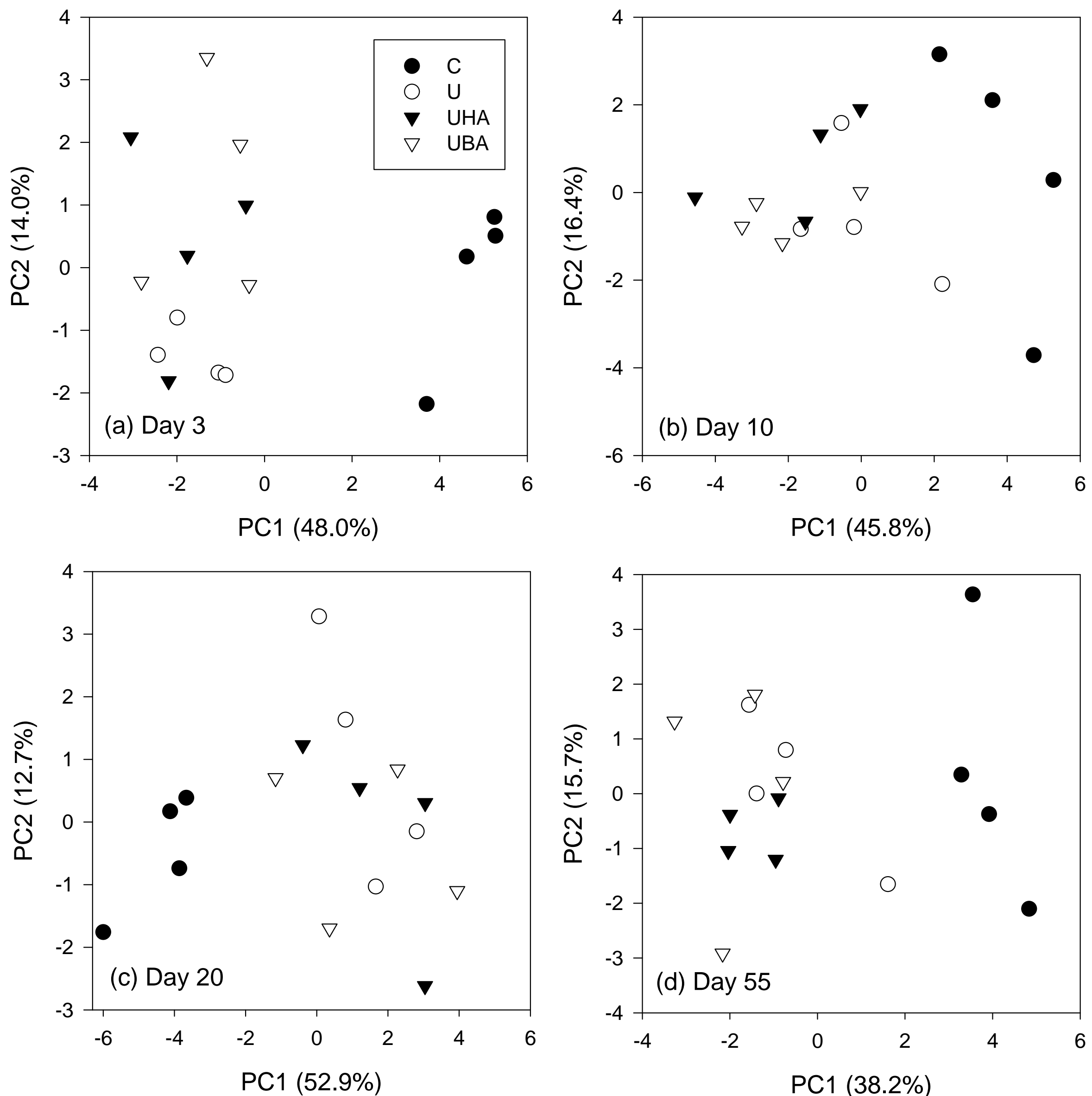


Figure 6.6 Results of principal component analyses for 18 PLFAs quantified on each sampling day.

The PCA of mol% values from all sampling days showed good separation between days on both axes (Figure 6.7). Day 3 C samples were closer to the day 20 and day 55 samples than to the day 3 urine-treated samples. Day 3 urine treatments were more positive on the PC1 axis than the other days, while days 20 and 55 were more positive on the PC2 axis than the earlier sampling days. Days 3 and 10 urine treatments and controls were clearly separated from each other and from both the other days, while days 20 and 55 were clustered together on the scores plot. The two principal axes were affected by both day and treatment.

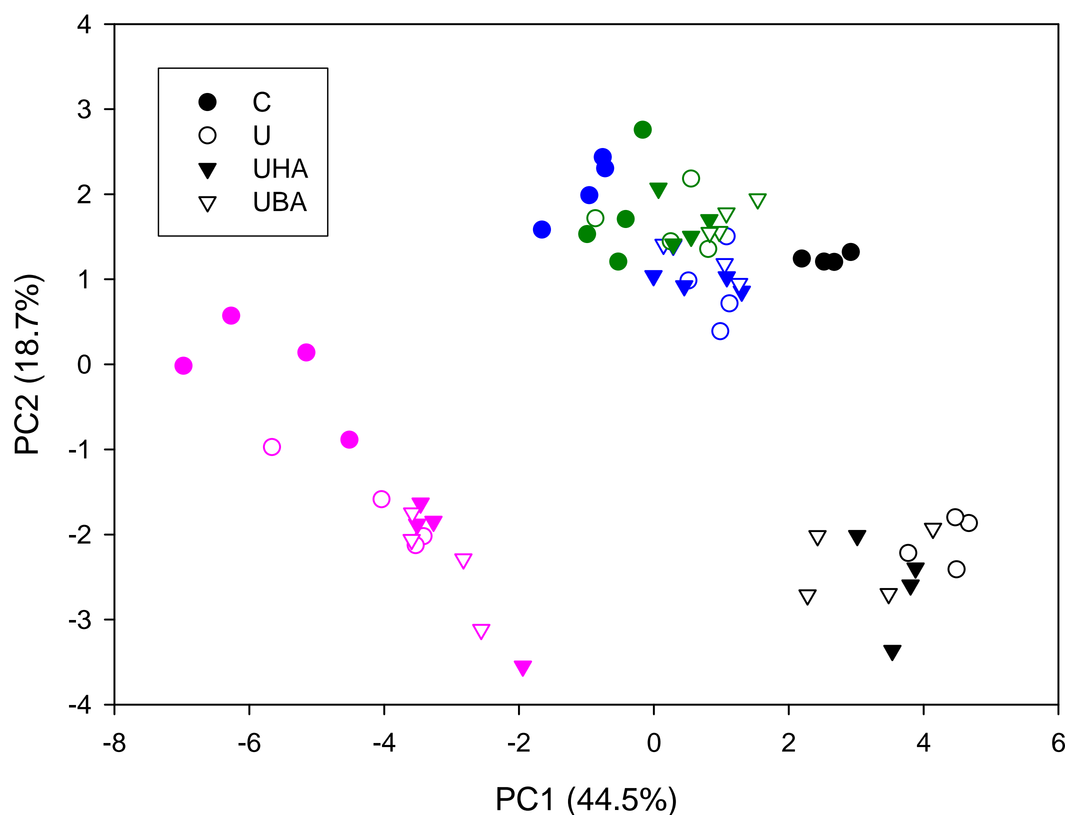


Figure 6.7 Principal component analysis for 18 extracted PLFA peaks for all days: Black = Day 3, Pink = Day 10, Blue = Day 20, Green = Day 55.

6.3.5 Microbial biomass and activity

Although initial MBC concentrations were elevated in the urine-treated soils, particularly in the UHA and UBA treatments, there were large variations within treatments and no statistically significant differences were seen over the course of the study (Figure 6.8).

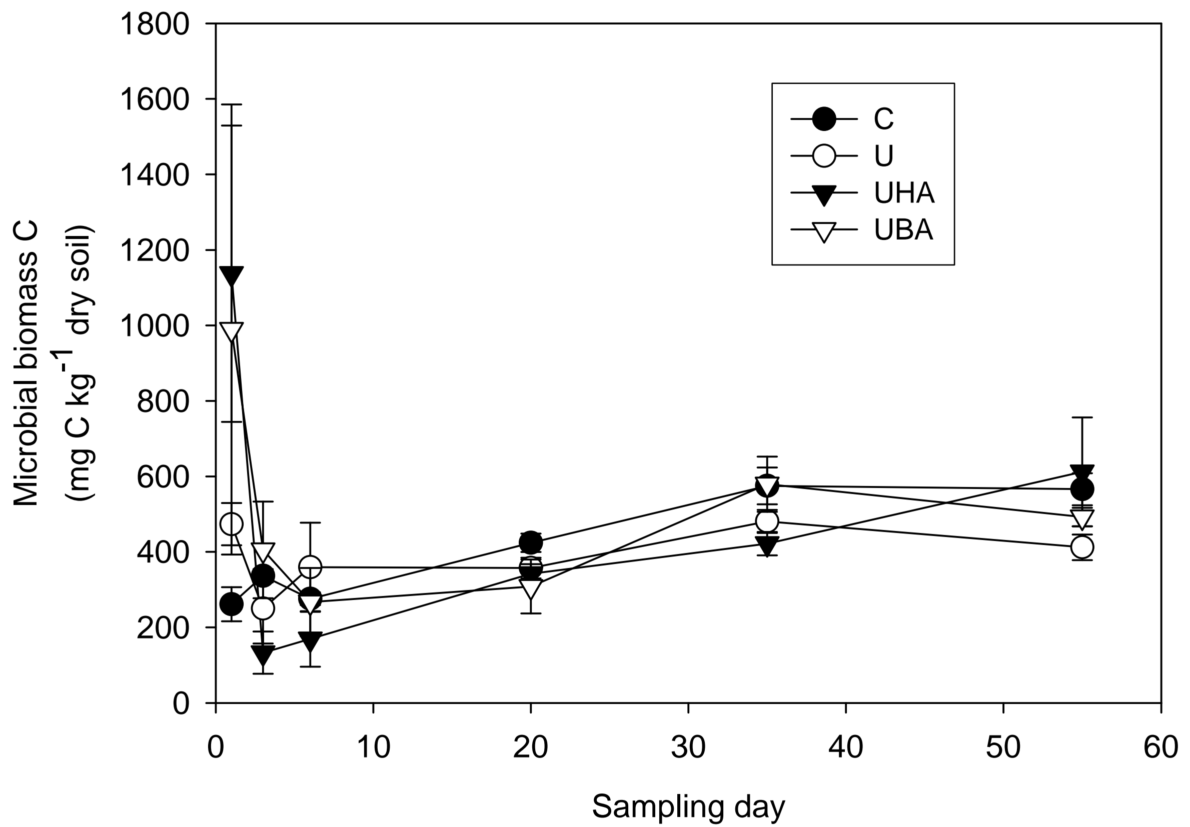


Figure 6.8 Mean microbial biomass C concentrations (mg C kg^{-1} dry soil) determined for all treatments (Error bars = SEM, n = 4).

Dehydrogenase enzyme activity (DHA) had increased by day 1 in all the urine treatments and was highest on day 3, decreasing rapidly thereafter (Figure 6.9). The highest value of 63.8 µg TPF g^{-1} dry soil h^{-1} was measured in the UHA treatment on day 3. Compared with the C treatment, DHA in the urine-treatments was higher on days 1–10, the same on day 20, and lower on days 35 and 55. DHA values were consistently lower in the U treatment than in the UHA and UBA treatments but the differences were significant only on days 20 and 55, despite the much lower activity in the U treatment on day 10. There were no differences between the UHA and UBA treatments during the study. Dehydrogenase activity was positively correlated with pH ($r > 0.84$, $P < 0.05$), NH_4^+-N ($r > 0.94$; $P < 0.01$) and WSC ($r > 0.94$; $P < 0.01$)

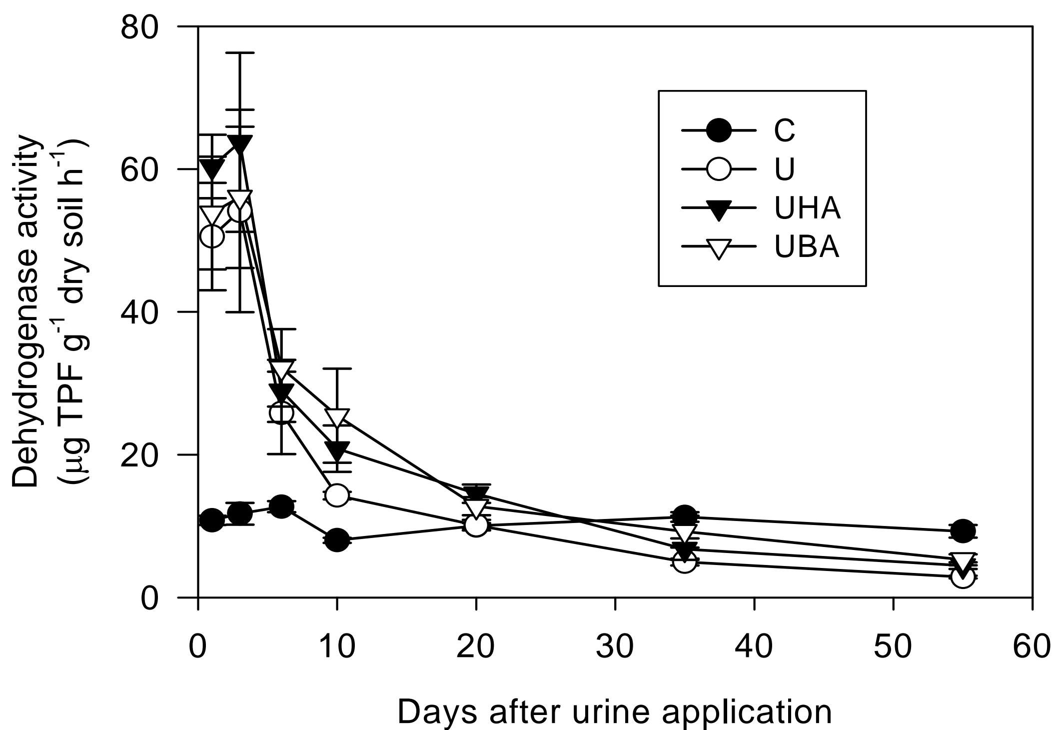


Figure 6.9 Mean dehydrogenase activity (µg TPF produced g^{-1} dry soil h^{-1}) determined for all treatments (Error bars = SEM, n = 4).

6.3.6 Headspace gas fluxes

Emissions of N_2O-N from the urine treatments had begun to increase by day 7 (Figure 6.10a), and were significantly higher than from the C treatment from day 13 onwards. The inhibition of N_2O-N emissions was apparent from day 9 until the end of the experiment, with N_2O-N fluxes from the UHA and UBA treatments remaining consistently lower than from the U treatment, and this was significant from day 25 onwards ($P < 0.05$). There was no difference between N_2O-N fluxes from the UHA and UBA treatments throughout the experiment. The increase in hippuric acid or benzoic acid concentration in the applied urine resulted in a 65% reduction in cumulative N_2O-N emissions. Cumulative N_2O-N emissions from the urine treatments were higher than expected, with an average of 16.8% of the N applied being lost as N_2O-N from the U treatment by the end of the experiment (Figure 6.10c). This was reduced significantly ($P <$ 0.01) to 5.9% and 4.7% by adding hippuric acid or benzoic acid respectively to the urine.

Ammonia fluxes from the U, UHA and UBA treatments peaked during the first 3 days and decreased to be the same as the C treatment by day 17 (Figure 6.10b). Although lower rates of NH_4^+-N oxidation were observed in the acid treatments (Table 6.2), cumulative N losses through NH_3-N volatilisation did not differ significantly between the urine treatments over the 17 days of NH_3-N sampling (16.7, 18.2 and 18.2% N applied, respectively) (Figure 6.10c), or between urine treatments on any day.

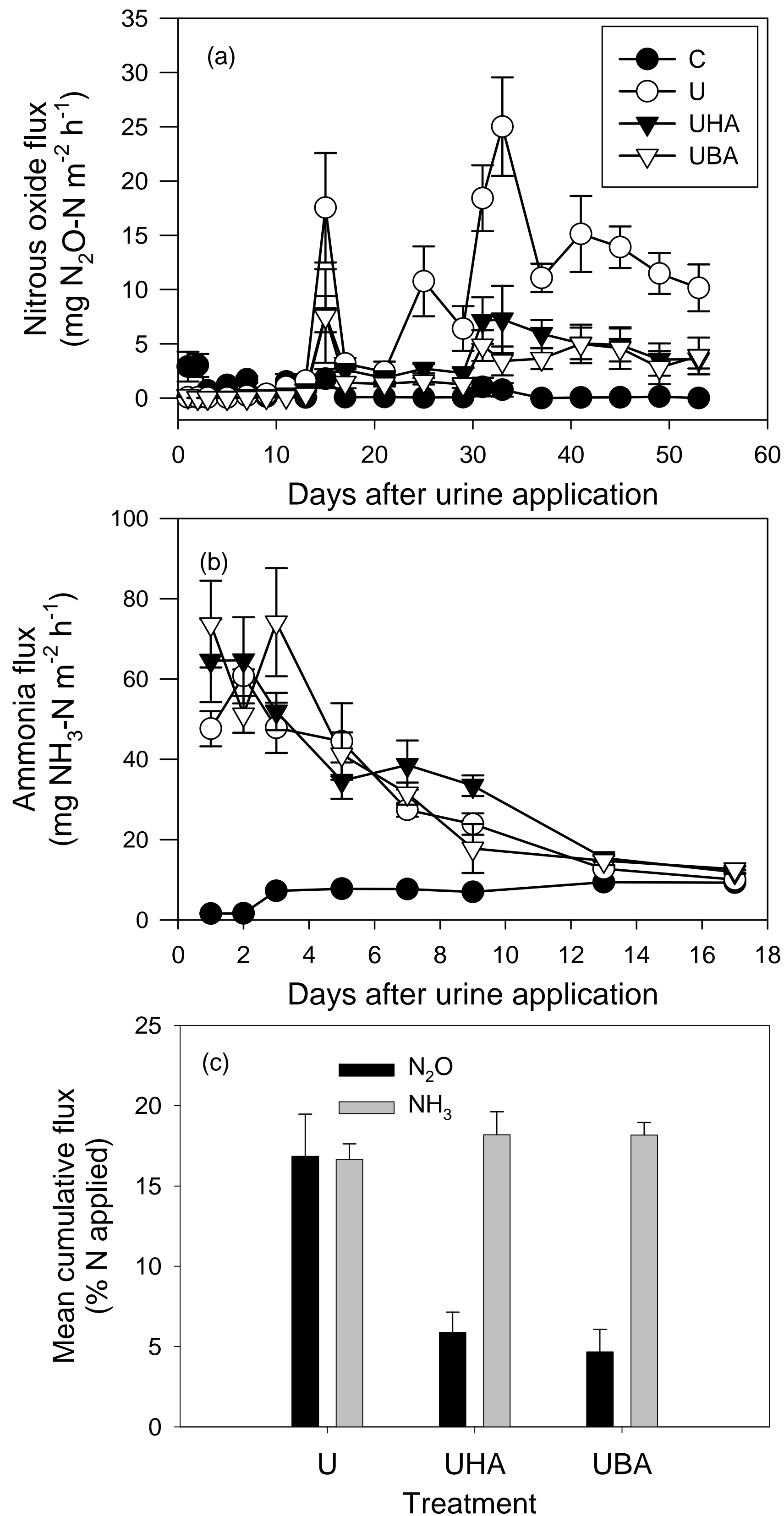


Figure 6.10 (a) Mean N_2O-N fluxes and (b) mean NH_3-N fluxes (mg N m^{-2} h^{-1}) for all treatments (<u>NOTE</u> differing scales on x and y axes); (c) Mean cumulative fluxes (% of N applied) of N_2O-N (to day 53) and NH_3-N (to day 17) for urine treatments (Error bars = SEM, n = 4).

Fluxes of CO_2-C decreased in all four treatments over the course of the experiment (Figure 6.11) and were higher from the urine-treated soils than from the C soils on days 5–33 and 49–53, although the differences were not consistently significant. The CO_2-C fluxes from the UHA and UBA treatments were only higher than those from the U treatment on days 13 and 29 ($P < 0.05$), with no differences between the two acid treatments.

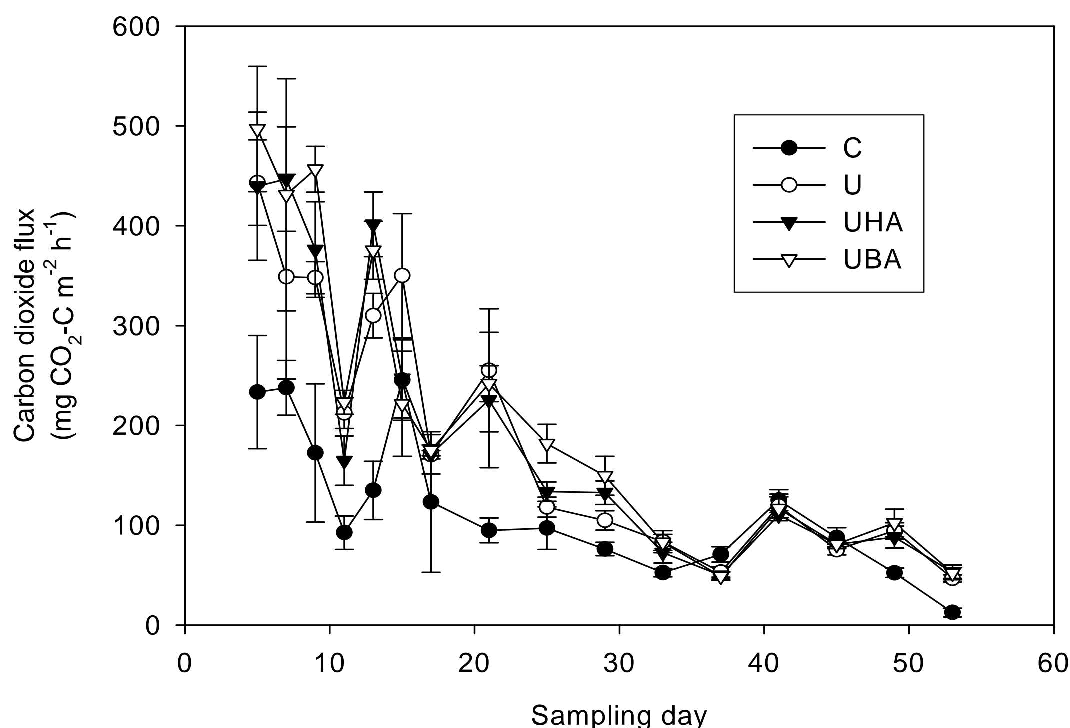


Figure 6.11 CO_2-C fluxes measured from all treatments over the experimental period (Error bars = SEM, n=4).

6.3.7 ^{15}N trace analyses

The ^{15}N enrichments of the applied urine are indicated by the small squares in Figure 6.12a. Reflecting these added enrichments, the ^{15}N atom% enrichment of the NH_4^+-N was higher in the U and UBA treatments than in the UHA treatment on days 6, 10 and 20. There were no differences in the NH_4^+-^{15}N enrichments between the different treatments by day 35 (Figure 6.12a). The ^{15}N atom% enrichment of the NO_3^--N increased rapidly until day 20 in the urine-treated soils (Figure 6.12b), and was higher in the U treatment than in the UHA and UBA treatments on all occasions ($P < 0.05$) except day 20 ($P = 0.07$). There was no difference in the NO_3^--^{15}N enrichment between the two acid treatments. The ^{15}N enrichment of the N_2O-N had increased by day 1 in the U treatment, and reached a maximum by day 15, staying constant at

ca. 5.4 atom% enrichment thereafter (Figure 6.12c), which was approximately the enrichment of the urine applied (Table 6.1).

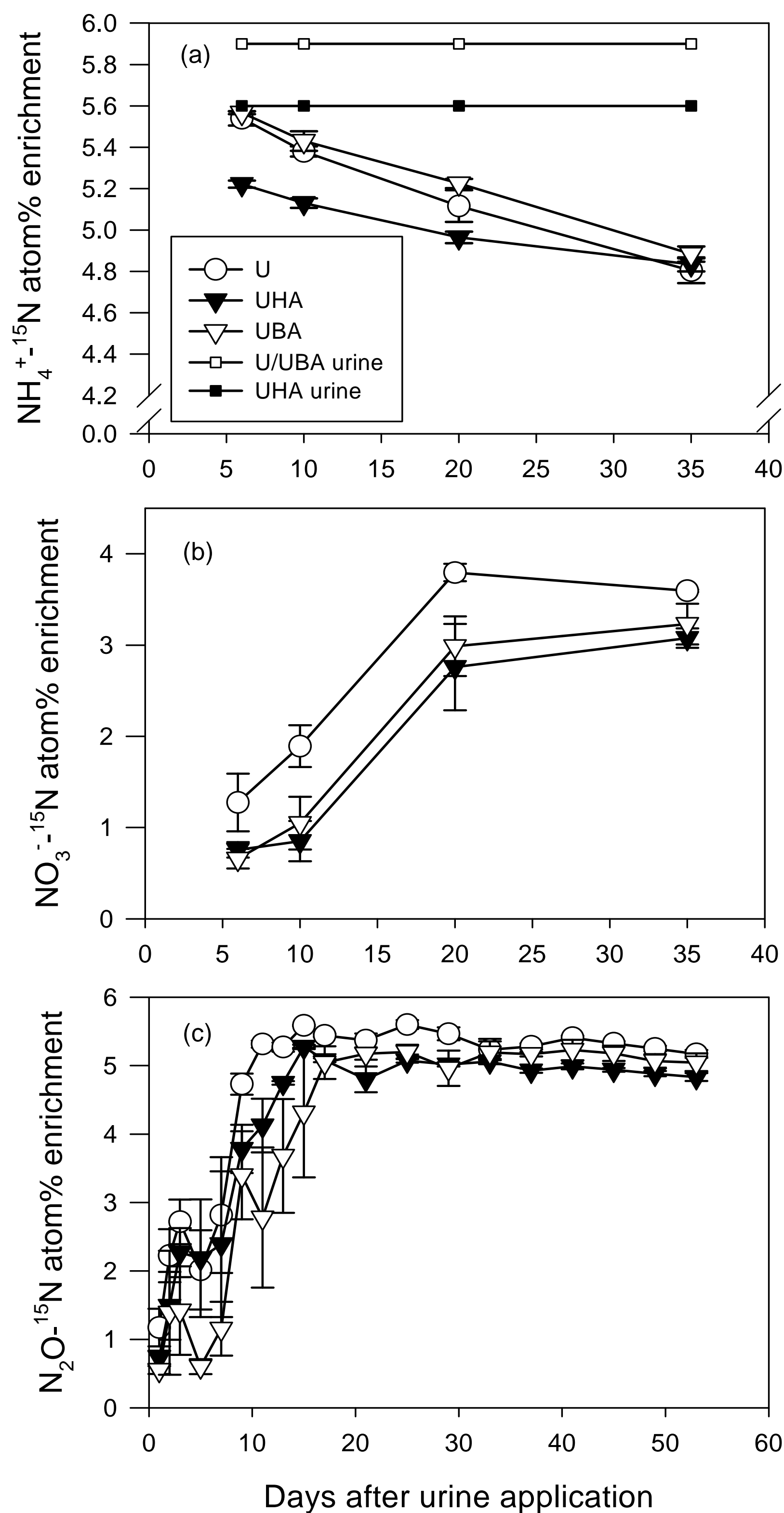


Figure 6.12 Mean ^{15}N atom% enrichment measured in urine treatments: (a) added urine and NH_4^+-N (days 6, 10, 20 and 35), (b) NO_3^--N (days 6, 10, 20 and 35), and (c) N_2O-N (days 1–55) (Error bars = SEM, n = 4).

6.4 Discussion

6.4.1 N_2O emissions

The results of this study, using real ruminant urine, supported the findings of the earlier synthetic urine studies (Kool et al., 2006b; van Groenigen et al., 2006) where increasing urinary hippuric acid or benzoic acid concentrations led to inhibition of N_2O-N emissions from cow urine. Kool et al. (2006b) approximately halved N_2O-N emissions by tripling the hippuric acid content of their synthetic urine. In this experiment the 65% reduction in cumulative N_2O-N emissions from real urine was achieved by doubling the hippuric acid content from 6.4% to 12.6% of total urinary-N. However, the hippuric acid concentration of the UHA treatment in this experiment (96 mmol L^{-1}/12.6% total N) was above the highest hippuric acid concentration (68 mmol L^{-1}/9% total N) used by Kool et al. (2006b), so greater N_2O-N reductions might be expected.

The experimental conditions for the current study were chosen to enhance N_2O-N emissions by promoting denitrification, since the earlier studies had hypothesised that hippuric acid inhibited denitrification (Kool et al., 2006b; van Groenigen et al., 2006). Kool et al. (2006b) achieved their highest N_2O-N emissions by changing the soil moisture content during the experiment to allow an accumulation of NO_3^--N at 70% WFPS to later be readily denitrified at 92% WFPS, whereas the reductions in the present experiment occurred with the soil moisture held at 70% WFPS. The soil used by Kool et al. (2006b) was a very sandy soil with 85% sand and 3% clay, whereas the Wakanui silt loam used in this current experiment had 6% sand and 27% clay. With the much lower sand content, it is likely that anaerobic microsites conducive to denitrification (Whitehead, 1995c) occurred in the Wakanui soil, without requiring manipulation of the soil moisture to the same extent to get high N_2O-N fluxes. However, the N_2O-N emissions recorded in the current experiment were much higher than those measured in field trials and in lab incubations in general; the quantitative effects of hippuric acid and benzoic acid under field conditions remain to be determined.

Although Kool et al. (2006b) measured high cumulative N_2O-N fluxes, the fluxes measured in the current experiment were higher, and this could have contributed to the greater inhibitory effect of the added acids observed here. This may have been influenced by higher nutrient inputs in the current study, both through N application rates (750 kg N ha^{-1} here vs. 250 kg N ha^{-1} (Kool et al., 2006b)) and through the use here of surface soil from 0–10 cm vs. soil from 5–10 cm (Kool et al., 2006b), where soil nutrients may be more limited. The higher pH in this current experiment (8.6 here vs. 5.8 (Kool et al., 2006b)), was probably due to the higher N

application rate, and is also likely to have enhanced N_2O-N emissions, as both nitrification and denitrification are optimal at pHs >7 (Sections 2.5.1.3 & 2.5.1.4). However, N_2O-N emissions may instead be reduced at high pH values (Clough et al., 2003a), possibly due to greater reduction of N_2O-N to N_2-N during denitrification (Firestone, 1982).

6.4.2 Denitrification

The decline in soil NO_3-N concentrations after day 35 may be partially attributable to diffusion down the soil column, but given the soil moisture content it is highly likely that denitrification was the predominant source of N_2O-N from day 20 onwards. This being the case, it appears that suppression of denitrification in the UHA and UBA treatments explains the significant difference in the cumulative N_2O-N fluxes observed. This effect was also observed by Kool et al. (2006b). The smaller and earlier NO_2^--N peak observed in the U treatment may be further evidence of the inhibition of denitrification by both hippuric acid and benzoic acid, indicating that denitrification was relatively uninhibited in the U treatment so that the NO_2^--N was utilised more rapidly. However, it may indicate that the hippuric acid and/or its derivative, benzoic acid, were also inhibiting nitrification in this experiment.

6.4.3 Nitrification

The results indicate that the UHA and UBA treatments inhibited nitrification in addition to denitrification. This supposition is supported by the inhibition of NH_4^+-N oxidation from days 6–20 and NO_3^--N production from days 10–20 in the UHA and UBA treatments. In addition, the delay in the utilisation of NO_2^--N in these treatments may have been due to inhibition of nitrification, and the continued elevation of the pH in the two acid treatments between days 10 and 20 suggests that nitrification had been inhibited.

The inhibition of NH_4^+-N oxidation became significant at days 6–10 for the UHA treatment and days 10–20 for the UBA treatment (Table 6.2). This 6 to 10 day delay was a function of the chemistry of the urine patch, as NH_4^+-N oxidation became the major NH_4^+-N transformation mechanism only when the pH had started to drop and NH_3-N volatilisation had decreased. Hydrolysis of the urinary urea (Section 2.5.1.1) resulted in the pH remaining elevated, at 8.6, from day 1 until day 6. The relatively high concentrations of NH_3-N present at this high pH could have strongly inhibited nitrification (Monaghan and Barraclough, 1992; Villaverde et al., 1997), so that any inhibitory effect of the UHA and UBA treatments would not become apparent until the pH had decreased. In addition, any benzoic acid present, as a hydrolysis product of hippuric acid (Bristow et al., 1992), would be ionised at pH 8.6 to benzoate (Chipley, 1983), which is not as toxic to bacteria as benzoic acid (Brul and Coote, 1999). Therefore if

benzoic acid was the inhibitory chemical, as has been hypothesised (Kool et al., 2006b; van Groenigen et al., 2006), and if nitrification occurred under the high pH conditions on days 1–6 (pH 8.6), benzoic acid would be unlikely to have had an inhibitory effect at this stage. However, once the pH began to decrease, a larger proportion of the benzoic acid would be present in the more toxic form, and it is likely that this was responsible for the inhibition of production of NO_3^--N and N_2O-N in the UHA and UBA treatments.

Inhibition of nitrification was also demonstrated by the lower rates of NO_3^--N production in both the UHA and UBA treatments (Table 6.2), although this only became statistically significant on days 10–20. Soil NO_3^--N concentrations were higher and peaked sooner in the U treatment due to the relative lack of nitrification inhibition, while in the UHA and UBA treatments the retardation of NH_4^+-N oxidation led to a slower increase in soil NO_3^--N concentrations. However, although NH_4^+-N oxidation was significantly inhibited in the UHA treatment over days 6–10, the only time when slower net NH_4^+-N oxidation in the acid treatments was matched by congruent net increases in NO_3^--N was over days 10–20. The difference between these trends was not due to differences in NH_3-N volatilisation, but it is possible that some of the NO_3^--N had leached further down the soil core and was therefore not sampled, or that N was immobilised by microbes. It should also be noted that although there were treatment differences in NH_4^+-N oxidation on several sampling days the decline over the 55 days of the experiment was the same in all the urine treatments, indicating that NH_4^+-N oxidation was slowed but not halted.

The decrease in the ^{15}N enrichment of the NH_4^+-N pool over time was a result of dilution by natural abundance NH_4^+-^{14}N, which became increasingly substantial as the NH_4^+-^{15}N pool became progressively smaller. The lower ^{15}N enrichment of the NH_4^+-N in the hippuric acid treatment was a consequence of the additional ^{14}N present in the hippuric acid, which likely formed NH_4^+-^{14}N as a result of deamination of the glycine released during hydrolysis of hippuric acid in soil (Bristow et al., 1992).

The peak ^{15}N enrichment of the soil NO_3^--N pool on day 20 (ca. 3.8 atom% enrichment) in the U treatment was not as high as the initial enrichment of the NH_4^+-N pool or the peak enrichment of the N_2O-N pool (both ca. 5.6 atom% enrichment). This indicates that the KCl extraction included a substantial natural abundance pool of NO_3^--N and suggests that the urine may not have been evenly distributed throughout the soil. This is further supported by the constant and high ^{15}N enrichment of the N_2O-N at ca. 5.4 atom% enrichment which clearly came from the applied urine-^{15}N pool.

6.4.4 Ammonia volatilisation

The lack of difference between NH_3-N emissions from the U treatment and the acid treatments supports the supposition that inhibition of NH_4^+-N oxidation would be negligible during the first six days when the soil pH was elevated at 8.6. Any inhibitory effect of the added acids should have resulted in elevated NH_3-N emissions. However, these results are in contrast to those of Whitehead et al. (1989), who found that adding hippuric acid to solutions of urea, at concentrations of up to 5% of urea-N, led to enhanced NH_3-N emissions. This may have been due to interactions with other constituents of the real cow urine used in this study, which were not present in the synthetic urine solutions used by Whitehead et al. (1989). Alternatively, the higher hippuric acid application rates used here (6.8–14.4% urea-N) may have nullified the stimulatory effect, as Whitehead et al. (1989) found that NH_3-N fluxes at the highest hippuric acid rate (5%) were reduced compared to those at lower acid rates (0.5-2.5%).

The glycine added in the UHA treatment (as a conjugate of benzoic acid in the hippuric acid molecule) may have been expected to enhance NH_3-N emissions, through deamination to NH_4^+-N and hence NH_3-N (Bristow et al., 1992). If this occurred, however, it was not of sufficient magnitude to be measured.

Ammonia volatilisation here was of similar magnitude to other studies (Sherlock and Goh, 1984; Whitehead et al., 1989). The warm soil temperature used in this study may have helped to promote NH_3-N loss, as low soil temperatures have been seen to retard NH_3-N volatilisation (Sherlock and Goh, 1984). Other studies have shown that increasing the hippuric acid concentration of urine can stimulate urea hydrolysis within two hours of urine application (Doak, 1952), and enhance NH_3-N volatilisation within 48 h of urine application (Bristow et al., 1992). Thus we may have expected to see faster production of NH_4^+-N, and subsequently a more rapid increase in NH_3-N emissions and potentially faster accumulation of NO_3^--N in the UHA treatment. However, in this study no early treatment differences between soil NH_4^+-N or NO_3^--N concentrations were observed.

6.4.5 Carbon dynamics

The strong correlation between the pH and WSC, was possibly due to the solubilisation of soil organic matter (SOM) in the high pH of the urine patch (Monaghan and Barraclough, 1993; Shand et al., 2000). If the C released from SOM was microbially available (Petersen et al., 2004b), the close relationship between WSC and DHA could be attributed to use of this WSC by soil micro-organisms. Another potential source of WSC was the urine constituents, and the additional C supplied in the hippuric acid and benzoic acid would have increased the WSC

concentrations, which were higher in the UHA and UBA treatments than in the U treatment on day 1, although the difference was not significant.

The elevated respiration of CO_2-C on days 13 and 29 in the UHA and UBA treatments suggests that C availability may have stimulated microbial growth. This is during the same period that inhibition of NH_4^+-N oxidation became apparent and potentially the immobilisation of inorganic-N could have contributed to the decline in measured inorganic-N. However, since WSC and NH_4^+-N concentrations were higher in the acid treatments than in the U treatment on day 20 and no significant differences were observed in MBC between the urine treatments throughout the experiment, it is unlikely that the additional acid stimulated the microbial biomass.

6.4.6 Soil microbial community

While the UHA and UBA treatments did not affect MBC, there was a stimulatory effect of hippuric acid and benzoic acid application on the bacterial PLFA biomarkers, with higher PLFA concentrations measured in the acid treatments than in the U treatment on days 3 and 10. No previous studies could be found on the effects of hippuric acid on soil microbes, but given the expected anti-bacterial properties of benzoic acid (Section 2.2.2.1), as evidenced by the decrease in N_2O-N emissions, it might be expected that the enhanced acid concentrations would have resulted in a reduction of bacterial biomass. That the opposite effect was observed may be because the benzoic acid was less toxic at the high pH (8.6) at the start of the experiment. Alternatively, the additional C in the added acids could have been utilised by benzoic acid degrading bacteria (Pumphrey and Madsen, 2008). The strong correlation between WSC and DHA supports this latter possibility by indicating the importance of available C to the microbial community.

The additional hippuric acid and benzoic acid did not appear to affect the fungal community, but there was a definite inhibitory effect of urine addition on the fungal PLFA biomarker. The means by which urine addition inhibited the fungal biomass is not known, but there are several possibilities. A decrease in the F/B ratio with increasing soil pH was measured by Frostegård and Bååth (1996), however, increasing the pH by liming did not affect the concentration of the fungal PLFA biomarker (Frostegård et al., 1993b). High concentrations of NH_3-N, as seen here after urine application, may inhibit the soil fungal community (Eno et al., 1955), but NH_3-N concentrations had returned to background levels by day 17 in this experiment, while the fungal biomass remained depressed until day 55. However, the small scale of the soil cores may have made it difficult for the fungal community to recover. Fungi have different tolerances to salinity

(Tresner and Hayes, 1971), and it is possible that the salt added in the urine caused inhibition of some types of fungi, resulting in the reduction in total fungal biomass observed here. The combined effect of urine addition in stimulating the bacterial biomass and inhibiting the fungal biomass caused the decrease in the fungal/bacterial ratio.

The G+/G- ratio declined over the course of the experiment, as a result of the G+ PLFA biomarkers declining between days 3 and 55, while G- PLFA biomarkers increased slightly after declining between days 3 and 10. As G- bacteria are believed to be more tolerant of stress (Section 2.4.2.4), the increasing G+/G- ratio throughout the experiment showed that the bacterial community was undergoing stress, imposed by urine application, with more G- than G+ bacteria tolerating urine application. The G+ and G- bacterial biomarkers also showed the initial stimulatory effect of urine addition (data not presented) that was apparent in the total PLFA biomarkers. However, the only effect of the additional acids was a higher concentration of G+ biomarkers in the UHA and UBA treatments on day 3, possibly due to the higher inputs of C as discussed above (Section 6.4.5).

This initial stimulation of the microbial community by urine addition was also apparent in the lower cy/ω7 ratio in the urine treatments on the first two sampling days. This ratio was used to indicate stress induced by urine application (5.6 and 9.6 g N L^{-1}) to a sandy loam pasture soil (Petersen et al., 2004a), with an increase in cyclopropyl fatty acids indicating cell dormancy (Kaur et al., 2005) (Section 2.4.2.4). That the cy/ω7 ratio was lower in the urine treatments than in the C treatment in this current experiment indicated that the initial impact of urine addition was stimulatory rather than inhibitory. The increase in the cy/ω7 ratio in the urine treatments after day 3 may have indicated an increase in microbial stress, but as the cy/ω7 ratio was never significantly higher in the urine treatments than in the C treatment, it was probably merely an indication that the initial stimulation had ended.

No initial stimulatory effect of urine addition on the microbial community was seen in the other two stress ratios monitored, however. The i/a ratio and t/c ratio both showed membrane responses due to environmental changes, and the higher t/c ratio and lower i/a ratio seen here in the urine treatments indicated that the microbial community was stressed by the addition of urine. The decrease in the t/c ratio and increase in the i/a ratio over time showed that the microbial communities in the urine treatments began to recover from the imposed stress, and moved towards the ratios found in the C treatment. However, by day 20 the communities appeared to reach a new equilibrium and then remained unchanged, but significantly different

from control values, until the last sampling on day 55. This shows that the effect of urine on the microbial community may be long-term under the conditions of this experiment.

The contrasting changes in the different ratios used probably show responses by different parts of the soil microbial community to urine application. The G+/G- ratio and cy/ω7 ratio are reflections of the same section of the microbial community, as cy17:0 and 16:1ω7 are also used as G- biomarkers. The best representation of the stress response by the total microbial community are the i/a and t/c ratios, which have previously been used to indicate microbial stress induced by urine addition (Petersen et al., 2004a) and osmotic stress (Heipieper et al., 1996; Chihib et al., 2003) (Section 2.4.2.4).

The overall PCA scores (Figure 6.7) also indicated that the microbial community had reached equilibrium, as the scores for days 20 and 55 show considerable overlap, while the scores for the first two sampling days were well separated. All the PCA graphs indicated that the urine treatments did not cause differences in terms of microbial community response, while on all days it was apparent that the urine treatments differed from the C treatment. This indicated an overall impact of urine application, but there were no changes measured by PLFA that were specifically caused by the enhancement of the hippuric acid or benzoic acid concentrations.

6.5 Discussion summary

- Adding hippuric acid or benzoic acid to real cow urine reduced N_2O-N emissions by at least 65%
- Benzoic acid had the same effect on N_2O-N emissions and N dynamics as hippuric acid
- The reduction in N_2O-N emissions did not lead to an increase in NH_3-N emissions
- Although the soil microbial community was stressed by the addition of urine, there were no measured direct effects of the additional acids on soil microbes that corresponded with the changes in N_2O-N emissions

6.6 Conclusions

Hypothesis 1: That the addition of hippuric acid or benzoic acid to real cow urine would reduce emissions of N_2O-N over the course of the experiment.

This hypothesis was supported, with a 65% reduction in cumulative N_2O-N emissions measured when the hippuric acid content of real cow urine was increased from 6.4% to 12.6% of the total

urinary N. Benzoic acid, a product of hippuric acid hydrolysis, had the same effect as hippuric acid, reducing N_2O-N emissions by 72%. Fluxes of N_2O-N were reduced from 16.8% of the total N applied to 5.9% and 4.7% for hippuric acid and benzoic acid, respectively. Unexpectedly, the additional acid inhibited not only denitrification but also nitrification. On the evidence of this study, it appears that manipulating urinary hippuric acid concentrations could be developed into an effective mitigation strategy for reducing N_2O-N emissions from pasture soils.

Hypothesis 2: That any reduction in N_2O-N would result in an increase in NH_3-N volatilisation.

This hypothesis was not supported by the results of this experiment, with no increase in NH_3-N volatilisation measured as a result of the reduction in N_2O-N emissions.

Hypothesis 3: That these changes would be reflected by measurable changes in the microbial community.

There was no support for this hypothesis, with few changes being apparent in the microbial community due to the additional hippuric acid or benzoic acid in the urine. There was no difference due to the acid amendments in either MBC or microbial activity. Although some of the PLFA indicators showed early differences between the acid treatments and the U treatment, these did not last beyond day 3, so the N_2O-N inhibition in the acid treatments from day 9 onwards could not be accounted for by overall microbial community changes. These short-term differences may have been due to the higher C added in these treatments compared with the U treatment. In contrast to the short-term effects of the acid treatments, urine application imposed changes on the microbial community that were still apparent after 55 days.

Future research

- Investigation of hippuric acid effects on N_2O-N emissions from cow urine at a range of soil temperatures corresponding to those found under field conditions
- Manipulation of ruminant diets to modify hippuric acid concentrations in urine
- Field trials using cow urine amended with hippuric acid

Chapter 7
The effects of urinary hippuric acid at varying temperatures on soil nitrogen transformations and N_2O-N emissions

7.1 Introduction

Experiment 3 and the previously published studies examining hippuric acid effects on N_2O-N emissions (Kool et al., 2006b; van Groenigen et al., 2006) were all carried out at relatively high temperatures (16 or 21°C), which do not occur throughout the year in the field. Moreover, fluxes of N_2O-N may be highest when soil temperatures are reduced (i.e. autumn–spring) because the soil moisture content is higher (Allen et al., 1996; van Groenigen et al., 2005). Thus the objective of experiment 4 was to investigate the effects of hippuric acid over a realistic range of pasture soil temperatures. The temperatures used in this experiment (5, 10, 15 and 20°C) were representative of the temperature range found at 10 cm soil depth at Lincoln, New Zealand, as can be seen from the data in Figure 7.1 (NIWA, 2009).

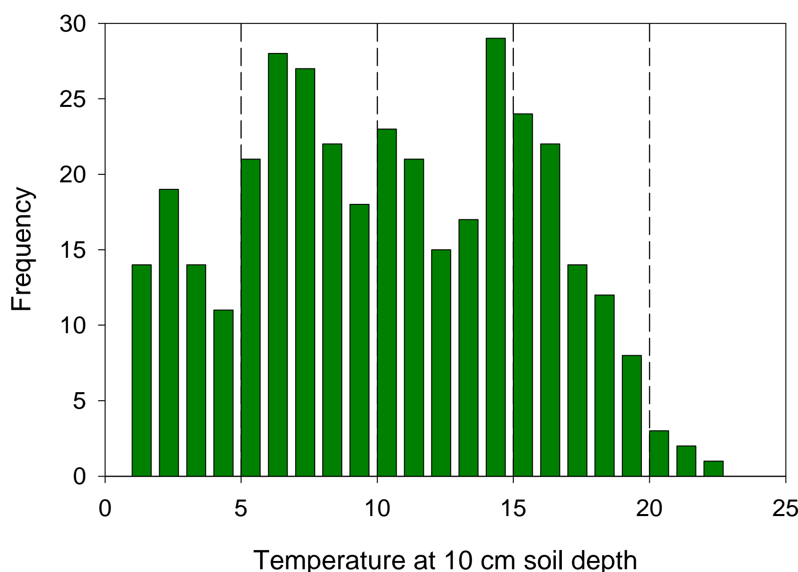


Figure 7.1 Frequency of annual soil temperatures at 10 cm depth at Lincoln, New Zealand. Vertical lines represent the temperatures used in this experiment (Section 7.2.2).

Previous studies have shown that soil temperature affects soil microbial processes, including nitrification and denitrification (e.g. Keeney et al., 1979; Malhi and McGill, 1982). Furthermore, elevated soil temperatures enhance enzyme activity, including DHA, and the rate of urea hydrolysis may also be faster due to increased urease activity (e.g. Dalal, 1975; Gong, 1997). Therefore, all things being equal, an increase in soil temperature could be expected to result in higher emissions of nitrogenous gases due to the higher rates of N transformations in the soil. However, previous studies have shown that increasing the soil temperature also increases the N_2-N/N_2O-N ratio (Keeney et al., 1979; Maag and Vinther, 1996), so that while total gaseous N emissions increase with temperature, the amount released as N_2O-N decreases. Moreover, degradation of benzoic acid (Pumphrey and Madsen, 2008) may occur faster at higher temperatures, thus reducing the amount in the soil and therefore reducing its inhibitory effect, as is known to occur with the nitrification inhibitor DCD (Kelliher et al., 2008).

The hypothesis to be tested was that hippuric acid inhibition of N_2O-N emissions would diminish as soil temperatures increased, due to enhanced microbial activity at higher temperatures.

7.2 Materials and Methods

7.2.1 Soil collection and soil core preparation

Temuka silt loam soil (Gley, New Zealand Soil Classification (Hewitt, 1998)) from 0–10 cm depth was collected from a continuously grazed pasture site at Lincoln, New Zealand (43°38.8′S, 172°29.1′E). The soil was sieved to < 4 mm and then packed into PVC tubes (70 mm high x 50 mm ID) to a depth of 60 mm and a bulk density of 0.79 g cm^{-3}. Nylon mesh fabric attached to the base of each core prevented soil loss. Each repacked soil core was covered with pierced Parafilm® M (Pechiney Plastic Packaging, Menasha, WI, USA) and incubated at its experimental temperature (Section 7.2.2) for one week prior to urine application.

7.2.2 Urine treatments and experimental design

Cow urine was collected from Friesian dairy cows, bulked, and stored overnight at 4°C. A subsample of the urine was analysed (Section 3.1), and contained 7.3 g L^{-1} of N and 6.3 g L^{-1} of hippuric acid (35 mmol L^{-1}; 6.7% total urinary N).

Two urine treatments were formulated. Urea (15.05 g) was added to 2.6 L of the collected urine to take the N content to 10.0 g L^{-1}. One half of the urine (1.3 L) received no further amendments, so the final hippuric acid content was 6.3 g L^{-1} (35 mmol L^{-1}; 4.9% of total urinary N; Treatment LHA). The other 1.3 L of urine received 9.32 g of hippuric acid,

increasing the hippuric acid content to 13.5 g L^{-1} (75 mmol L^{-1}; 9.9% of total urinary N; Treatment HHA) and the final urinary-N content to 10.6 g L^{-1}. Each soil core had 15.0 mL of urine pipetted onto its soil surface at a rate equivalent to 764 kg N ha^{-1} for the LHA treatment and 810 kg N ha^{-1} for the HHA treatment.

The experiment was a 2 x 4 factorial design, with two urine treatments (LHA and HHA, as described above) and four temperature treatments (5, 10, 15 and 20°C (±1°C)). Each treatment was replicated four times, giving a total of 32 cores for each destructive sampling (see below). This was further replicated to allow for five destructive sampling events on days 2, 7, 15, 29 and 77, with a grand total of 160 cores. The final set of 32 soil cores (4 replicates x 8 treatments), destructively sampled on day 77, was used for gas sampling and surface soil pH measurements throughout the duration of the experiment. The 5°C treatment was incubated in a fridge, the 10 and 20°C treatments were placed into incubators, and the 15°C treatment was incubated in a temperature-controlled laboratory. Air temperatures were monitored using maximum-minimum thermometers.

Acid traps were placed in the headspace of each soil core to remove any NH_3-N emitted while the cores were being incubated. This simulated NH_3-N diffusion away from the urine patch in a pasture system. These consisted of strips of filter paper moistened with 1–2 drops of orthophosphoric acid, and suspended above the soil surface beneath the Parafilm®. The acid traps were put in place immediately after urine application and were replaced at regular intervals, until being removed on day 48. These were substituted during gas sampling for temporary acid traps to allow timed sampling of NH_3-N emissions (Section 3.4.4).

The soil cores were adjusted to a gravimetric water content of 0.45 g water g^{-1} soil (47% WFPS), to promote nitrification, because there was evidence during experiment 3 of hippuric acid inhibition of nitrification (Section 6.4.3). This initial moisture adjustment was carried out one day after urine application to prevent leaching of urine constituents. The soil moisture was maintained at the same gravimetric water content by adding water to the cores every 3–4 days until day 17. On day 17, the gravimetric water content was increased to 0.55 g water g^{-1} soil (62% WFPS), to encourage denitrification, as there had been no indication of hippuric acid inhibition of N_2O-N fluxes or nitrification by this stage. The soil moisture content was then maintained at this level for the remainder of the experiment, by adjusting the moisture every 3–4 days as before.

7.2.3 Gas and soil sampling

Sampling for N_2O-N (Section 3.4.2) was performed on 28 occasions between day 0 (8 hours after urine application) and day 77, initially daily after urine application on day 0 and after altering the soil moisture content at day 17, and then at increasing intervals, to be once a week by the end of the experiment. Headspace NH_3-N sampling (Section 3.4.4) was performed on 17 occasions, at the same time as N_2O-N sampling, but was discontinued after day 45 when NH_3-N concentrations in all treatments were negligible. The soil surface pH (Section 3.2.3) was measured on 23 occasions between day 1 and day 76, immediately after the soil moisture was adjusted.

Destructive soil core sampling entailed transferring all the soil from the PVC tubes into labelled resealable plastic bags. After thorough mixing, soil sub-samples were removed to be analysed for gravimetric soil moisture, bulk soil pH, inorganic-N concentrations, and DHA (Sections 3.2.1, 3.2.2, 3.2.3 & 3.3.2).

7.3 Results

7.3.1 Soil pH

The bulk soil pH was highest immediately after urine application, and remained at ca. pH 7.0 in all treatments until day 7 (Figure 7.2a), after which time it decreased to < 6.0 in all treatments, but to a greater extent in the higher temperature treatments. The mean pH (averaged over all temperature and hippuric acid treatments) was significantly lower at each successive sampling ($P < 0.001$) until day 29. There were significant differences in pH due to temperature on all sampling occasions. The pH values of the 5 and 10°C treatments were higher than the pH of the 20°C treatment on all days ($P < 0.001$), and the pH of the 15°C treatment was higher than that of the 20°C treatment on days 7 and 15. On days 29 and 77, the pH in the 5°C treatment was higher than all the other treatments, while the pH in the 10°C treatment was greater than the 15 and 20°C treatments, which did not differ from each other. The average pH values over the experimental period for the 5, 10, 15 and 20°C treatments all differed from each other ($P < 0.001$), and were 6.3, 6.0, 5.6 and 5.5 respectively. There was a significant interaction between time and temperature on the bulk soil pH ($P < 0.001$), with pH decreasing at a faster rate with increasing temperature (Figure 7.2b). The hippuric acid treatment did not affect the bulk soil pH.

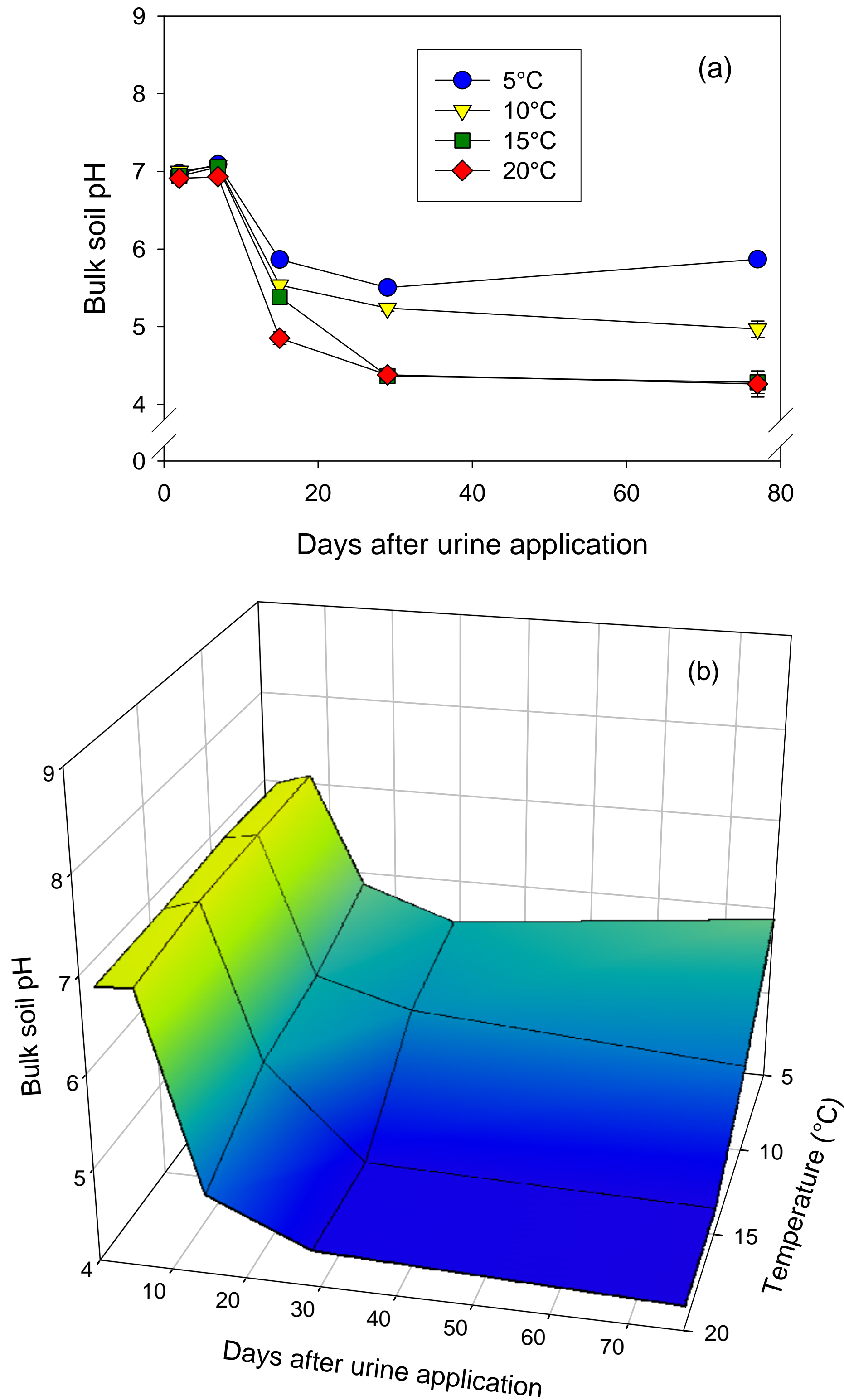


Figure 7.2 Mean bulk soil pH: (a) bulk soil pH for each temperature treatment measured over time (Error bars = SEM, n = 8) and, (b) interaction of time and temperature on bulk soil pH. (NOTE: Fig. 2b; y axis labels (temperatures) are 20–5°C.)

The surface soil pH showed the same trends as the bulk soil pH over the course of the experiment (Figures 7.3 & 7.4a). However, the pH values were generally higher on the soil surface (Figure 7.3), and at day 1 were 0.4–1.4 pH units higher than the day 2 bulk soil pH

values. The surface soil pH began to decline after day 1, in contrast to the bulk soil pH, which increased up until day 7. At this time, the surface soil pH was lower than the bulk soil pH in the 15 and 20°C treatments. The surface soil pH was higher than the bulk soil pH on days 15 and 29, but was lower or the same as the bulk soil pH by the end of the experiment (Figure 7.3).

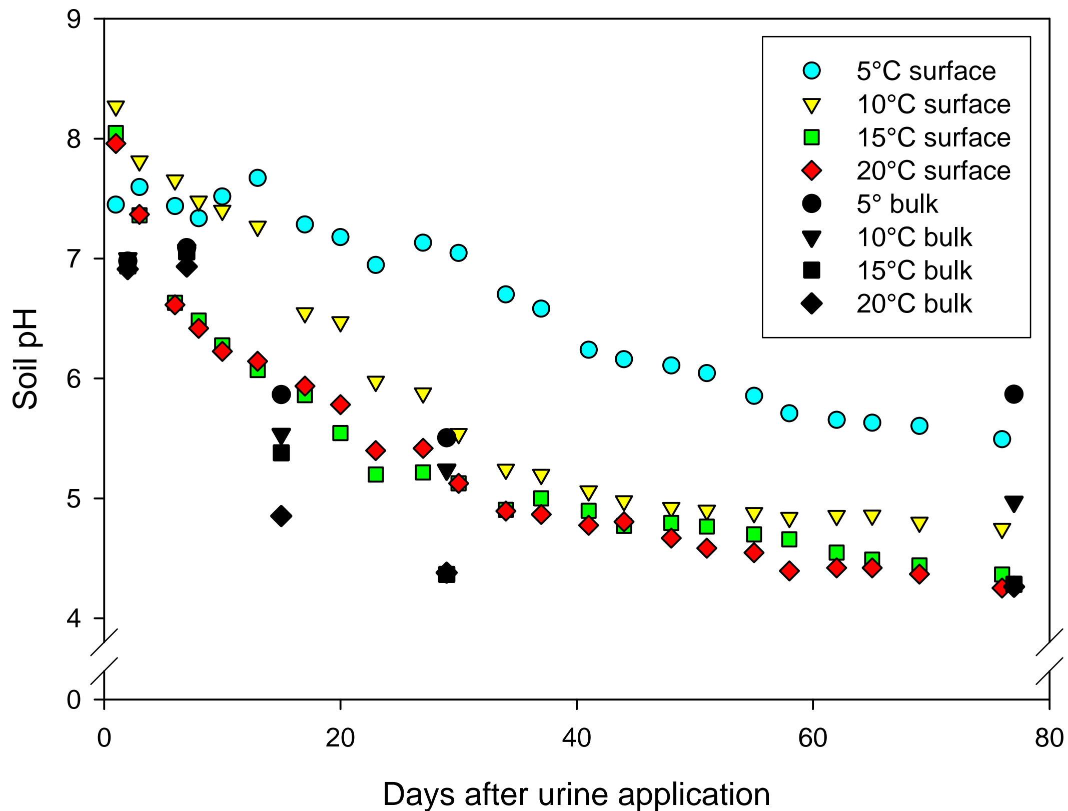


Figure 7.3 Surface and bulk soil pH measured over the experimental period (n = 8).

On day 1 the surface soil pH in the 5°C treatment was lower than the pH in all the other temperature treatments ($P < 0.001$), which did not differ from each other (Figure 7.4a). However, the surface pH in the 5°C treatment was significantly higher than that in the 15 and 20°C treatments from day 6 onwards ($P < 0.001$), and was higher than that in all the other temperature treatments from day 13 onwards ($P < 0.001$). The surface pH in the 10°C treatment was higher than in the 15 and 20°C treatments on days 3–34 and 55–76 ($P < 0.01$), while the pH in the 15 and 20°C treatments differed only on days 27, 51 and 58 ($P < 0.001$).

Averaged over the experimental period, the surface soil pH in the 10°C treatment was lower than in the 5°C treatment, but higher than in the 15 and 20°C treatments ($P < 0.001$). The 15 and 20°C treatments did not differ from each other. The surface pH was affected by both a time x temperature interaction (Figure 7.4b), where the pH declined faster at higher temperatures,

and a temperature x hippuric acid interaction, where the pH was enhanced in the HHA treatment at 5–15°C. However, hippuric acid treatment alone did not affect the pH.

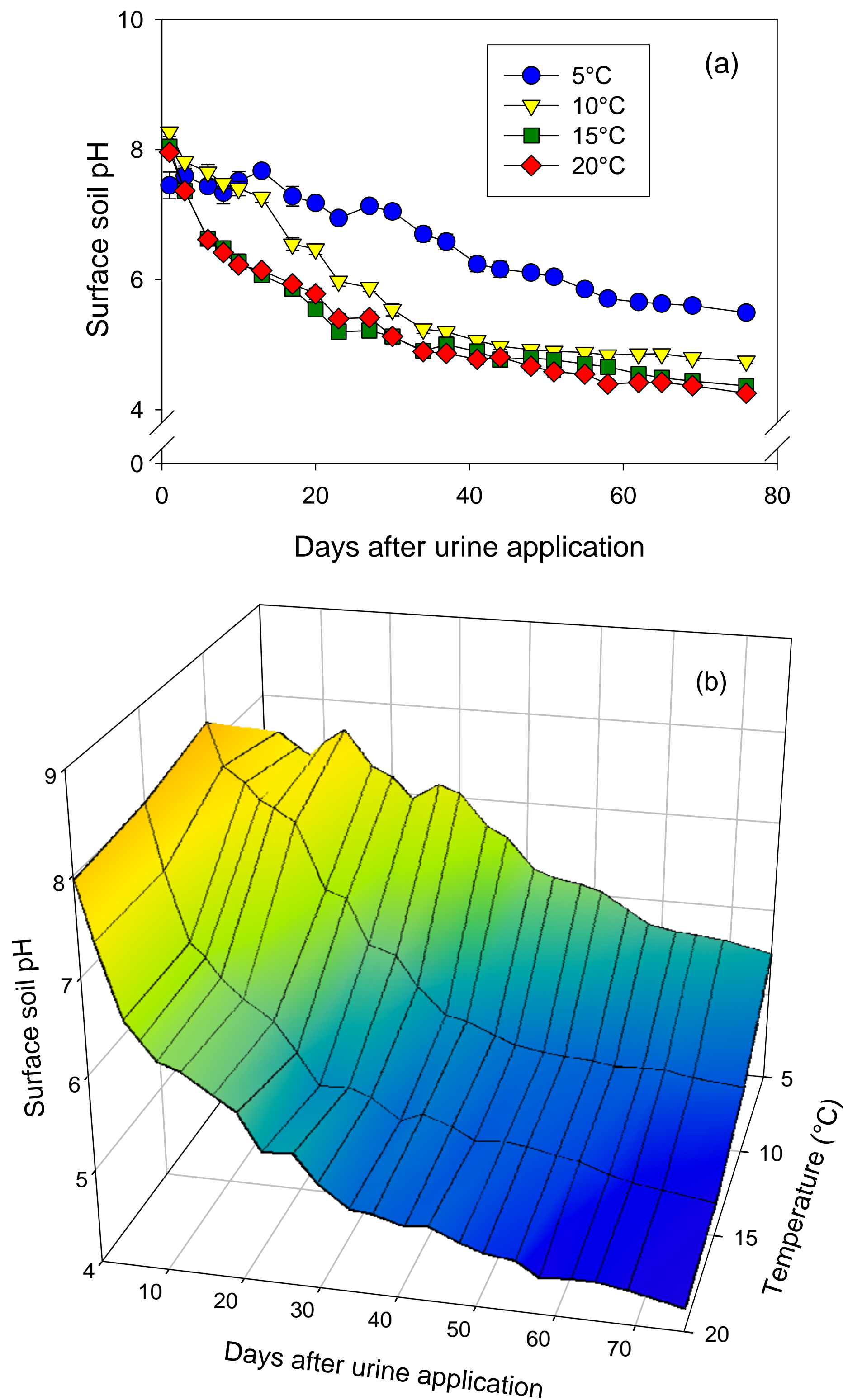


Figure 7.4 Mean surface soil pH: (a) surface soil pH for each temperature treatment measured over time (Error bars = SEM, n = 8) and, (b) interaction of time and temperature on surface soil pH. (NOTE: Fig 4b; y axis labels (temperatures) are 20–5°C.)

7.3.2 Inorganic N

Soil NH_4^+-N concentrations peaked on day 2 in all treatments and declined as the experiment progressed (Figures 7.5a–c). On day 2, the highest mean concentration (1471 mg NH_4^+-N kg^{-1}) was measured in the 10°C treatment, and this was higher than concentrations in both the 15 and 20°C treatments ($P < 0.01$) (Figure 7.5a). The NH_4^+-N concentration in the 20°C treatment was also lower than that in the 5°C treatment on day 2 ($P < 0.01$). On days 15–77, NH_4^+-N concentrations were lower with each increase in temperature ($P < 0.001$). Significantly higher concentrations of soil NH_4^+-N occurred in the HHA treatment than in the LHA treatment on days 15 and 77 ($P < 0.01$) (Figure 7.5b). There was also a time x temperature effect ($P < 0.001$), with NH_4^+-N concentrations decreasing faster over time at the higher temperatures (Figure 7.5c). Soil NH_4^+-N was positively correlated with bulk soil pH in the 15 and 20°C treatments ($r \geq 0.91$, $P < 0.05$) and in the 10°C, LHA and HHA treatments ($r \geq 0.83$, $P < 0.10$).

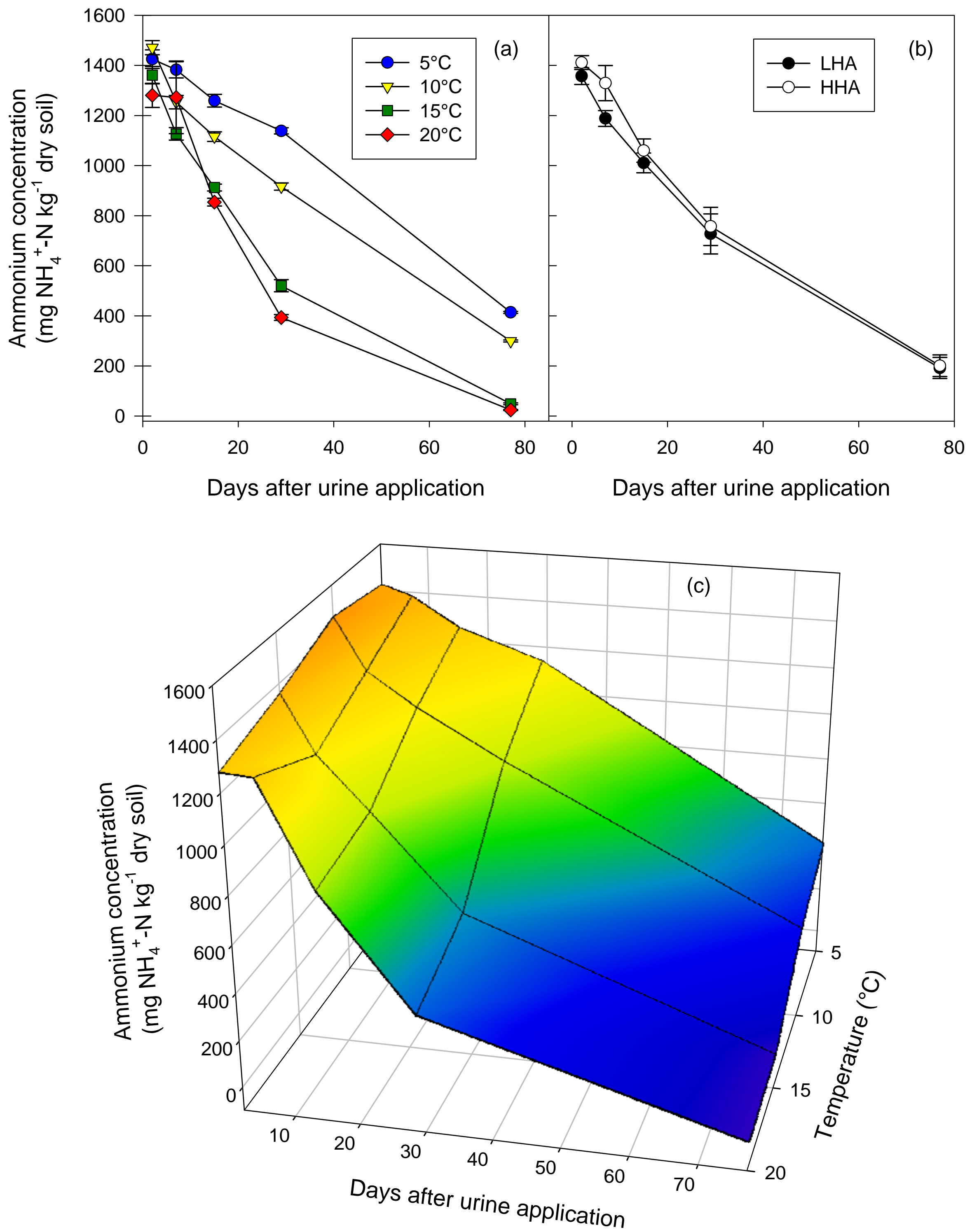


Figure 7.5 Soil ammonium concentrations measured over time: (a) effect of temperature treatment (Error bars = SEM, n = 8), (b) effect of hippuric acid treatment (Error bars = SEM, n = 16), (c) interaction effect of time and temperature. (NOTE: Fig. 5c; y axis labels (temperatures) are 20–5°C.)

On day 2, NO_2^--N concentrations were higher in the 5 and 10°C treatments (> 5.0 mg kg^{-1}) than in the 15 and 20°C treatments (< 2.0 mg kg^{-1}) ($P < 0.001$) (Figure 7.6a). Nitrite concentrations had decreased by day 7, but remained higher in the 5°C treatment compared to the 10 and 15°C treatments ($P < 0.05$). Concentrations were still low on day 15, but all treatments differed significantly ($P < 0.001$), and the 10°C treatment had the highest concentration (0.81 mg kg^{-1}). On day 29, the concentration of NO_2^--N in the 20°C treatment had increased to nearly 20 mg kg^{-1} and was significantly higher than that in all the other temperature treatments ($P < 0.001$). Within the hippuric acid treatments, the elevated concentration of NO_2^--N measured in the HHA treatment was only significant on day 2 ($P < 0.05$) (Figure 7.6b). There was a significant temperature x hippuric acid effect on days 2 and 7 ($P < 0.01$), where NO_2^--N concentrations were proportionately higher in the HHA treatment than in the LHA treatment as the temperature decreased. Averaged across all treatments, the concentration of NO_2^--N on day 2 was higher than on all other sampling occasions, except day 20, which had the highest NO_2^--N concentration ($P < 0.001$). The temperature also had a significant effect on NO_2^--N, with concentrations being higher on day 20 than on all other days ($P < 0.001$). The concentration of NO_2^--N was higher in the HHA treatment than in the LHA treatment overall, but this was only significant at the $P < 0.10$ level. The time x temperature interaction also had a significant effect on NO_2^--N concentrations ($P < 0.001$), with lower concentrations of NO_2^--N in the higher temperature treatments on day 2 and a higher concentration in the 20°C treatment on day 29 (Figure 7.6c).

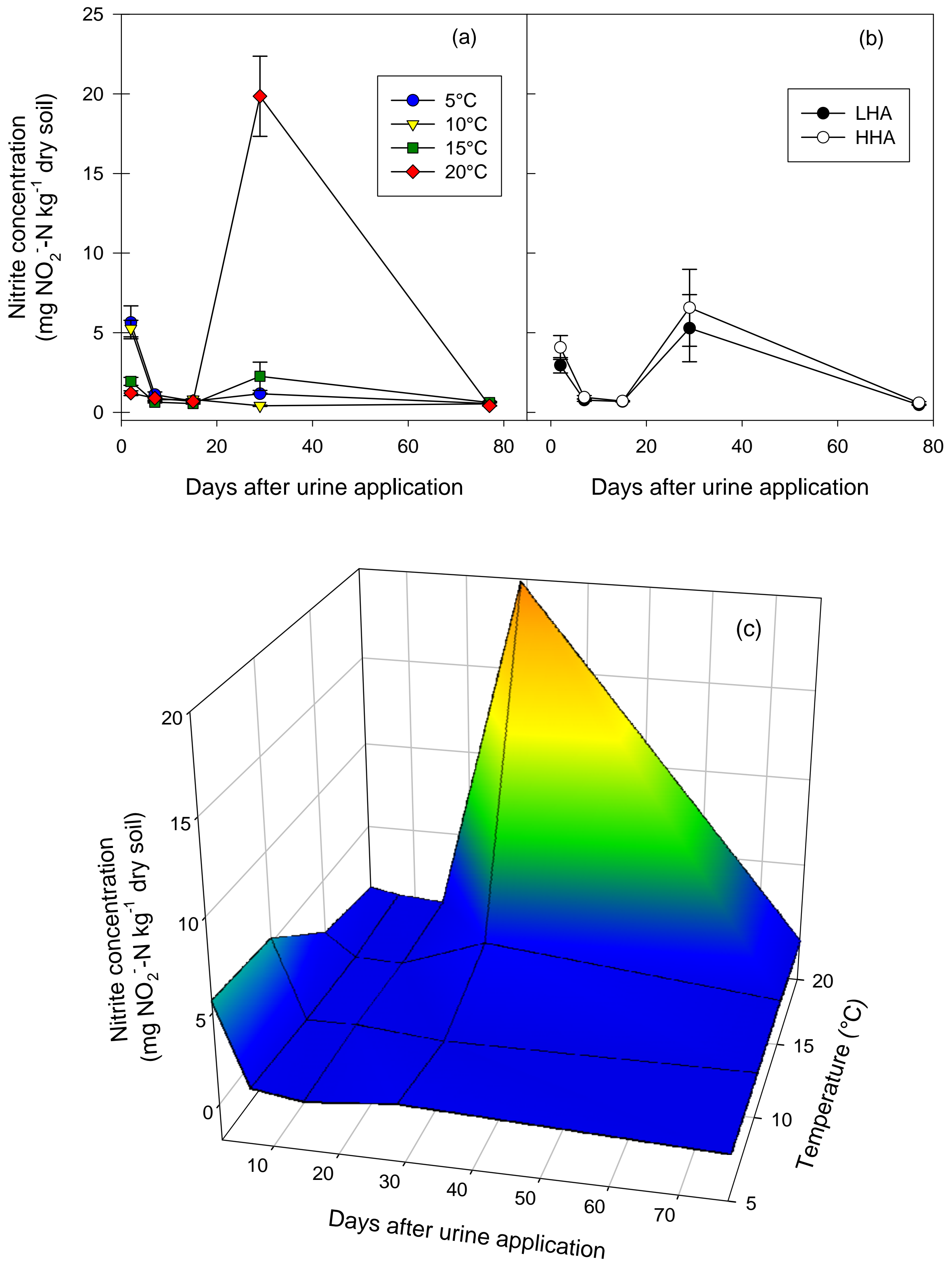
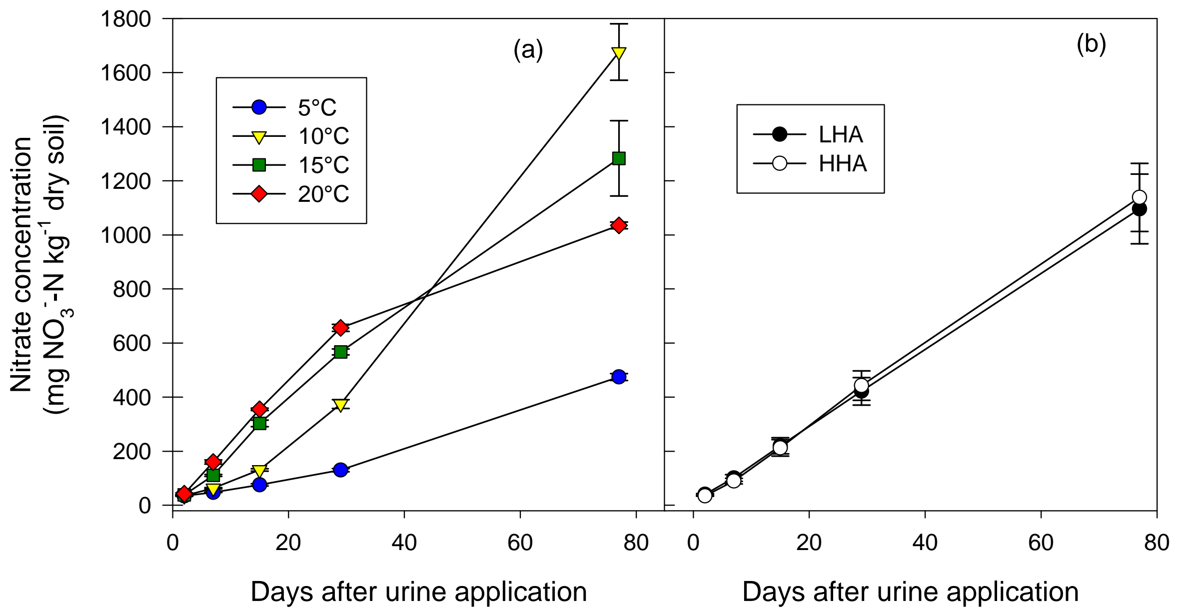


Figure 7.6 Soil nitrite concentrations measured over time: (a) effect of temperature treatment (Error bars = SEM, n = 8), (b) effect of hippuric acid treatment (Error bars = SEM, n = 16), (c) interaction effect of time and temperature. (<u>NOTE</u>: Fig. 6c; y axis labels (temperatures) are 5–20°C.)

Soil NO_3^--N concentrations were at their lowest on day 2 in all treatments, and increased throughout the experimental period (Figures 7.7a–c), reaching 1676 mg kg^{-1} in the 10°C treatment on day 77. The NO_3^--N concentrations differed with temperature on all sampling occasions (Figure 7.7a). The 20°C treatment had higher concentrations than the 10 and 15°C treatments on day 2 ($P < 0.01$). On days 7–29 the NO_3^--N concentration in any given temperature treatment was significantly higher than in any lower temperature treatment ($P < 0.001$). By day 77, the concentration of NO_3^--N was higher in the 10°C treatment than in the 15 and 20°C treatments and lower in the 5°C treatment than in all other treatments ($P < 0.001$). On days 2 and 7 the NO_3^--N concentrations were higher in the LHA than in the HHA treatment ($P < 0.01$), but there was no difference due to hippuric acid treatment thereafter (Figure 7.7b). Averaged over all treatments, NO_3^--N concentrations were lower on days 2 and 7 than on subsequent sampling occasions, and were then higher at each consecutive sampling ($P < 0.001$). Over the experimental period, the mean NO_3^--N produced in the 5°C treatment was significantly lower than all the other temperature treatment means ($P < 0.001$). There was also a time x temperature effect over the course of the experiment ($P < 0.001$) (Figure 7.7c), where rates of NO_3^--N increase differed between temperature treatments. There was a negative correlation between NO_3^--N and bulk soil pH in the 15 and 20°C treatments ($r \leq -0.83$), and a negative correlation between NO_3^--N and NH_4^+-N in all treatments ($r \leq -0.95$).



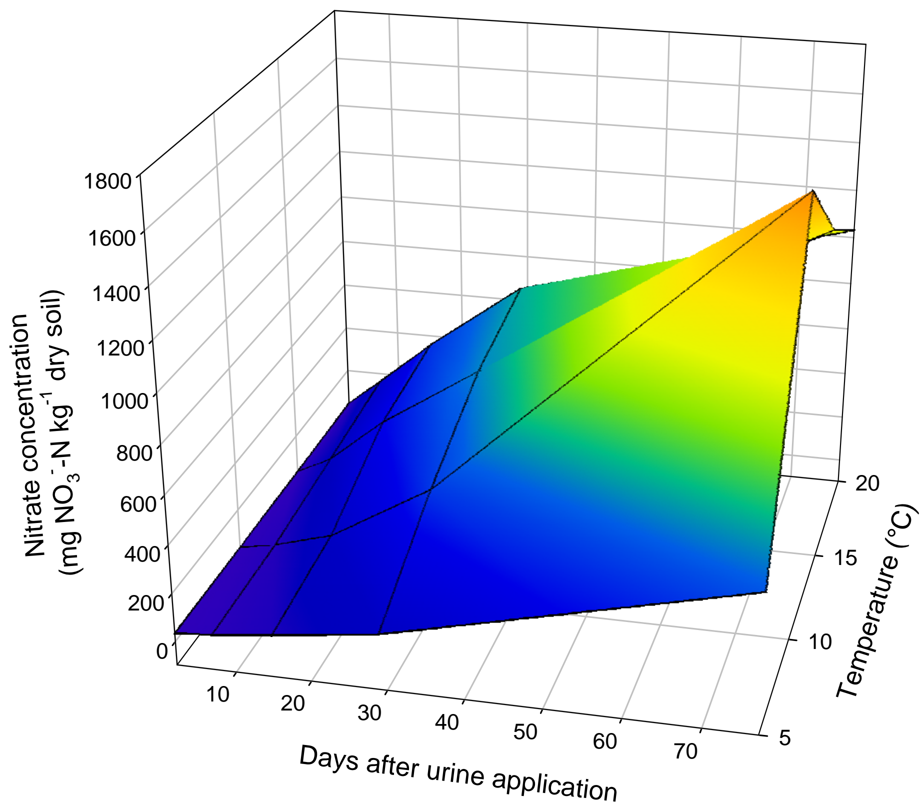


Figure 7.7 Soil nitrate concentrations measured over time: (a) effect of temperature treatment (Error bars = SEM, n = 8), (b) effect of hippuric acid treatment (Error bars = SEM, n = 16), (c) interaction effect of time and temperature. (NOTE: Fig. 7c; y axis labels (temperatures) are 5–20°C.)

On days 0–2, net NH_4^+-N production rates were higher in the 5 and 10°C treatments than in the 20°C treatment ($P < 0.01$), while the net rates at 5 and 15°C did not differ (Table 7.1). On days 7–29, net rates of NH_4^+-N depletion increased with increasing temperature, with significant differences between all treatments on days 15–29 and between the 20°C treatment and the 5 and 10°C treatments on days 7–15 ($P < 0.05$). On days 29–77 the reverse was seen, with the net rates of NH_4^+-N depletion increasing with decreasing temperature, and significant differences between all treatments ($P < 0.001$). There was no significant effect of the hippuric acid treatments. Overall, net NH_4^+-N depletion rates on days 2–29 were higher than on days 29–77 but lower than on days 0–2, when the net rate was positive ($P < 0.001$). Net rates of NH_4^+-N transformation (formation and depletion) over the experimental period were higher at 5 and 10°C than at 15 and 20°C ($P < 0.01$). There was also a time x temperature interaction effect ($P < 0.001$), with net rates of NH_4^+-N depletion varying in the different temperature treatments over the course of the experiment.

The net rates of NO_3^--N production on days 7–77 were higher than on days 2–7, but lower than on days 0–2 ($P < 0.001$) (Table 7.1). Net NO_3^--N production was highest at 20°C between days 0 and 29, and was lowest on all occasions at 5°C. These two treatments differed significantly between days 0 and 29 ($P < 0.01$), and net NO_3^--N production was also lower at 5°C than at 10 and 15°C on days 2–77 ($P < 0.001$). On days 29–77, net NO_3^--N production was higher at 10°C than at 15°C, and both these treatments had higher net production rates than the 5 and 20°C treatments ($P < 0.001$), which did not differ from each other. Overall, net rates of NO_3^--N production increased with temperature, and did not differ between the 15 and 20°C treatments, while rates in the 5°C treatment were lower than in all other treatments ($P < 0.001$). The hippuric acid treatments only had an effect on days 0–2, when net NO_3^--N production rates were higher in the LHA treatment ($P < 0.001$). An interaction occurred between time and temperature ($P < 0.001$), where net NO_3^--N production rates varied with temperature over the experimental period, with lower rates on days 29–77 at > 15°C. There was a time x hippuric acid treatment effect ($P < 0.05$), due to the higher rate of NO_3^--N production in the LHA treatment on day 2.

Table 7.1 Net rates of change in the soil NH_4^+-N and NO_3^--N concentrations (mg N kg^{-1} dry soil d^{-1}). Negative rates indicate declining concentrations.

	Time (days)				
	0–2	2–7	7–15	15–29	29–77
	NH_4-N (mg N kg^{-1} dry soil d^{-1})				
Treatment					
5°C	713	-8.5	-15.4	-8.6	-15.1
10°C	735	-43.5	-17.2	-14.3	-12.8
15°C	681	-46.9	-26.7	-28.0	-9.8
20°C	640	-1.6	-52.3	-32.9	-7.7
significance	**	NS	*	***	***
LSD (0.05)	51.3		26.2	4.8	1.0
df = 24, n = 8					
HHA	706	-16.4	-33.6	-21.6	-11.6
LHA	679	-33.9	-22.2	-20.2	-11.1
significance	NS	NS	NS	NS	NS
LSD (0.05)					
df = 24, n = 16					
	NO_3-N (mg N kg^{-1} dry soil d^{-1})				
Treatment					
5°C	17.4	2.6	3.5	3.9	7.2
10°C	17.5	5.6	8.4	17.4	27.1
15°C	19.1	14.4	24.1	18.9	14.9
20°C	21.3	23.5	24.4	21.5	7.9
significance	**	***	***	***	***
LSD (0.05)	2.3	2.4	2.8	3.2	5.5
df = 24, n = 8					
HHA	17.1	11.1	15.3	16.5	14.5
LHA	20.5	12.0	14.8	14.4	14.0
significance	***	NS	NS	NS	NS
LSD (0.05)	1.6				
df = 24, n = 16					

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS = not significant

7.3.3 Microbial activity

On all sampling days DHA was highest at 5°C and, at any given time, decreased with increasing temperature. The highest rate of DHA (58 µg TPF g^{-1} dry soil h^{-1}) was measured in the 5°C treatment on day 2. All temperature treatments were significantly different from each other on days 2, 15 and 29 ($P < 0.001$), but the 15 and 20°C treatments did not differ on days 7 and 77 (Figure 7.8a). Within the 15°C treatment, DHA was higher in the HHA than the LHA treatment only on day 2 ($P < 0.001$), while, at 10°C, the HHA treatment had higher DHA than the LHA treatment on days 2 and 7 ($P < 0.001$) (data not shown). In the 5°C treatment, DHA was higher in the HHA treatment on days 2–15 but higher in the LHA treatment on day 77 ($P < 0.001$) (data not shown). The significance of the hippuric acid effect at different temperatures is discussed in Section 7.4.3. Averaged over all temperatures, the DHA was significantly higher in the HHA treatment than the LHA treatment on days 2 and 7 ($P < 0.001$), but was unaffected by hippuric acid treatment thereafter (Figure 7.8b).

Dehydrogenase activity differed over time, being lower at each consecutive sampling time ($P < 0.001$), and was reduced by each temperature increment over the experimental period ($P < 0.001$). The HHA treatment had higher total DHA than the LHA treatment ($P < 0.001$), and there was an interaction effect of time x hippuric acid ($P < 0.001$), with the HHA treatment declining to a greater extent than the LHA treatment. An interaction between time x temperature x hippuric acid was seen ($P < 0.001$), with DHA decreasing with both time and temperature, and the higher DHA in the HHA treatment being longer-lasting at the lower temperatures. These effects were also reflected in significant interactions of time x temperature (Figure 7.8c) and temperature x hippuric acid (both $P < 0.001$). Dormaar et al. (1984) noted that enzyme assays measure potential activities, rather than reflecting the reality of the field situation, and this is further discussed in Section 7.4.3.

Dehydrogenase activity was positively correlated with bulk soil pH ($r \geq 0.88$, $P < 0.05$) and NH_4^+-N concentration ($r \geq 0.97$, $P < 0.01$) and was negatively correlated with NO_3^--N concentration ($r \leq -0.90$, $P < 0.05$), in both the hippuric acid treatments and in the 10–20°C temperature treatments. The same correlations in the 5°C treatment (r =0.81, 0.87 and -0.83 respectively) were only significant at the $P < 0.10$ level.

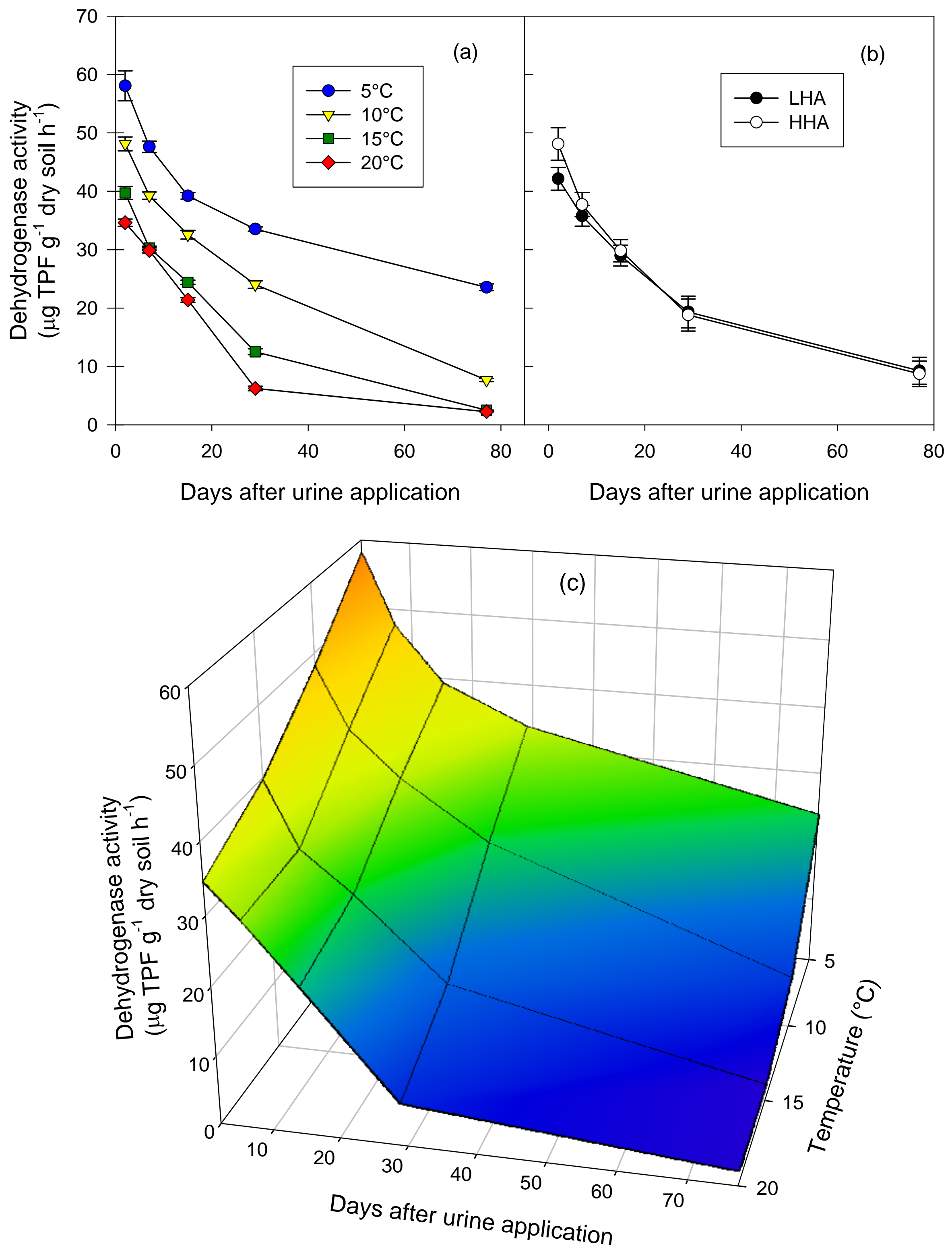


Figure 7.8 Dehydrogenase activity measured over time: (a) effect of temperature treatment (Error bars = SEM, n = 8), (b) effect of hippuric acid treatment (Error bars = SEM, n = 16), (c) interaction effect of time and temperature. (NOTE: Fig. 8c; y axis labels (temperatures) are 20–5°C.)

7.3.4 Headspace gas fluxes

Nitrous oxide fluxes were elevated on day 0, 8 h after urine application, in all treatments, and were highest in the 20°C treatment, where the mean concentration was > 1500 µg N_2O-N m^{-2} h^{-1} (Figures 7.9a, 7.9b & 7.10). Fluxes had reduced by day 1 and on days 2–14 were ≤ 100 µg N_2O-N m^{-2} h^{-1}. From day 14 onwards fluxes of N_2O-N were significantly higher from the 15 and 20°C treatments than from the 5 and 10°C treatments on all days ($P < 0.001$), but did not always differ from each other (Figure 7.9a). The increase in the soil moisture content on day 17 resulted in an immediate increase in N_2O-N fluxes at all temperatures, reaching > 11000 µg N_2O-N m^{-2} h^{-1} in the 20°C treatment (Figure 7.9a). Fluxes had declined by day 19, but then increased again in all treatments before the end of the experiment. After day 19 the highest mean fluxes in the 5, 10, 15 and 20°C treatments were 143, 156, 4987 and 7017 N_2O-N m^{-2} h^{-1} respectively.

Averaged over the experimental period, mean N_2O-N fluxes were highest from the 20°C treatment, followed by the 15°C treatment, with lower fluxes measured from the 5 and 10°C treatments, which did not differ from each other ($P < 0.001$). On days 0–3, N_2O-N fluxes were higher from the HHA treatment than from the LHA treatment ($P < 0.05$), while on day 8 fluxes were higher from the LHA treatment ($P < 0.05$) (Figure 7.9b), which was reflected in a significant time x hippuric acid interaction ($P < 0.001$). There were no differences between mean N_2O-N fluxes from the hippuric acid treatments when averaged over the experimental period. Time had a significant effect on N_2O-N fluxes ($P < 0.001$) and there was an interaction effect of time x temperature ($P < 0.001$), where fluxes increased with increasing temperature over time (Figure 7.10). There was also a temperature x hippuric acid interaction ($P < 0.01$), with the difference between HHA and LHA treatments being greater in the lower temperature treatments. Cumulative N_2O-N fluxes are discussed below.

The calculated Q_{10} values (temperature coefficients; Section 3.4.2.1) for N_2O-N production on days 20–49, were 56 for the 5–15°C temperature range, 150 for the 10–20°C temperature range, and 2.0 for the 15–20°C temperature range.

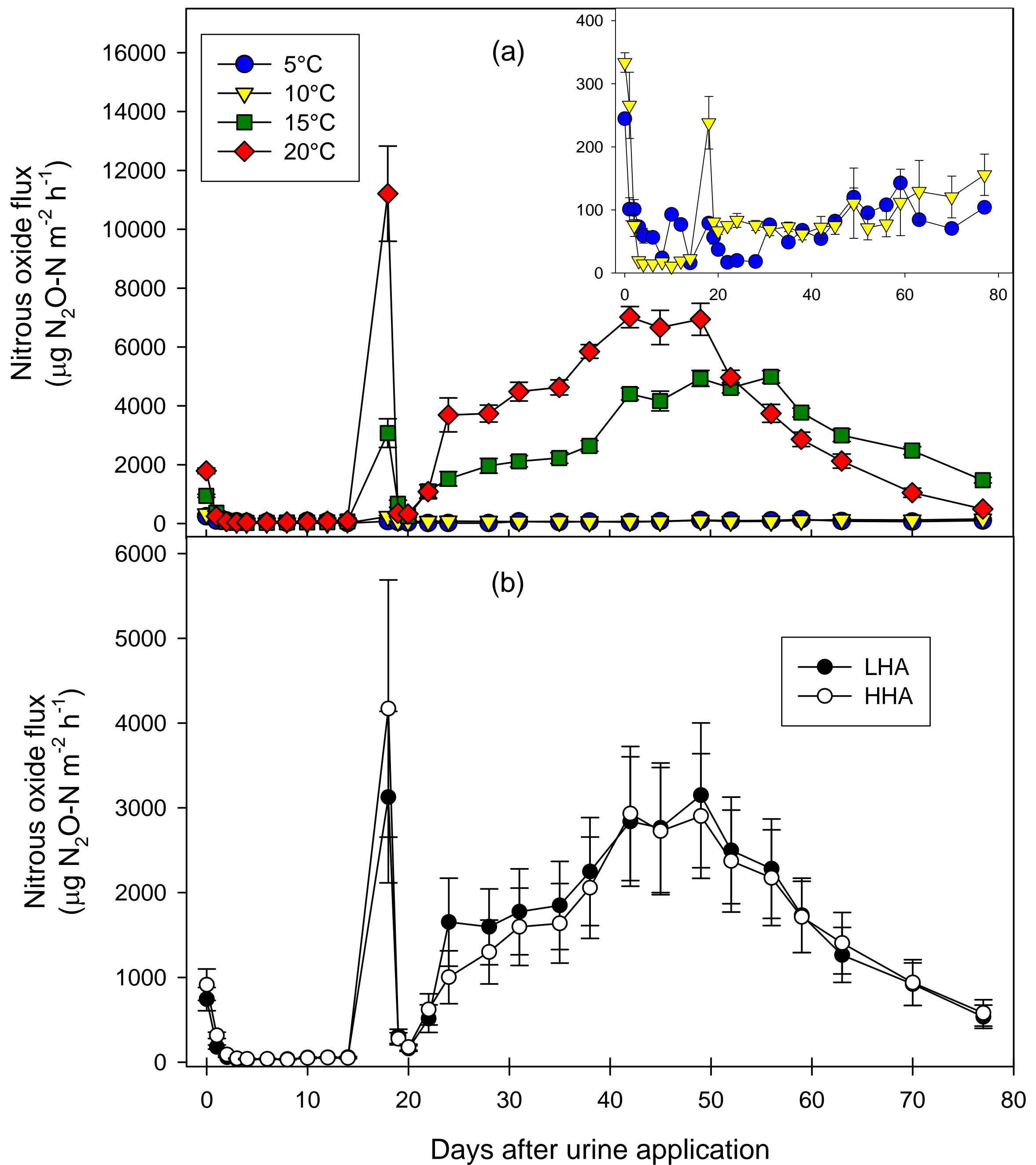


Figure 7.9 Nitrous oxide emissions measured from (a) all temperature treatments, with (inset) 5°C and 10°C temperature treatments only, and (b) hippuric acid treatments (Error bars = SEM, n = 8 (a) or 16 (b)). <u>NOTE</u>: differing scales on y axes.

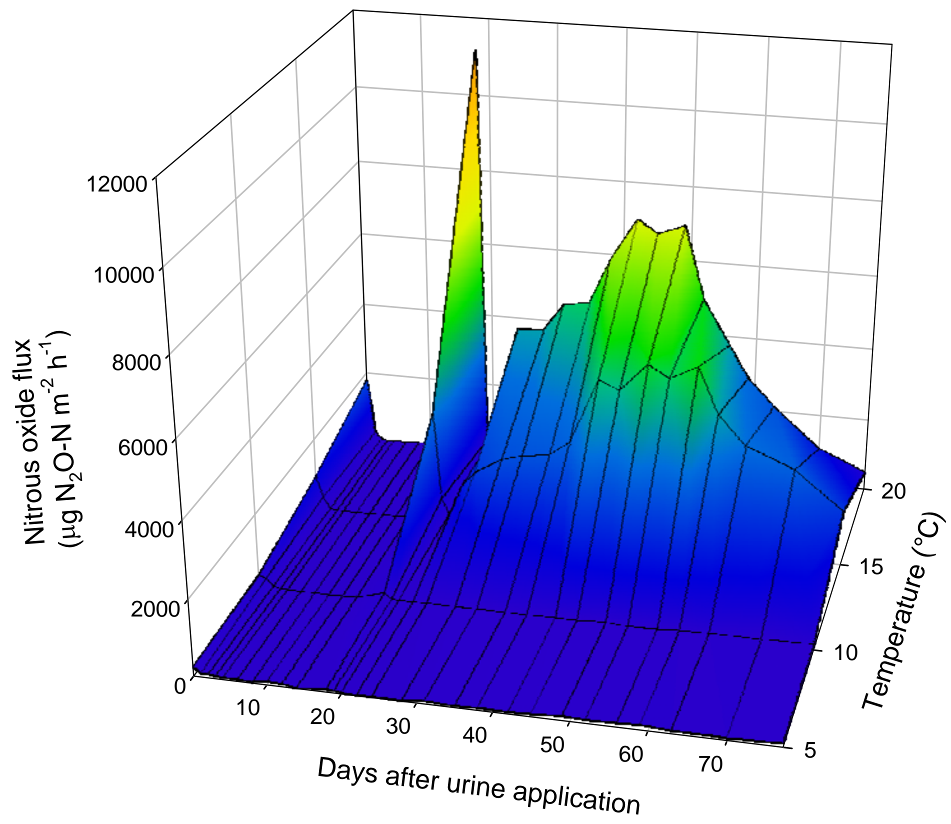


Figure 7.10 Effect of interaction of time and temperature on nitrous oxide fluxes. (<u>NOTE</u>: y axis labels (temperatures) are 5–20°C.)

Ammonia emissions peaked on day 1 in the 10, 15 and 20°C treatments at 25280, 48630, and 43240 μg m^{-2} h^{-1} respectively, while the highest flux of 39200 μg m^{-2} h^{-1} from the 5°C treatment was measured on day 4 (Figures 7.11a & 7.12). After the early peak, concentrations decreased in all treatments, but increased briefly on day 18 when the soil moisture content was increased, before declining to < 4000 μg m^{-2} h^{-1} by day 20. Emissions of NH_3-N were significantly higher from the 5°C treatment than from the other treatments on 8 out of 17 sampling occasions (P < 0.01). Ammonia emissions from the 15 and 20°C treatments differed only on day 3, when the 15°C treatment had higher emissions (P < 0.01). Emissions from the 15 and 20°C treatments were lower than from the 5 and 10°C treatments from day 4 onwards, and this was significant on days 8, 20, 22, 28, 31 and 38 (P < 0.001). The exception to this was day 18, after the soil moisture adjustment, when emissions increased with increasing temperature. Averaged across the sampling period, NH_3-N emissions were higher from the 5°C treatment than from the other temperature treatments (P < 0.001).

The hippuric acid treatments had a significant effect on NH_3-N emissions only on days 22 and 38, when emissions from the LHA treatment were higher (P < 0.05) and on day 24, when emissions from the HHA treatment were higher (P < 0.05) (Figure 7.11b). There was a significant interaction of time x temperature (P < 0.001) (Figure 7.12), with NH_3-N emissions declining faster and to a greater extent as the temperature increased. There was a significant interaction between temperature x hippuric acid (P < 0.01), with NH_3-N emissions differing between hippuric acid treatments in the 5 and 10°C treatments but not in the 15 and 20°C treatments. An interaction between time x temperature x hippuric acid was also seen (P < 0.001), where NH_3-N emissions were higher in the HHA treatment early in the experiment, and declined with time and increasing temperature. In all the temperature treatments, there were no strong correlations between NH_3-N and N_2O-N, but NH_3-N was positively correlated with the surface soil pH ($r \geq 0.60$, P < 0.05). Although NH_3-N emissions were also positively correlated with the bulk soil pH ($r \geq 0.83$) in all treatments, this was significant only at 10°C (P < 0.05). Cumulative NH_3-N losses are discussed below.

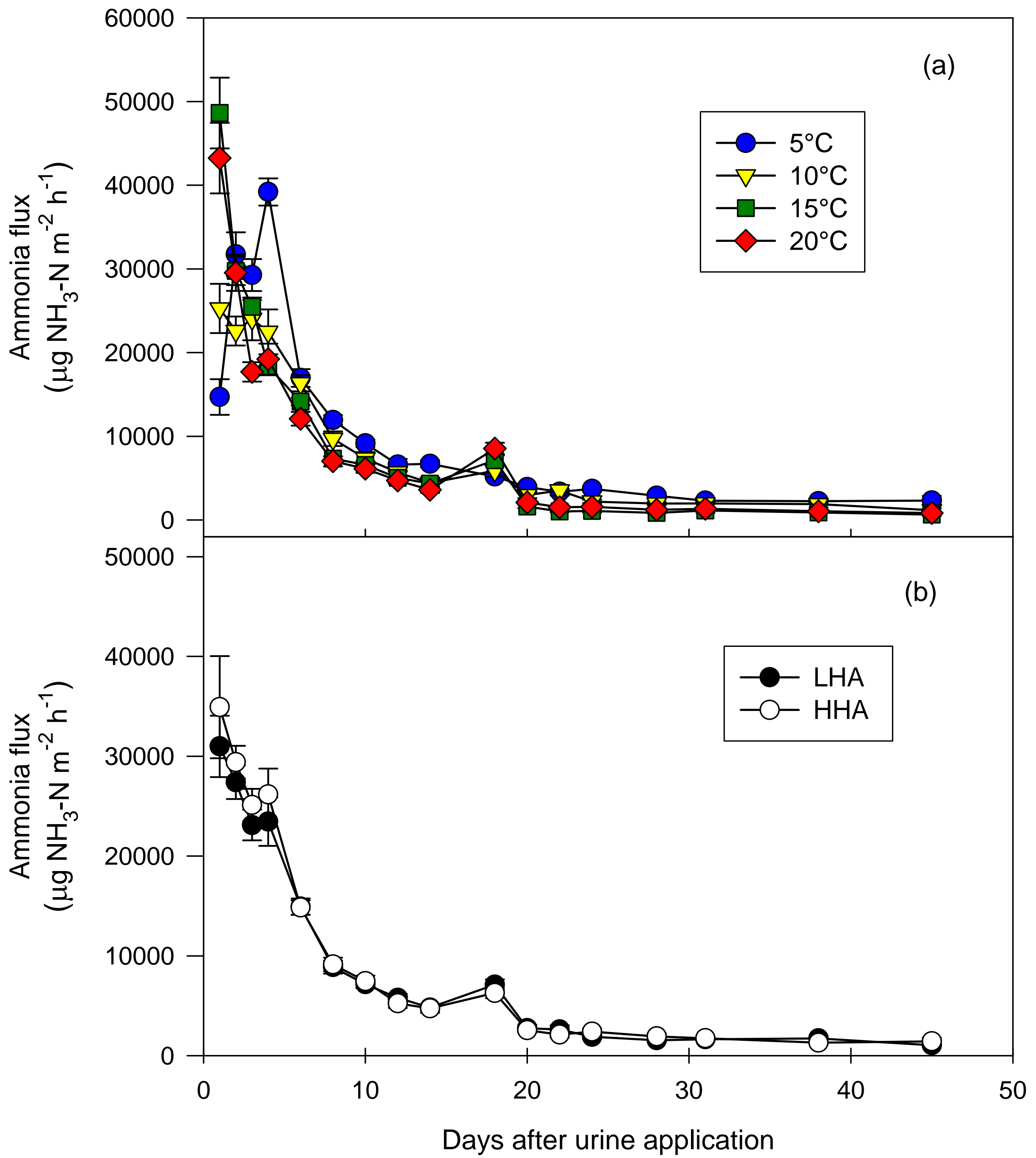


Figure 7.11 Ammonia emissions from (a) all temperature treatments, and (b) hippuric acid treatments (Error bars = SEM, n = 8 (a) or 16 (b)). NOTE: differing scales on y axes.

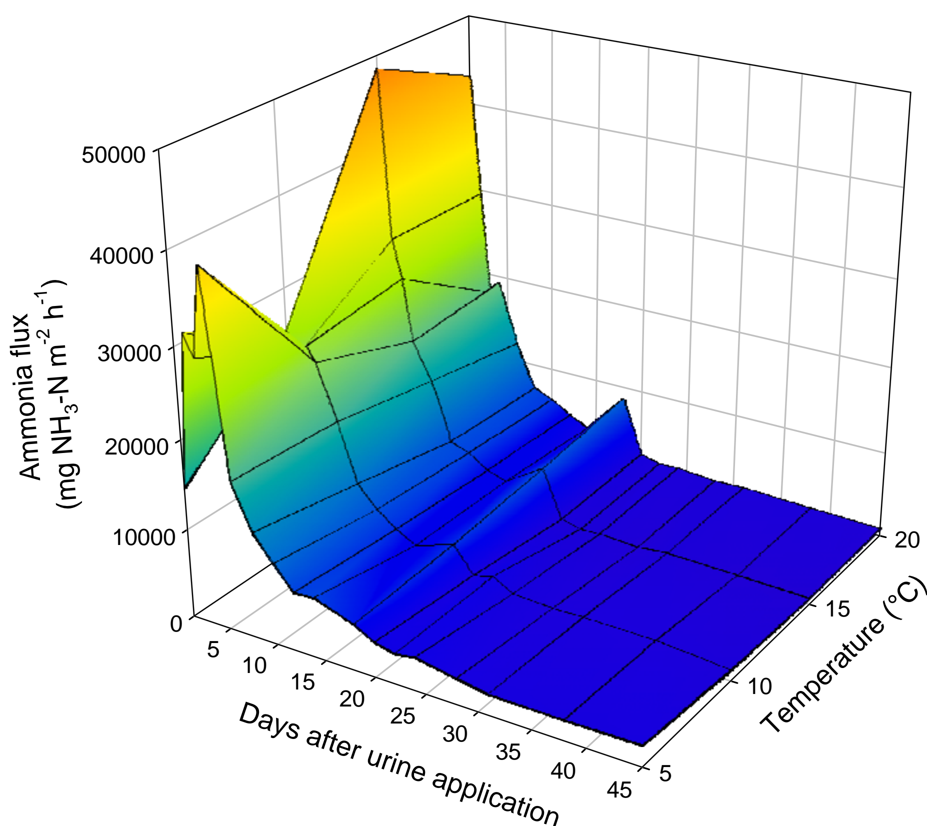


Figure 7.12 Effect of interaction of time and temperature on ammonia emissions. (NOTE: y axis labels (temperatures) are 5–20°C.)

Cumulative gaseous N losses over the experimental period differed due to temperature but not due to hippuric acid rate (Table 7.2). Cumulative N_2O-N fluxes from the 20°C treatment were significantly higher than those from the 15°C treatment, while fluxes from the 5 and 10°C treatments did not differ from each other but were significantly lower than those from the 15 and 20°C treatments ($P < 0.001$). Ammonia-N losses showed the opposite trend, being lowest in the 20°C treatment, and increasing as the temperature decreased, although the difference was significant only between the 5°C treatment and the other temperature treatments ($P < 0.001$). Total N loss was lowest in the 10°C treatment (9.8% total N applied) because both NH_3-N and N_2O-N losses were low at this temperature. The highest overall loss (17.5% total N applied) was from the 20°C treatment. Nitrogen losses from the two hippuric acid treatments did not differ for either N_2O-N or NH_3-N, and there was also no difference when the loss was calculated as a percent of total N applied, to account for the different N rates used (Section 7.2.2).

Table 7.2 Cumulative emissions of N_2O-N (to day 77) and NH_3-N (to day 45) as total N emitted (mg) and percent N applied. Means followed by same letter in each column do not differ significantly.

Treatment	Mean cumulative N emitted per core					
	mg N			% total N applied		
Temperature	N_2O-N	NH_3-N	Total#	N_2O-N	NH_3-N	Total#
5°C	0.31 c	18.18 a	18.48 c	0.20 c	11.76 a	11.96 c
10°C	0.37 c	14.75 b	15.12 d	0.24 c	9.56 b	9.80 d
15°C	9.96 b	14.17 b	24.13 b	6.45 b	9.18 b	15.63 b
20°C	13.36 a	13.67 b	27.03 a	8.67 a	8.84 b	17.51 a
significance	***	***	***	***	***	***
Acid						
LHA	6.07	14.93	21.00	4.05	9.95	14.00
HHA	5.93	15.45	21.38	3.73	9.72	13.45
significance	NS	NS	NS	NS	NS	NS

*** $P < 0.001$, NS = not significant #Sum of N_2O-N + NH_3-N

7.4 Discussion

7.4.1 Effects of hippuric acid

In contrast to the results of experiment 3, there was no reduction in cumulative N_2O-N emissions due to hippuric acid amendment. This was unlikely to have been because the difference between the two hippuric acid concentrations was insufficient, as the variation between the acid treatments here (0.57 g HA-N L^{-1}) was similar to the treatment difference in experiment 3 (0.70 g HA-N L^{-1}) and to that used by Kool et al. (2006b) (0.63 g HA-N L^{-1}). However, the soil moisture contents used in this current experiment (47–62% WFPS) were lower than those used in either experiment 3 or in the studies of Kool et al. (2006b) and van Groenigen et al. (2006) (70–92% WFPS). This suggests that the inhibitory effect of hippuric acid (or benzoic acid) is highly dependent on the soil moisture content. It is possible that any benzoic acid produced by hippuric acid hydrolysis in this current study was rapidly degraded in the more aerobic soil conditions (Wibbertmann et al., 2005), before the pH had declined to a level where sufficient benzoic acid would be present in its toxic unionised form to induce inhibition. Alternatively, the results can be interpreted as indicating that nitrification, which was demonstrably occurring, as shown by the increasing NO_3^--N concentrations, was not inhibited by benzoic acid, despite the indications seen in Experiment 3 (Section 6.4.3).

Although cumulative N_2O-N emissions were unaffected by hippuric acid addition in this current study, there were some differences between N_2O-N emissions from the two hippuric acid

treatments on a daily basis early in the experiment, with fluxes initially higher from the HHA treatment than from the LHA treatment. In experiment 3, a significant inhibitory effect of hippuric acid was not seen until day 25, after the surface soil pH had declined to < 7.2. In this current experiment, the only day that N_2O-N fluxes were lower from the HHA treatments was day 8, when the surface and bulk soil pH in both hippuric acid treatments had declined to ≤ 7.1. This suggests that, despite benzoic acid having a pKa of 4.2 (Wibbertmann et al., 2005), there was sufficient benzoic acid unionised at about pH 7.0–7.2 to inhibit microbial activity. After day 8 no significant differences were measured between N_2O-N fluxes from the two acid treatments. Therefore there may have been a transient inhibitory effect of the high hippuric acid content when the pH began to decrease in the 15 and 20°C treatments, but with insufficient acid present at this stage to have a longer-lasting effect.

As mentioned above, it is possible that any benzoic acid produced was destroyed by aerobic benzoic acid degrading bacteria (Philippe et al., 2001; Gescher et al., 2002; Pumphrey and Madsen, 2008) due to the low moisture content of the soil. It is also possible that the amount of acid remaining by day 8 had been reduced, perhaps due to the presence of a larger population of benzoic acid degrading bacteria in the experiment 4 soil than in the experiment 3 soil, or perhaps the microbial population in the experiment 4 soil had been primed for benzoic acid degradation as a result of previous urine deposition in the field prior to soil collection. The latter is unlikely since stock were excluded from the site for several weeks prior to soil collection and visible urine patches were avoided. Alternatively, the hippuric acid may have become bound to organic matter and thus inactivated (Cecchi et al., 2004; Inderjit and Bhowmilk, 2004), although this does not explain the lack of inhibition in this experiment compared to experiment 3. The higher DHA in the HHA treatment on days 2 and 7 may have been caused by the higher inputs of N and C in these treatments due to the additional hippuric acid. A stimulatory effect of acid addition on DHA was also seen in experiment 3 (Section 6.3.5), suggesting that some of the added acid in that experiment was being used as a substrate by the microbial community. The differences between the HHA and LHA treatments within each temperature treatment in this current experiment are discussed in Section 7.4.3.

As with experiment 3, urinary hippuric acid content had no effect on cumulative NH_3-N emissions, and unlike the N_2O-N emissions, NH_3-N emissions did not differ early in the experiment. This is because NH_3-N loss is a physicochemical process and is not directly dependent on microbial activity rates. Since the two hippuric acid treatments alternated in producing the higher concentration of NH_3-N, there was no clear overall effect of the hippuric acid rate. The lack of any difference between either of the nitrogenous gases measured meant

that the total N loss (NH_3-N + N_2O-N) did not differ over the experimental period as a result of urinary hippuric acid amendment.

The higher NH_4^+-N concentrations in the HHA treatments at all destructive soil sampling times could have been a consequence of the higher N concentrations in these treatments due to the additional hippuric acid, through deamination of the conjugated glycine (Bristow et al., 1992), although again this effect was not previously observed in experiment 3. Since an increase in hippuric acid concentrations has previously been seen to enhance urea hydrolysis (Doak, 1952), the higher soil NH_4^+-N concentration may instead have been a result of this effect. An increase in NH_4^+-N concentrations can lead to enhanced emissions of NH_3-N, as observed by Whitehead et al. (1989) when urinary hippuric acid concentrations were increased from 0–5% of urea-N. However, as discussed above, NH_3-N emissions were not enhanced by increasing the hippuric acid concentration in this current study from 4.9% up to 9.9% of the total urinary N, similar to the results of experiment 3. This may have been due to the same process noted by Whitehead et al. (1989), whereby the highest concentration of hippuric acid used (5% of total urea-N) caused a reduction in cumulative NH_3-N emissions compared with hippuric acid concentrations of 0.5–2.5% of total urea-N.

The higher net rates of NO_3^--N production in the LHA treatment on days 0–2 resulted in higher concentrations of NO_3^--N in the LHA treatment than in the HHA treatment on the first two sampling days. It could be postulated that this difference was due to inhibition of nitrification in the HHA treatment, as was seen in experiment 3 (Section 6.4.3). However, at the high soil pH present at this stage of the experiment, very little of the benzoic acid present in the soil would have been in the toxic unionised form (Chipley, 1983; Brul and Coote, 1999). The inhibition may instead have been due to a combination of the higher concentrations of NH_3-N and NO_2^--N in the HHA treatments at this time. Although not always significant, concentrations of NH_4^+-N were higher in the HHA treatment than the LHA treatment throughout the experiment, and in conjunction with the initial high pHs, would produce high NH_3-N concentrations, which can inhibit NO_2^--N oxidation (Villaverde et al., 1997). This is supported by the higher fluxes of NH_3-N from the HHA treatments on days 1–4. Any differences in NH_3-N concentrations between the two acid treatments could not have been a function of soil pH, as there were no measurable differences between the HHA and LHA treatments. Inhibition of the nitrite-oxidising bacteria (NOB) by NH_3-N was also indicated by the significantly higher concentrations of NO_2^--N in the HHA treatment on day 2, which could have further inhibited NO_2^--N oxidation. Inhibition of nitrifying bacteria due to elevated NO_2^--N concentrations has previously been measured at $pH < 7.5$ (Hunik et al., 1992, 1993), corresponding to the bulk soil

pH measured here on day 2. After day 2 there was no further evidence of inhibition of nitrification by hippuric acid, as net rates of NO_3^--N production and net rates of NH_4^+-N depletion did not differ. The higher net NO_3^--N production rates in the LHA treatment on days 0–2 were coincident with lower net rates of NH_4^+-N production. This may have been due to either lower actual rates of NH_4^+-N production or to higher rates of NH_4^+-N depletion in this treatment, some of which would have undergone nitrification. However, at this stage in the experiment, N_2O-N emissions were lower from the LHA treatment, so the higher rates of NO_3^--N production were not enhancing production of N_2O-N.

7.4.2 Effects of temperature

Although urease activity, and thus urea hydrolysis, increases measurably with increasing temperature (e.g. Dalal, 1975; Vlek and Carter, 1983; Moyo et al., 1989), this was not apparent either in the rates of NH_4^+-N production on days 0–2 or in the concentrations of NH_4^+-N on day 2. Hydrolysis of urea can be very rapid, even at low temperatures (Holland and During, 1977), so that any rate differences due to temperature treatment may not have been detectable by the first destructive sampling at day 2, by which time nearly all of the N applied was in the form of NH_4^+-N in all the temperature treatments. Agehara and Warncke (2005) found that different incubation temperatures affected the rate of urea hydrolysis, but did not affect the net amount of N mineralised, although, unlike this current study, their experiment showed rate differences two weeks after urea application. The incubation temperatures used in the current experiment are unlikely to have resulted in urease destruction, as urease activity is stable at temperatures up to 60°C (Zantua and Bremner, 1977). Optimal urease activity may be acclimatised to the site of origin of the soil (Agehara and Warncke, 2005), so the soil used in this experiment, exposed to annual temperatures of 1–22°C (NIWA, 2009) (Figure 7.1), would contain urease that was active across all the experimental temperatures used.

Nitrification rates are also temperature dependent (Section 2.5.1.3), as evidenced by the increasing production of NO_3^--N on days 0–29 as the temperature increased from 5 to 20°C, an effect also observed in other studies (Maag and Vinther, 1996; Russell et al., 2002). The higher concentrations of NH_4^+-N in the 5 and 10°C treatments on day 2 (and subsequently) were largely a consequence of the higher nitrification rates, and thus faster depletion of NH_4^+-N, in the 15 and 20°C treatments. This higher rate of nitrification in the higher temperature treatments was maintained until day 29, after which time the supply of NH_4^+-N became exhausted and production of NO_3^--N became higher in the 10°C treatment between days 29 and 77. Higher denitrification rates in the 20°C treatment may also have contributed to the reduction in NO_3^--N. However, although the pattern of higher rates of change in the high temperature treatments was

seen in both the rates of net NH_4^+-N depletion and net NO_3^--N production, the amounts of N depleted and produced were not equivalent. By measuring only net production rates, any NO_3^--N denitrified would not be accounted for. Some of the NH_4^+-N may also have been assimilated into the microbial biomass.

The higher concentrations of NO_2^--N in the 5 and 10°C treatments on day 2 could have been due to slower activation of NOB than of ammonia-oxidising bacteria (AOB) or to a greater degree of inhibition of NOB during nitrification at the low temperatures. The latter is more probable, as NO_3^--N production in the low temperature treatments continued to lag behind production in the high temperature treatments, as has been noted in previous studies (Justice and Smith, 1962; Russell et al., 2002). Accumulation and persistence of NO_2^--N has also been measured at low temperatures (< 12°C) in other experiments (Tyler and Broadbent, 1960; Justice and Smith, 1962; Russell et al., 2002), and although concentrations were lower in this current study, the cause of the accumulation was potentially the same.

The large NO_2^--N peak in the 20°C treatment on day 29 was probably the result of the stimulation of denitrification caused by the increase in soil moisture content (to 62% WFPS) on day 17. The previous low soil moisture content (47% WFPS) would have suited nitrification, with denitrification limited to anaerobic microsites, resulting in the low N_2O-N emissions measured until day 14 (Figure 7.9) (Wrage et al., 2004; Bateman and Baggs, 2005). The peak N_2O-N flux at day 18 indicated that denitrification had been stimulated by the onset of anaerobic conditions, and suggested that there was inadequate N_2O-N reductase present to immediately reduce all the N_2O-N generated (Firestone and Tiedje, 1979; Dendooven and Anderson, 1994). The ensuing decline in N_2O-N may have been due to a higher N_2-N/ N_2O-N ratio, which is known to increase as the soil moisture content rises (Maag and Vinther, 1996). A N_2O-N spike was also measured at the start of experiment 1, following urine application, and this phenomenon has been observed previously in both field and laboratory studies when anaerobic conditions are imposed (Firestone, 1982; Sherlock and Goh, 1983). After day 20, the increased production of N_2O-N in the 15 and 20°C treatments was an indication that denitrification was occurring in these treatments, and the high concentration of NO_2^--N in the 20°C treatment on day 29 may possibly have been caused by a lag in the synthesis of NO_2^--N reductase. Synthesis of *de novo* NO_2^--N reductase usually starts 3–6 h after the commencement of anaerobic conditions (Smith and Tiedje, 1979; Dendooven and Anderson, 1994), but rapid denitrification of NO_3^--N may have created a large pool of NO_2^--N (still apparent by day 29), that required more time to be further denitrified.

It is also possible that inhibition of nitrification contributed to or was the cause of the NO_2^--N peak. Accumulation of NO_2^--N at 20–40 days after urine application was also measured by Monaghan and Barraclough (1992), who conjectured that the cause was inhibition of NOB by high pH and high NH_4^+-N concentrations. However, this was unlikely to be the case in this current study, as both pH and NH_4^+-N concentration were lower in the 20°C treatment than in the other temperature treatments. Nitrite accumulation in batch reactor systems was believed to be a result of insufficient C for complete denitrification to take place, so that the NO_2^--N produced could not be further denitrified (Her and Huang, 1995). In this present experiment, the DHA was lowest in the 20°C treatment on day 29, so it is plausible that the available soil C substrate had been consumed, and that incomplete denitrification was the cause of the NO_2^--N accumulation. If this was the case, the continued production of N_2O-N may indicate that autotrophic nitrifying bacteria were reducing the accumulated NO_2^--N to N_2O-N via nitrifier denitrification at this time (Wrage et al., 2001). Alternatively, NOB may have been outnumbered by AOB in the 20°C soil, creating the accumulation of NO_2^--N that was measured (Burns et al., 1995); however, this would only be likely if the substrate was unevenly distributed (Monreal et al., 1986).

The change in bulk soil and surface soil pH over the course of the experiment closely followed the changes in NH_4^+-N concentrations, as shown by the strong correlations between the bulk soil pH and NH_4^+-N in the 10, 15 and 20°C treatments. The extended elevation of bulk soil and surface soil pH in the lower temperatures reflected the lower rates of nitrification in the 5 and 10°C treatments. The higher rate of NO_3^--N production in the 10°C treatment on days 29–77 corresponded with the declining pH in the 10°C treatment at that time, so that the 10°C pH had declined to levels close to the 15 and 20°C treatments by the end of the experiment while the pH in the 5°C treatment remained elevated. The surface pH would be expected to be higher than that of the bulk soil if the urine had not leached very far, because concentrations of urea would then be higher at the surface so that urea hydrolysis, with its resulting increase in pH, would also be higher. The methods of measurement of pH may have contributed to the differences between surface soil and bulk soil pH, with the soil surface probe measuring only a small area, while the bulk soil method required 10 g of soil, in which the applied urine may not have been uniformly mixed.

As noted above, increasing temperatures have previously been shown to increase both nitrification (e.g. Malhi and McGill, 1982; Russell et al., 2002; Di and Cameron, 2004) and denitrification (e.g. Keeney et al., 1979; George and Antoine, 1982; Maag and Vinther, 1996). This might be expected to result in higher emissions of N_2O-N, as was observed in this

experiment, with the 15 and 20°C treatments producing higher N_2O-N emissions than the 5 and 10°C treatments. However, Maag and Vinther (1996) found that N_2O-N emissions from nitrification decreased with increasing temperature, which they attributed to an accumulation of NO_2^--N at low temperatures, an effect that was not seen in this current experiment. Studies have also shown that the ratio of N_2-N to N_2O-N produced by denitrification increases with increasing temperature between 5 and 25°C (Nõmmik, 1956; Bailey, 1976; Maag and Vinther, 1996), although at temperatures close to 0°C severe inhibition of N_2O-N reductase may result in enhanced emissions of N_2O-N (Holtan-Hartwig et al., 2002). Thus, in this experiment, the calculated proportion of applied N emitted as gaseous N during denitrification would probably have been even higher in the high temperature treatments compared with the low temperature treatments if N_2-N losses had also been measured.

The relatively high fluxes of N_2O-N measured in the high temperature treatments in this experiment would be unlikely to occur in a pasture situation (Section 2.5.1.5) due to removal of NH_4^+-N and NO_3^--N via macropore flow, leaching, and plant uptake (Section 2.3.1). Cumulative N_2O-N emissions did not differ between the 5 and 10°C treatments, and the very low N_2O-N emissions from these treatments, at the same time that N_2O-N fluxes increased in the 15 and 20°C treatments, suggests that denitrification was severely inhibited at the low temperatures. This is supported by the findings of Stanford et al. (1975) and George and Antoine (1982), who measured very little denitrification at either 5 or 10°C.

With denitrification apparently very low at 5 and 10°C, the high Q_{10} values calculated for the 5–15°C and 10–20°C temperature ranges (> 50) may have been the result of N_2O-N production via denitrification in the 15 and 20°C treatments being compared with N_2O-N production via nitrification in the 5 and 10°C treatments. The Q_{10} calculation is used to measure the change imposed on a biological process by a change in temperature, not to measure differences between two separate processes. Therefore, the Q_{10} for the 15–20°C temperature range, comparing two systems where denitrification was high, was more comparable to that calculated by Stanford et al. (1975) for denitrification between 15 and 35°C, measured via changes in NH_4^+-N and NO_3^--N concentrations. The value measured here (2.0) indicated that a 10°C temperature increase would double the denitrification rate.

The decrease in NH_3-N emissions with increasing temperature was in contrast to previous research, where increases in NH_3-N emissions with increasing temperature have been reported (e.g. Doak, 1952; Whitehead and Raistrick, 1991), although Holland and During (1977) measured no differences. The increased NH_3-N emissions in the low temperature treatments in

this current experiment were probably due to the higher concentrations of NH_4^+-N (Figure 7.5a), because of lower net rates of nitrification, as suggested by Whitehead and Raistrick (1991). The continued presence of high concentrations of NH_4^+-N and elevated pH at low temperatures could further inhibit nitrification, via high concentrations of free NH_3-N (Section 2.5.1.3).

7.4.3 Dehydrogenase activity

In contrast to the nitrification rates (net NH_4^+-N depletion and NO_3^--N formation), the DHA rates were highest in the 5°C treatment and decreased with increasing temperature. This was possibly due to the DHA results being confounded by C substrate availability at the different temperatures.

The DHA assay measures the rate of biological transformation of triphenyl tetrazolium chloride (TTC) into triphenyl formazan (TPF). The TTC is used as an electron acceptor in the oxidation of organic compounds by heterotrophic microorganisms, which is catalysed by dehydrogenase enzymes (Tabatabai, 1982; Alef, 1995). Thus, a higher level of microbial activity requires greater amounts of organic substrate. In previous studies, the measured DHA has increased with increasing temperature (e.g. Gong, 1997; Brzezińska et al., 1998), so that C depletion would be expected to be faster at higher temperatures, in agreement with the findings of Cookson et al. (2007).

If, as expected, rates of DHA were lower at the lower experimental incubation temperatures in this current study, there would be higher concentrations of remaining C substrates in these treatments, since there were no ongoing nutrient inputs by plants. Thus the heightened microbial activity at the assay temperature of 30°C would be enhanced by the greater availability of C in the low temperature treatments. Therefore, rather than being a measure of actual microbial activity under the experimental conditions, the DHA results indicated the potential enzyme activity, as suggested by Dormaar et al. (1984).

This is the same effect as that discussed by Kirschbaum (2006), when investigating the impact of temperature on rates of SOM degradation. Using previous studies as examples, Kirschbaum (2006) illustrated how the availability of labile substrates can decrease rapidly, even after short incubation periods. In a study by Koepf (1953) pre-incubation of the soil at an elevated temperature resulted in a lower flux of CO_2-C than when the pre-incubation temperature and the experimental temperature were the same. This was due to enhanced depletion of the C substrate at the higher temperatures. Similarly, Nicolardot et al. (1994) found that an added substrate

declined faster as the temperature increased. Kirschbaum (2006) concluded that, when different incubation temperatures are being used, the potential for changes in substrate availability must also be considered.

In this current experiment, the faster depletion of substrate in the higher temperature treatments resulted in lower DHA when these soils were assayed at 30°C. Thus the assay was not measuring actual DHA under experimental conditions, but rather was providing a measure of the supply of organic C remaining in the soil

This indicates that microbial activity actually increased with increasing incubation temperature. The effect would have been exacerbated by the one week equilibration period at experimental temperatures prior to urine application. This would have encouraged higher microbial activity in the high temperature treatments during this period, so that soil C concentrations would already have been lower in these treatments by the time urine was applied.

The positive correlation between DHA and available C, which was noted in all the previous experiments (Chapters 4–6), emphasises the importance of C availability to microbial activity. However, it also implies that there should be a caveat on this method, which is that the method is not suitable when comparing different temperature treatments, or that, at least, the soil C availability must also be taken into account.

In this present study, the slower rates of DHA at the lower incubation temperatures also explain the differences in DHA seen between the HHA and LHA treatments within each temperature treatment (Section 7.3.3). As the temperature increased the stimulatory effect of the HHA treatment was reduced. At 15, 10 and 5°C the HHA had a stimulatory effect on DHA at one, two or three samplings times, respectively, after urine application, but had no effect at 20°C. This indicates that utilisation of the additional hippuric acid was delayed at the low temperatures, and so the additional substrate was still present at a high enough concentration in the 5°C treatment to stimulate DHA on day 15. Thus, to reiterate, the assay was not measuring actual DHA under experimental conditions, but rather was providing a measure of the supply of organic C remaining in the soil.

7.5 Discussion summary

- Over the course of the experiment there was no consistent inhibitory effect of hippuric acid on N_2O-N emissions, possibly due to rapid degradation of benzoic acid in the aerobic soil conditions.

- There was no difference in cumulative NH_3-N emissions due to hippuric acid treatment.

- Cumulative N_2O-N emissions were higher from the 15 and 20°C treatments than from the 5 and 10°C treatments, probably because of lower rates of denitrification at the lower temperatures.

- Total gaseous N losses (NH_3-N + N_2O-N) were higher from the 15 and 20°C treatments than from the 5 and 10°C treatments.

- Stimulation of microbial processes occurred in the high hippuric acid treatment, probably due to the acid being utilised as a substrate.

- The stimulatory effects of the enhanced hippuric acid concentration included higher mean concentrations of NH_4^+-N, and NO_2^--N and lower concentrations of NO_3^--N on days 2 and 7 in the HHA treatments.

- Concentrations and rates of change in NH_4^+-N and NO_3^--N were generally higher in the high temperature treatments and reduced with temperature.

- Although the DHA results appeared to indicate higher enzyme activity at lower temperatures, this was actually a reflection of the faster depletion of organic substrates in the high temperature incubations, and demonstrated that DHA decreased as the temperature decreased.

- Due to the DHA declining as the temperature increased, the stimulatory effect of the additional hippuric acid-C on DHA was extended as the temperature decreased.

7.6 Conclusions

Hypothesis: That hippuric acid inhibition of N_2O-N emissions would diminish as soil temperatures increased, due to enhanced microbial activity at higher temperatures.

The hypothesis was not proven, as there was no evidence of an effect on cumulative N_2O-N emissions. This was unexpected, but might be explained by the aerobic soil conditions being more conducive to benzoic acid degradation than the more anaerobic conditions present in previous studies. There was some evidence for this, with the higher initial N_2O-N emissions and DHA activity in the HHA treatment suggesting that the added hippuric acid was being used as an additional substrate by the microbes, in the absence of rhizodeposits by plants. There was no

indication that either nitrification or denitrification were inhibited by the increased urinary hippuric acid concentration, and no apparent effect of hippuric acid at any temperature.

However, there was support for the second part of the hypothesis, that microbial activity would be reduced at lower temperatures. The higher potential DHA in the low temperature soils showed that higher rates of DHA had occurred in the high temperature soils. There was also evidence for higher rates of both nitrification and denitrification at higher temperatures, as shown by the faster NH_4^+-N and NO_3^--N transformations, and by the greater amounts of N_2O-N produced in the 15 and 20°C treatments. Although urease activity (which is both an intra- and extra-cellular process) appeared to be higher in the 5 and 10°C treatments on days 0–2, this effect was due to higher rates of nitrification in the 15 and 20°C treatments, resulting in faster depletion of the NH_4^+-N produced.

Future research

- Effects of hippuric acid and/or benzoic acid on N_2O-N emissions at a range of soil moisture contents, and including measurements of the acids in the soil to determine how rapidly they are degraded.
- Use of isotopically labelled substrate to trace the fate of added hippuric acid and/or benzoic acid.
- Influence of plants on the degradation and fate of added hippuric acid and/or benzoic acid, because all experiments to date have used bare soil.
- Investigation of the effects of hippuric acid in a range of soil types.

Chapter 8
General discussion, conclusions and future research recommendations

8.1 Introduction

In this chapter the major findings of all the experiments carried out during this study are brought together and discussed in the context of previous work. In Section 8.2 the overall results of the study are discussed, and in Section 8.3 conclusions are drawn from these results. Section 8.4 lists recommendations for future research priorities, based on the work in these experiments.

8.2 General discussion

8.2.1 Assessment of effects of cow urine on soil nutrients

8.2.1.1 Release of existing soil nutrients due to urine application

The few previous studies into soil nutrient release after urine application focused on organic C, N and P (Shand et al., 2000; Shand et al., 2002). Extant soil nutrients may be contained on soil exchange sites and in SOM, including the microbial biomass, as well as in the soil solution or more permanently bound in soil components (Stevenson and Cole, 1999). The increased concentrations of trace elements after urine application in experiment 2 (Chapter 5) were probably released from SOM dissolved in the high pH soil environment formed by urea hydrolysis (Haynes and Sherlock, 1986; Jarvis and Pain, 1990). Soil organic matter was the most likely source of these elements because (1) urinary concentrations of trace elements are generally low, (2) all the measured elements followed the same trends, and (3) Cu was less responsive, as would be expected by its stronger binding to SOM. Since trace elements are required by both plants and microbes, the release of these elements by urine application could be of consequence. However, no previous research on trace element dynamics in cow urine patches could be found. The additional PO_4^{3-}-P measured after urine application during experiment 1 (Chapter 4) was more likely to have come from lysed microbial cells than from the SOM, with accumulation only possible because plants were absent. When plants were present, in experiment 2 (Chapter 5), there was no accumulation of P and in fact soil DRP was reduced in the urine affected soil. However, the decline in the microbial biomass in experiments 1 and 3 (Chapters 4 and 6), without plants, indicated that inputs of plant nutrients are necessary in order for soil microbes to flourish.

8.2.1.2 Urinary components in soil

As in earlier studies, the concentrations of nutrients measured in the soil immediately after urine application in these experiments corresponded well with those previously measured in bovine urine (e.g. Haynes and Williams, 1992; Williams and Haynes, 1994). The early appearance of NH_4^+-N in these experiments and previously (e.g. Sherlock and Goh, 1984; Petersen et al., 2004b) was due to hydrolysis of the urinary urea (Doak, 1952; Holland and During, 1977). As nitrification progressed, concentrations of NH_4^+-N declined while NO_3^--N increased. This occurred to a greater extent at temperatures > 10°C, in wet soils, and when N application rates were high. The relationship between soil NH_4^+-N concentrations and NH_3-N fluxes is considered below (Section 8.2.1.3), and accumulation of soil NO_2^--N is discussed in Section 8.2.2.1. The soil pH was positively correlated with NH_4^+-N concentration, as expected from Equation 2.1. There was apparently little lateral movement of urine through the soil, in agreement with previous work (Monaghan et al., 1999).

8.2.1.3 Fate of added and released nutrients

Use of ^{15}N (Chapters 4 and 6) allowed the progression of the added urea-N to be traced through NH_4^+-N, NO_2^--N and NO_3^--N to the emitted N_2O-N. Since N_2-N / N_2O-N ratios increase with increasing temperature (Nõmmik, 1956; Maag and Vinther, 1996), the proportion of applied N emitted during the 15–20°C incubations would have been much higher than was indicated by measuring the N_2O-N flux alone. Nitrogen was also emitted as NH_3-N and, unlike previous studies (Doak, 1952; Whitehead and Raistrick, 1991), fluxes were higher at low temperatures. This was probably because nitrification was slower at the low temperatures so that NH_4^+-N concentrations and the soil pH remained elevated for longer. However, total gaseous N emissions were still higher at 15–20°C than at 5–10°C.

In addition to reducing nitrification rates, incubation at 5 or 10°C also reduced denitrification rates, so that N_2O-N emissions were lower at these temperatures. However, denitrification was stimulated by increasing the soil moisture content, and production of NO_3^--N and N_2O-N was higher at 70% WFPS than at 35% WFPS. This was anticipated (Bremner and Shaw, 1958; Bateman and Baggs, 2005), but the higher rate of nitrification seen at 70% WFPS compared with 35% WFPS was unexpected, as nitrification is generally optimal at lower soil moisture contents (Bateman and Baggs, 2005). However, these findings supported the assertion of Schjønning et al. (2003) that WFPS is not the best predictor of nitrification rates.

Apart from gaseous emissions of N, plant uptake of soil nutrients also occurs in the urine patch. Higher concentrations of N and K^+ in plants growing in urine patches have been measured in

several studies (Ledgard et al., 1982; Fraser et al., 1994). In experiment 2 (Chapter 5), the observed increase in plant biomass in the urine patch indicated that plant nutrient uptake had risen, and the increase in total PLFAs showed that microbial assimilation of nutrients had also increased. The plants and microbes present would also have utilised the released trace elements, Cu, Fe and Mo, which are required for metabolic processes, including enzyme synthesis (Römheld and Marschner, 1991; Zumft, 1997). Leaching of nutrients may also have contributed to the decline in soil nutrients, including N; however, leaching losses were not quantified during this study.

Plant P concentrations may be higher, lower or unchanged by urine application (Ledgard et al., 1982; Williams et al., 1999a; Shand et al., 2002), and in experiment 2 (Chapter 5) the reduced soil DRP in the urine patch was matched by a reduction in plant P concentrations. However, the higher total PLFAs in the urine treatments indicated that the P had been assimilated by soil microbes. The lower plant P in the urine treatments was probably due to reduced uptake in the presence of high soil N concentrations (Dijkshoorn, 1958; Riley and Barber, 1971; Joblin and Keogh, 1979), but may have been the result of competition from soil microbes. However, there do not appear to be any studies on the potential for competition between plants and microbes for soil P.

8.2.2 Assessment of effects of cow urine on soil microbiology

8.2.2.1 Inhibition of soil microbes

Accumulation of NO_2^--N indicates inhibition of nitrite oxidising bacteria (NOB), which are known to be inhibited under a variety of conditions (Section 2.5.1.3), so that NO_2^--N accumulation may occur at various stages of the nitrification process. High NO_2^--N concentrations soon after urine application were caused by a combination of high soil pH and high NH_4^+-N concentrations which can elevate concentrations of free NH_3-N to inhibitory levels (Aleem and Alexander, 1960; Smith et al., 1997). Accumulation of NO_2^--N several days after urine application may have been due to suppression of NO_2^--N reductase synthesis in aerobic soils or to a lag in the synthesis of *de novo* NO_2^--N reductase enzymes coupled with persistent NO_3^--N reductase activity in anaerobic conditions (Smith and Tiedje, 1979; Dendooven and Anderson, 1994). High concentrations of salt or NO_3^--N also appeared to inhibit NOB during these experiments, with transient NO_2^--N accumulation measured under these conditions.

Microbial stress after urine application was also shown during this study by monitoring changes in PLFA stress indicator ratios. Only two studies had previously used these ratios to directly

examine the effects of urine application on the microbial community (Petersen et al., 2004a; 2004b). However, this current study was the first to use these three recommended ratios (Section 2.4.2.4) together, and to compare their differing responses to urine addition. The cy/w ratio had previously been used to study changes in osmotic pressure, but here it gave a temporary response, at best, to urine application. It was, however, the only ratio used that responded to urinary salt concentration. The t/c ratio, previously used to monitor microbial responses to changes in osmotic pressure, nutrient supply or pH, varied between experiments, but indicated increased microbial stress due to both high soil moisture content and urine addition. The most reliable respondent to urine application was the i/a ratio, which had been used in other studies to indicate microbial stress caused by high salinity (Chihib et al., 2003). These stress ratios indicated that the soil microbes responded to urine addition by creating PLFAs that allowed them to survive in unfavourable conditions (Section 2.4.2.4).

The ratio of G+/G- bacteria can also be used to indicate microbial stress (e.g. Frostegård et al., 1993b), and the lower ratios measured 10 days after urine application in this present study, showed that the more resilient G- bacteria were surviving better in the soil conditions. Since this response was delayed, the stress indicated may have been due to exhaustion of soil substrates following stimulation of the microbial community by urine addition. This was not a robust indicator of microbial stress because results were not consistent between the different experiments.

Principal component analyses (PCAs) of the PLFA data also showed that the microbial community altered in response to the soil moisture content and urine addition, and these changes largely corresponded to the PLFA stress ratio results. A decrease in the F/B ratios was observed and was evidence of urine inhibition of fungi in the soil cores, although an increase in the fungal PLFA biomarker after urine application in experiment 2 (Chapter 5) indicated that this inhibition may be short-lived in a field situation.

The stress response to high soil moisture indicated by the t/c ratio was supported by an increased *q*CO2 in experiment 1 (Chapter 4). Increasing the urinary salt content resulted in significantly lower cumulative N_2O-N emissions and possibly caused transient inhibition of urease activity, which can be induced by high salinity (Inubushi et al., 1999). Furthermore, osmotic stress following urine application may have been responsible for the increase in PO_4^{3-}-P discussed above (Section 8.2.1.1).

Many of the urine-imposed changes to the soil microbial community were still apparent 30 or 55 days later, in the field and laboratory experiments, respectively, so that the influence of urine

application on soil microbes may continue longer than some of the chemical changes monitored. Therefore longer-term studies of the effects of urine application on the soil microbial community are still required.

8.2.2.2 Stimulation of soil microbes

Urine addition resulted in increased rates of DHA, nitrification and denitrification compared with untreated soils. These microbially mediated processes were also stimulated by temperatures $\geq$ 15°C, high soil moisture (70% WFPS) and elevated urinary N concentrations. It is possible that the trace elements released into soil solution immediately after urine application were used for enzyme synthesis, thus also contributing to these microbial processes. Current knowledge of trace element dynamics in urine patches is lacking.

Addition of urine stimulated the microbial biomass via increased concentrations of soil DRP and WSC and through the addition of moisture, although this was temporary when plants were absent. In contrast to the soil core studies (Chapters 4 and 6) and the findings of Petersen et al. (2004a), urine addition to pasture soil in the field (Chapter 5) resulted in an increase in the fungal biomass, possibly due to fungi recolonising from outside the urine patch.

Overall, although some stimulation of the soil microbial community was observed after urine application, particularly in the field study (Chapter 5), the increase in microbial stress indicated that the population was under constraints, possibly due to soil resources becoming scarce following a population increase.

8.2.3 Emissions of N_2O, NH_3 and CO_2 following urine application

Urine application stimulated N_2O-N production under all the experimental conditions applied. However, the higher fluxes from wet (70% WFPS) non-urine-treated soils than from dry (35% WFPS) urine-treated soils, indicates the importance of denitrification, which dominates in wet conditions, to the production of N_2O-N in these experiments. The higher rates of nitrification and denitrification at high temperatures meant that more N_2O-N was produced at 15 or 20°C than at temperatures $\leq$ 10°C, where denitrification is strongly inhibited (Stanford et al., 1975; George and Antoine, 1982).

Increased NH_3-N volatilisation was not an inevitable consequence of reduced N_2O-N emissions, as discussed below (Section 8.2.4). Where NH_3-N volatilisation was enhanced, the cause was lower rates of nitrification at low temperatures, so that concentrations of NH_4^+-N remained higher. The equilibrium between soil NH_4^+-N and NH_3-N volatilisation described in equation

2.2 shows that where NH_4^+-N concentrations and the soil pH are high, as occurs when nitrification is retarded, there will be a greater flux of NH_3-N from the soil.

Although urine application caused CO_2-C emissions to immediately increase by orders of magnitude, this was primarily due to urea hydrolysis (Equation 2.1). However, as this process is usually complete approximately 4 d after urine application (e.g. Holland and During, 1977; Sherlock and Goh, 1984; Williams and Haynes, 1994) the continuing high fluxes in the urine treatments after this time can be attributed to increased microbial respiration, indicating increased microbial activity.

8.2.4 Hippuric acid effects

This study demonstrated for the first time that enhancing the hippuric acid content of real cow urine could reduce N_2O-N fluxes, as had previously been seen with artificial cow urine (Kool et al., 2006b; van Groenigen et al., 2006). As with van Groenigen et al. (2006), the same effect was attained using benzoic acid, showing that this is the inhibitory component of hippuric acid. Emissions of N_2O-N were reduced by ca. 65% when either acid was added to cow urine at a rate that increased the acid concentration towards the upper natural limit. Both nitrification and denitrification appeared to be inhibited by the increased urinary acid content. There was no measured increase in NH_3-N emissions when N_2O-N emissions were reduced, so that total gaseous N emissions were lowered by enhancing the hippuric acid concentration. This has important implications in the management of gaseous N losses from urine patches, as hippuric acid concentrations in urine may be manipulated by altering the feed of the animals (Martin, 1969; Martin, 1970; Kreula et al., 1978).

There were few indications that increasing the urinary acid concentrations had affected the greater microbial community, with no changes measured in either microbial C or DHA as a consequence of adding the acid. There was an apparent stimulatory effect, shown by the bacterial PLFA biomass being higher in the enhanced acid urine treatments compared with the urine-only treatment (Chapter 6), which may have been due to the acids being used as substrates, but this was only measured up to 3 d after urine application. However, in experiment 4 (Chapter 7) increasing the urinary acid content resulted in increases in DHA and higher initial N_2O-N emissions. In this experiment, which had a lower soil moisture content, the apparent degradation by aerobic soil microbes meant there was no inhibition of nitrification, denitrification or N_2O-N emissions at any of the experimental temperatures. Therefore, these experiments present strong evidence that the inhibitory effect of hippuric acid is strongly dependent on soil moisture conditions.

8.2.5 Evaluation of methods used

Most of the methods employed during these experiments were successfully applied and do not require further comment. However, a number of the methods raised points of interest which are discussed here.

The HPLC method used to analyse hippuric acid and benzoic acid in urine and soil extracts required dilution of the liquid samples by up to 2000-fold, and the use of very low standard concentrations. However, this method gave reproducible results providing the hippuric acid standards were renewed for each set of analyses, since the hippuric acid separated into its benzoic acid and glycine constituents in storage.

Attempts to measure the trace elements (Cu, Fe and Mo) by atomic absorption spectrometry and graphite furnace spectrometry following extraction with $Ca(NO_3)_2$ or water were unsuccessful because the concentrations in many samples were below the detection limits of the instruments. While both $Ca(NO_3)_2$ and water extracts were prepared for ICP-OES analysis, the water extract results were used for data analysis as they were consistently higher and less variable than the $Ca(NO_3)_2$ extract results.

The unsuitability of the DHA assay as a measure of *actual* enzyme activity under some experimental conditions became evident in the final experiment (Chapter 7) where temperature effects were assessed, although its use as an indicator of *potential* enzyme activity was still informative, providing the results were evaluated with care.

Although PLFA analysis gave valuable insight into changes in the soil microbial community after urine addition, reconsideration of sample storage became necessary following the recent publication of Lee et al. (2007). Subsequently, samples of fresh soil were stored at 4°C for extraction within 2 weeks of collection. Analysis of additional experiment 1 replicates, that were in storage at -80°C, indicated that there had been degradation of the PLFAs, and thus fewer analyses were carried out for the earlier experiments than was originally anticipated.

The importance of measuring NH_3-N emissions became apparent when evaluating reductions in N_2O-N emissions in the final two experiments, to ensure that a reduction in one nitrogenous gas did not result in an increase in another. Although derived from two similar methods (Section 3.4.4), this particular use of phosphoric acid diffusion traps appeared to be new. Assessment of the method showed that it was a simple and effective way of collecting the NH_3-N volatilised.

8.3 Conclusions

8.3.1 Hippuric acid effects

- Addition of extra hippuric acid or benzoic acid to real cow urine resulted in a significant decrease (65%) in cumulative N_2O-N emissions when the urine was applied to Wakanui silt loam soil maintained at 70% WFPS, similar to the reduction previously measured when synthetic cow urine was used.

- There was no increase in NH_3-N emissions as a result of the decrease in N_2O-N emissions, so that there was a net decrease in gaseous N losses from soil at 70% WFPS.

- Addition of extra hippuric acid to urine subsequently applied to Temuka silt loam soil at 40–60% WFPS had no inhibitory effect, probably because the acid had been hydrolysed in the soil and subsequently utilised by aerobic benzoic acid degrading bacteria.

- In this latter case, the added acid had a stimulatory effect on the soil microbial community, with the length of the stimulatory period being related to the soil incubation temperature.

8.3.2 Microbial stress

- There was evidence of stress in microbial communities in soil with a high moisture content (70% WFPS), in urine-treated soil, and due to high urinary salt concentrations.

- Phospholipid fatty acid (PLFA) analysis was a useful gauge of microbial stress, with the i/a ratio being the most consistent indicator of stress due to urine application.

- Although both the PLFA t/c stress ratio and the qCO_2 indicated increased microbial stress in the high moisture soil, the higher N_2O-N emissions from this soil than from the low moisture soil showed that the measured stress did not necessarily affect denitrifying microbes.

8.3.3 Assessing the soil microbial community

- In addition to being good stress indicators, PLFAs were also used to determine the soil microbial biomass (total PLFAs) and the microbial community structure (G+ and G- bacteria, fungi, and bacteria).

- The PLFA fungal biomarker decreased after urine application in laboratory experiments, but increased under field conditions, possibly due to recolonisation by unaffected fungi from outside the urine patch.

- Results of the DHA assay are best treated as potential microbial activity, as the results depend on the amount of substrate in the soil, and this can be affected by experimental incubation conditions.

8.3.4 Soil nitrogen dynamics

- Nitrogen dynamics in the soil were greatly enhanced by high soil incubation temperature, high soil moisture and high N application rates, resulting in higher soil NO_3^--N concentrations and higher N_2O-N fluxes.

- Fluxes of NH_3-N were higher at low temperatures (≤ 10°C), because the soil NH_4^+-N concentration remained elevated due to the lower nitrification rate.

- Overall, gaseous N emissions were higher when soil incubation temperatures were ≥ 15°C.

- Fluxes of N_2O-N were reduced by enhanced concentrations of urinary hippuric acid, benzoic acid or KCl salt.

8.3.5 Soil phosphorus and plants

- In the absence of plants, concentrations of PO_4^{3-}-P, possibly sourced from microbial cells lysed by changes in the soil osmotic pressure, increased to maximum values approximately one week after urine application.

- When plants were present, concentrations of soil DRP and plant P were lower in the urine patch, while PLFA content was higher, suggesting that soil microbes were utilising the available soil P, possibly at the expense of the growing plants.

8.3.6 Trace element dynamics

- As well as increases in macronutrients, trace element concentrations increased after urine application.

- The additional Al, Cu, Fe and Mo were most likely sourced from SOM dissolved in the high pH soil conditions after urine application, since concentrations of trace element and

other metals are low in urine, and an increase in pH should yield lower concentrations of all the elements except Mo.

8.4 Priorities for future research

- Although hippuric acid proved to be a potentially useful mitigation tool for greenhouse gas emissions, the effect was not consistent between the final two experiments. More work is therefore needed to investigate its effect at different soil moisture levels and in different soil types, including measurements of the amounts of free and bound hippuric acid and benzoic acid remaining in the soil. The fate of the added acid could be traced using isotopically labelled substrates. In addition, studies into realistic strategies for manipulating stock feed to maximise hippuric acid content should be carried out. Since completing these studies, a field trial using hippuric acid amended cow urine has been carried out and the results of this experiment have been published (Clough et al. (2009) Soil Biology and Biochemistry 41:2222-2229).

- This study found contrasting responses of soil fungi to urine application. More work should be carried out investigating the effects of urine on fungi, particularly in the field, since fungi are believed to participate in both nitrification and denitrification in pasture soils. The contribution of fungi to these processes was not investigated during this study, but could be studied in future work using specific inhibitors in urine affected soil.

- There has been very little previous work on trace element and P dynamics in urine patches. Studies comparing the nutrients added in urine with those present in the soil immediately after urine application and those released later, as well as measurements of nutrient concentrations in plants and microbes are required, to complete the picture of urine effects on pasture systems. This would also clarify the relationship between plants and fungi in regards to the potential for competition for these resources in urine patches.

- The use of PLFA stress indicator ratios gave a good insight into the urine components that had the main effects on the microbial community. However, since the PLFA measurements were only used for a limited duration (up to 55 days), studies on urine-affected soils over a longer time period (e.g. up to a year) should be carried out to investigate if urine induced effects are still measurable after this time.

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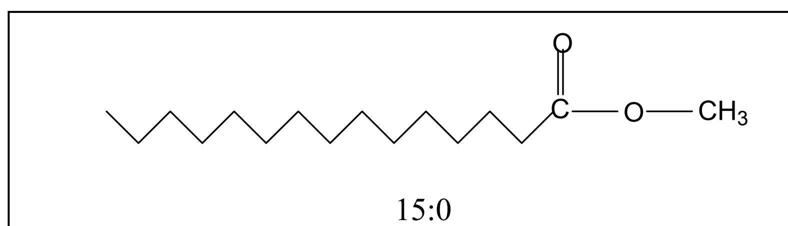
Appendix

Structures of some of the Fatty acid methyl esters (FAMEs) used for PLFA analysis



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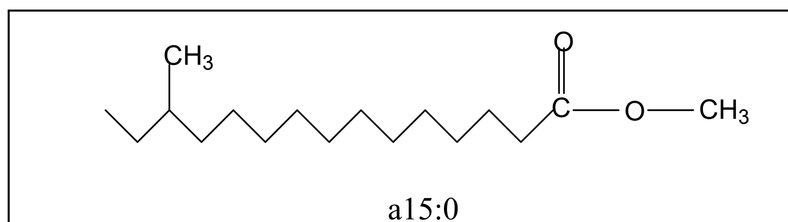

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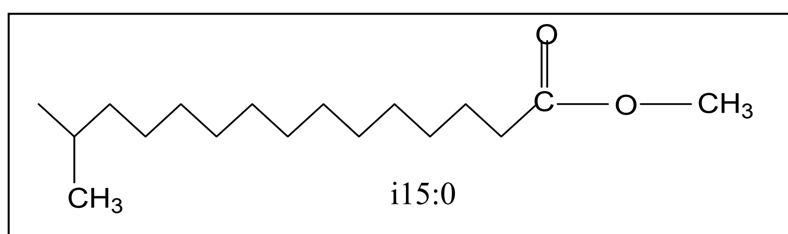

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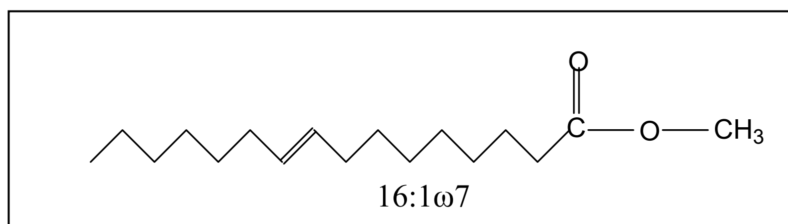

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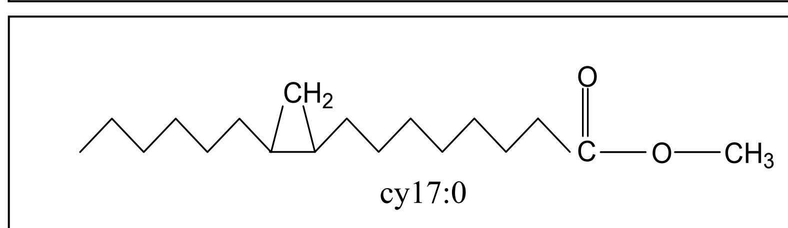

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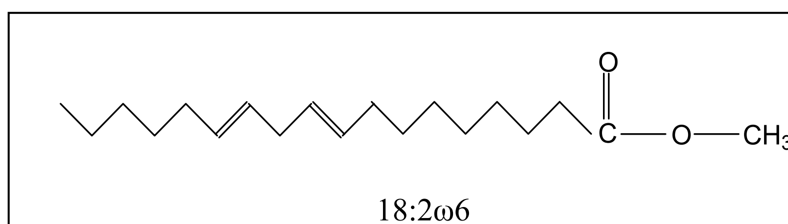

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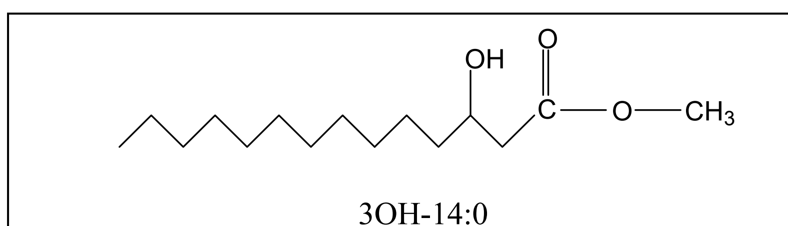

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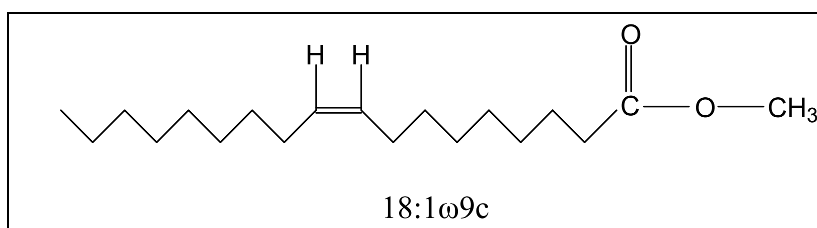

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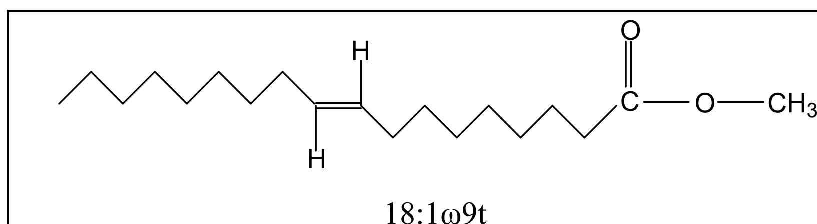

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