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Simulated sheep urine causes the formation of acidic subsurface layers in soil under field conditions

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Abstract

This study aimed to ascertain whether application of sheep urine led to the development of acidic subsurface layers of a pasture soil. Deionised water or simulated urine solution delivering urea-N at 44.8 g m^{-2} and K^+ at 25 g m^{-2} was applied to soil in either winter or spring. Treatments were applied to the soil surface within 10.3 cm internal diameter PVC tubes inserted 20 cm into the soil either under ryegrass or kept bare. Main sampling times corresponded to the completion of various soil N transformations as determined by periodic sampling. Main samplings involved the collection of above ground plant material and soil sampling in 2 cm depth increments in 0-10 cm and 5 cm intervals in 10-20 cm depths.

Following treatment application, Urea and NH_4^+-N moved to a depth no greater than 20 cm but the extent of movement was greater in winter than spring due to the influence of initial soil moisture. Following urea hydrolysis, soil pH increased in the 0-15 cm depth. Subsequent nitrification significantly acidified soil under pasture by 0.8 to 1.0 pH units in the 2-8 and 2-6 cm depths in winter and spring, respectively. This created a net acidic subsurface layer of 0.2-0.4 pH units compared with soil at the beginning of the experiment. Subsurface acidification was 0.5-0.7 pH units greater in bare soil compared with the presence of pasture.

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Additional keywords: nitrogen transformations, pastures, stratification, acidification, acidity, urine

Introduction

Soil acidification is one of the major factors limiting agricultural production in much of Australia (Helyar *et al*. 1990; Ridley *et al*. 1990) and internationally (Von Uexkull and Mutert, 1995). It is estimated that 15 % (13.7 million hectares) of Australia's agricultural soil is strongly acidic (pH < 4.8 in 0.01 M $CaCl_2$) and another 41 % (37.4 million hectares) is mildly acidic with pH between 4.6 and 5.5 (Anon 2001).

In a review of soils under crop and pasture systems around the world, Paul *et al.* (2003) reported that within the surface soil (0-20 cm), layers of low pH occur with higher pH soil above and below. These low pH layers are termed acidic subsurface layers. In soils of southern New South Wales (NSW), differences in soil pH between these layers have been observed to range between 0.4 and 2 pH units (Black 1992; Young *et al*. 1995; Evans *et al*.

1998; Paul *et al*. 2001; Condon *et al*. 2004). These acidic subsurface layers have been shown to limit growth of crop and pasture species (Black and Cameron 1984**,** Pinkerton and Simpson 1986a, 1986b). The spatial extent of acidic subsurface layers in Australian soils remains largely unknown due to the large (10 cm) depth increments used in routine commercial soil sampling and landscape modelling.

The processes of the nitrogen (N), carbon (C) and sulphur (S) cycles are known to initiate soil pH changes (Helyar and Porter 1989; Kennedy 1992; Conyers *et al*. 1995). Of these, the N cycle processes have the greatest effect (Helyar 1976; Bolan *et al*. 1991; Conyers *et al*. 1995).

In grazed systems, areas subjected to urine return from stock (cattle or sheep) contain very high concentrations of N returning rates of up to 500 to 1100 kg ha^{-1} in localised patches (Doak 1952, Wachendorf *et al.* 2005, Selbie *et al.* 2015). Under glasshouse conditions, Black (1992) and Condon *et al.* (2004) found evidence that sheep urine resulted in the formation of acidic subsurface layers within 6 weeks of application. Simulated urine solutions of urea were found to replicate the changes in soil pH due to N transformations occurring in urine patches (Condon *et al*. 2004) enabling application of repeatable concentrations in experiments.

The biological processes that alter the net release and loss of protons, such as mineralisation, urea hydrolysis, nitrification, denitrification and plant uptake, are influenced by environmental conditions, especially moisture and temperature (Haynes 1986). Hence the extent of the development of acidic subsurface layers following urine return may vary according to season due to changes in vertical distribution of added N and environmental conditions. The extent of subsurface acidification by N transformations following urine input to field soils has not been evaluated. It is hypothesised that the development of acidic subsurface layers under urine patches in situ will vary according to

prevailing conditions especially moisture and temperature regimes which influence the vertical distribution and plant utilisation of urinary N.

Methods and materials

The Site

The field experiment was conducted on the Charles Sturt University farm, Wagga Wagga, NSW (-35.064026°, 147.349469°). The soil at this site was characterised as a Red Chromosol (Isbell 1996), Haplustalf (Soil Survey Staff, 2014) derived from granite and existed on a slope of 8 %. The soil (0-10cm) had a pH of 5.7 (1:5 soil : 0.01 M $CaCl_2$) and a pH buffering capacity (Conyers *et al.* 1995) of 1.28 cmol H^+ kg^{-1}.pH. The site had not been subject to any previous agricultural activity other than occasional grazing by sheep. The soil was a coarse sandy loam in the depths sampled for this experiment and other properties are shown in Table 1.

Insert Table 1

Pasture Establishment

To reduce variability in the 30 x 30 m experimental area, an annual pasture was established in the year prior to the experiment and animals were precluded from grazing the site for a period of 2 years until the completion of the experiment. After existing plant residues were removed the site was sprayed with glyphosate and the soil was mixed to a minimum depth of 10 cm with 3 passes of a tractor-mounted rotary hoe. Annual ryegrass (*Lolium rigidum* cv.Wimmera) was sown at 1 g m^{-2} along with 20 g m^{-2} single superphosphate. No other fertiliser additions were made and no nutrient deficiencies were observed.

In the year of the experiment, the dead and dry pasture residue was removed from the site to minimise any effect of the oxidation of organic residues on soil pH (Paul *et al*.

2001). During the first week of May, the site was rotary hoed with a total of 5 passes made in different directions to ensure that the surface 10 cm of the profile was uniformly mixed. The resultant pH was constant through the surface 10 cm (Table 1). On the 10th of May annual ryegrass (*Lolium rigidum* cv.Wimmera) seed was broadcast onto the soil surface at a rate of 1 g m^{-2} which, together with the germinating self-sown seed, resulted in the establishment of 400 plants m^{-2}. The site was sprayed with Bromoxynil (2.0 L ha^{-1}) and MCPA® (0.7 L ha^{-1}) on the 13th of July to kill *Echium* spp. and *Arctotheca calendula* in early growth stages. On 12th of September, the pasture was cut to a height of 10 cm and vegetation removed to simulate grazing and restrict flowering of the ryegrass to maximise pasture uptake of applied N. Within an area of 18 x 22 m, locations on a 1m grid were marked. Each location was a centre of a subsequent treatment application.

Design

The randomised block design included: 2 treatments applied to 3 experimental systems and at 2 application times, all replicated in 9 blocks. Each block was 2 x 22 m on a grid running north-south, at right angles to the direction of the slope. Each experimental unit was randomly applied to locations on the 1 m grid within the experimental site.

The two treatments applied were a control of deionised water and a simulated urine treatment (urine). The urine solution contained 10.5 mg urea-N L^{-1} and 6 mg KCl-K L^{-1} which, when applied at 4.26 L m^{-2}, was equivalent to an application of urea-N at 44.8 g m^{-2} and K^+ at 25 g m^{-2} as used in a previous simulated urine study (Condon *et al.* 2005). Black (1992) and Condon *et al*. (2004) showed both simulated and natural urine produced subsurface acidity in glasshouse studies. Simulated urine was used so the N content would at the concentration required. Urea-^{15}N with an enrichment of 1.98 atom % was used in the

urine treatment solution applied in systems containing plants that were to be sampled at the completion of each experimental period.

The three experimental systems were:

1. Plants within Tubes (PT). Tubes made from 100 mm diameter PVC (internal diameter 10.3 cm x length 23 cm) were inserted into the ground so that approximately 0.3 cm of the tube remained above the soil surface. Each core contained an average of 3 actively growing plants. Treatment solutions were applied to the soil surface of each core. The 0.3 cm of the core above ground contained the solution preventing any run off or run in. The cores remained in place until they were removed for destructive sampling at various sampling times. The PT system was the primary system used to facilitate comparison with glasshouse data (Condon *et al.* 2004, 2005). The PVC cores provided a physical barrier to inhibit lateral movement of treatment solution and plant roots, thereby decreasing the number of variables acting in the experiment.

2. No plants within Tubes (NP). On the 13th of July sites for the NP system were surrounded with a galvanised iron spray shield (diameter 1 m x height 30 cm) and sprayed with glyphosate to kill all plants in the area. PVC tubes were then inserted into the centre of the sprayed zone. The NP system was included to provide a contrast to the PT system in order to assess the impact that plants had on the system.

3. Plants Unconfined (PU). A galvanised iron nail (1.5 cm diameter head) was inserted into the soil at each grid site selected for the PU system. A PVC ring (internal diameter 24 cm x height 4 cm) was then placed on the soil surface so that the nail was located in the centre of the ring. The PVC ring was then gently pressed 0.5 cm into the surface soil to avoid any surface run off of treatment solution. Once the solution was applied, the PVC ring was removed. Nails remained in the soil for the purpose of locating the application site during and at the end of the experimental periods. The PU system represents an

unconfined simulated stock urine event. The influence of the enclosure system (PT) on the normal field processes that occur when no physical barrier to treatment or plant growth exists was determined by comparison with this PU system.

Treatment solutions were applied in winter, W, (28th of July) and spring, S, (21st of September). Following each application time there were 4 sampling times corresponding to the stages of N transformations: time 0 (designated W0 and S0 for winter and spring respectively), occurred within minutes of treatment application; W1 and S1 were conducted when the daily NH_3 volatilisation rate decreased to less than 1 % of applied N; W2 and S2 were performed when the NH_4^+-N content in the respective cores was less than 5 % of applied N; and, W3 in winter was carried out when the total inorganic N content of the cores containing plants had decreased to less than 5 % of applied N. On the 5th of November, sheep accidentally grazed the site so S3 in spring of PT and NP was conducted on that day to minimise the impact of the sheep. The timing of the second and third samplings were determined by periodic sampling of additional urine treated cores located randomly within the experimental grid.

Environmental conditions

During the experiment, daily rainfall was measured in a gauge located at the site. Soil temperature was monitored hourly using a Starlogger® model 6004-21 data logger and 2 thermistor probes which were inserted at 1 and 9 cm depths into two cores adjacent to the experimental grid.

Soil sampling and analysis

During sampling of the PT and NP systems, cores were excavated from the field. For the PU system, tubes (24 cm in diameter x 23 cm length) were inserted at sampling time and

175 excavated to then remove a 24 cm diameter soil core. Soil was removed in 2 cm intervals
176 in 0-10 cm and 5 cm intervals in 10-20 cm depths. Soil for each depth interval was mixed.
177 This moist soil was used in extractions and for measurement of soil pH, NH_4^+-N, NO_3^--N
178 and gravimetric moisture content.

179 Soil pH was measured from an extract solution of 40 ± 2 g of moist soil in 200 mL of 1
180 M KCl. The soil/KCl solution was shaken end over end for 1 hour and allowed to settle for
181 30 minutes prior to pH measurement using a glass combination electrode. An aliquot of
182 approximately 40 mL of this solution was collected after filtration through a number 5
183 Whatman paper. This was stored at below 4 °C for no longer than 48 hours until analysis
184 of urea (Mulvaney and Bremner, 1979), exchangeable NH_4^+-N (Crooke and Simpson
185 1971) and NO_3^--N (Henriksen and Selmer-Olsen 1970) using a Perstorp Alpkem
186 segmented flow auto analyser. For the purposes of this paper, the term NH_4^+-N includes
187 both exchangeable and soluble NH_4^+-N unless specified and NO_3^--N refers to the
188 combination of NO_2^--N and NO_3^- -N as they were not separated by analysis.
189 Concentrations of N are reported as mg/layer to enable simple comparison of N mass
190 recoveries to pH change and to avoid erroneous interpretation if mg/kg were to be used
191 where different layer depths occur. Gravimetric moisture content was calculated following
192 the drying of approximately 50 g of field moist soil at 105 °C for at least 24 hours.
193 Volatilisation from cores, treated as in the PT system but located adjacent to the
194 experimental grid, was measured daily using the dynamic enclosure method (Black *et al.*
195 1985) until the daily rate of NH_3 loss decreased below 0.5 % of applied N.

196 Plant tops within a core were cut at ground level prior to destructive sampling at the
197 various sampling times. For the PT system plant material was also collected from a
198 concentric ring (24 cm diameter) outside the cores to account for uptake from N that may
199 have leached below the core and been utilised by surrounding plants. For the PU system

plant material was collected from the area to which treatments were applied (24 cm diameter) and two concentric rings (43 and 57 cm in diameter) outside the site of application to capture any plant uptake of applied N outside the area of initial application.

The plant tops were dried at 60 °C for at least 48 hours and weighed. The material was ground to pass a 1 mm sieve and the total N content determined using a Kjeldahl acid digest of 200 mg of plant material.

Plant material of both treatments from W3 winter samplings was ground to a finer size using a puck and ring grinder before analysis of ^{15}N content in triplicate using a Europa Scientific Roboprep/ Tracermass mass spectrometer. Total N analyses by Kjeldahl acid digests were conducted on plant samples from all samplings and treatments, including those that underwent ^{15}N analysis.

Data analysis

The influence of treatment on soil and plant parameters over time and between treatments for each experimental period was determined by analysis of variance (ANOVA). Soil samples taken over various times were considered independent because they were taken from different sites within blocks at each sampling time. ANOVA was used to compare soil properties of treatments within each layer. Throughout the subsequent discussion of results, the net change in a soil property associated with the urine treatment is presented as the calculated difference between the urine treatment and the control at a given sampling time. Throughout the text this is designated as Δ "soil property". For example, the net change in the NH_4^+-N concentration would be the numerical difference between the NH_4^+-N concentration of the urine treatment and that of the control for a given layer at a given sampling time. This would be expressed as the Δ NH_4^+-N concentration.

ANOVAs were used for Δ soil pH and Δ gravimetric moisture content. Data were transformed where skewed distributions existed. Standard error of the mean was used to show relative differences in Δ urea and Δ mineral N content within layers of PT in winter and spring as the data could not be normalised by transformation.

For calculation of net acidification and for the comparison of the NP and PT systems, the values of the control at the initial sampling, for each season, were subtracted from the respective measurements of the final sampling. Therefore, the data presented as δ "soil property" in any comparison of the NP and PT systems, or net acidification, are those between the urine treatment and control during the experiment and are not influenced by differences that may have existed prior to treatment application.

Results

Environmental conditions

Daily soil temperature and rainfall are shown in Figure 1. For the winter application, the mean daily temperature recorded in the surface 10 cm of soil within cores was below 10 °C for all but one day of the first 3 weeks and remained below 12 °C until W2. After W2 the average daily soil temperature increased to an average of approximately 17 °C until W3. Total rainfall of 176 mm was recorded during winter with 110 mm falling between W1 and W2.

Insert Figure 1

For the spring application, the mean daily temperature in the surface 10 cm over the 6 days from S0 to S1 was relatively constant at 16 °C. Temperature fluctuated from 13-20 °C between S1 and S3. A total rainfall of 103 mm was recorded during spring with 41 mm between S1 and S2.

Plants in tubes

Soil Water

During the first 3 samplings in winter, the gravimetric moisture contents were at approximately field capacity (0.2 g g^{-1}) within the cores. There were no significant differences in the moisture content due to treatment at these times (W0, W1, W2) (Figure 2a). By the completion of the experiment (W3) the urine treatment had caused significant drying of the surface layers by 0.04 g g^{-1} of gravimetric moisture content.

Insert Figure 2

At the start of the spring period the soil moisture contents were similar to the drier conditions at the end of the winter period. No significant changes in soil moisture occurred between the first samplings of spring (S0 and S1). In the time between S1 and S2, the soil lost up to 0.08 g g^{-1} soil moisture in the surface 10 cm and approximately 0.04 g g^{-1} in the 10-20 cm layers due to the urine treatment relative to the control (Figure 2a). The soil profile then remaining unchanged until the completion of the experiment (S3).

Urea

At W0, urea moved into all layers of urine treated cores but was concentrated in the surface 2 cm (38 % of the recovered urea) and the 2-8 cm layers, which contained another 50 % of recovered urea (Figure 2b). The concentration then decreased with depth to negligible concentrations. In contrast, the majority (65 % of recovered) of urea applied in spring was present in the 0-2 cm depth and less than 5 % was found in each layer below this. At the time of the initial sampling 90 ± 2.2 and 97 ± 6.6 % of applied N was recovered as urea in the 20 cm profiles sampled in winter and spring, respectively.

NH_3 volatilisation

Maximum volatilisation rates occurred on days 3 and 2 after treatment application for winter and spring applications, respectively, with daily volatilisation loss decreasing to less than 0.5 % per day by days 5 (W1) and 6 (S1) respectively. Volatilisation loss between W0, W1 was to 7.2 ± 0.8 % of N applied for winter and 7.9 ± 1.7 % between S0 and S1 for spring.

Ammonium

The NH_4^+-N content of the controls at all samplings and both application times (W0 and S0) in the urine treatment was less than 0.3 mg layer^{-1} (data not shown).

By W1 and S1, the Δ NH_4^+-N concentration had increased in all layers. In winter, the Δ NH_4^+-N concentrations were evenly distributed throughout the surface 8 cm (Figure 2c) and decreased with depth below 8 cm. The apparent recovery of applied N as NH_4^+-N in the surface 20 cm was 81 ± 1.6 %. In contrast in spring, the Δ NH_4^+-N content was highest in the 0-2 cm layer and decreased steadily with depth. The total apparent recovery of NH_4^+-N at S1 was 57 ± 2.8 % of applied N.

In both seasons, once the NH_4^+-N concentration in the profile decreased to less than 5 % of applied N (W2 and S2) the remaining NH_4^+-N was located below 10 cm depth. At W3 and S3, negligible NH_4^+-N remained to 20 cm depth in cores treated in either season.

Nitrate

At the time of treatment application in both seasons (W0 and S0) the NO_3^--N concentration in all layers was approximately 0 mg layer^{-1} (data not shown). The soil Δ NO_3^--N content remained below 2 and 5 mg layer^{-1} in all layers of the profile throughout the winter and spring experiments, respectively (Figure 2d). The apparent recovery of applied N as NO_3^--

298 N at the completion of the winter (W3) and spring (S3) experiment was 1.9 ± 0.5 and 7.6 ±
299 1.6 % respectively.
300
301 *Plant growth and N uptake*
302 In both winter and spring, the urine treated soil produced significantly more dry matter
303 (1733 and 933 g m^{-2}) compared to the control (513 and 382 g m^{-2}), respectively. The N
304 yield of the plant tops was also significantly greater in the urine treatment (31.5 and 25.3 g
305 N m^{-2}) than the control (6.4 and 4.0 g N m^{-2} for winter and spring, respectively). Apparent
306 recovery of applied N in plant tops at W0, W1, W2 and W3 was 0.6 ± 0.5 %, 3 ± 0.5, 44 ±
307 4 and 56 ± 4 % respectively. Corresponding data for S0, S1 and S2 were 0 ± 1, 7 ± 1 and
308 47 ± 4 % respectively. For the winter period, a total of 98 ± 1 % of recovered ^{15}N came
309 from plant material harvested from plants within the cores where synthetic urine was
310 applied to soil enclosed in tubes.
311 Total apparent recovery of applied N (urea, mineral N, NH_3 volatilised and plant uptake) at
312 W0, W1, W2 and W3 was 92 ± 3 %, 92 ± 3, 57 ± 7 and 64 ± 5 % respectively, and 97 ± 8,
313 73 ± 7 and 73 ± 10 % for S0, S1 and S2, respectively.
314
315 *Soil pH*
316 In winter, the soil pH profiles of the control cores did not change significantly below the
317 surface 2 cm layer during the experiment. However, the 0-2 cm layer of the control
318 exhibited an acidification of only 0.1 pH units during the experiment. In spring, the 2-15
319 cm layer of the control exhibited significant acidification of less than 0.15 pH units (data
320 not shown).
321 The Δ pH significantly increased in both seasons in the time between the urine
322 treatment application (W0, S0) and when volatilisation decreased to negligible rates (W1,

S1) (Figure 2e). In winter, the Δ pH increased by approximately 1.2 units in the layers of the surface 10 cm and 0.8 units in the 10-15 cm layer. In spring, Δ soil pH increased by 0.9 pH units in the 0-2 cm layer, 0.8 units from 2-8 cm and 0.6 units in the 8-10 cm layer.

The Δ pH in both seasons decreased significantly following W1 and S1 samplings (Figure 2e). The magnitude of the Δ pH decreases in the winter experiment were approximately 1.25, 1.4, 1.35 and 0.8 pH units in the 0-2, 2-8, 8-10 and 10-15 cm layers respectively. This resulted in significantly more acidity than the initial soil (W0) in the surface 0-8 cm. At the completion of the winter experiment (W3), significant net acidification of 0.23 and 0.24 units had occurred in the 2-4 and 4-6 cm layers, respectively. In spring, significant acidification occurred in the surface 0-10 cm following the S1 sampling. The magnitude of this acidification was greater than 1 pH unit in the 0-8 cm layers and 0.7 units in the 8-10 cm layer. This resulted in the 0-8 cm layers having Δ pHs that were significantly less than the initial sampling (S0) with the greatest net acidification (0.3 pH units) occurring in the 2-4 cm layer. There was no further acidification observed following W2 or S2 samplings for the respective winter and spring experiments.

Effect of plant growth

The presence of plants lowered the soil moisture content of the measured soil profiles during the experiment compared to where plants were absent (NP system) (Figure 3a). The effect was greater in spring than in winter.

Insert Figure 3

The δNH_4^+-N contents in the PT and NP system did not exceed 11 mg $layer^{-1}$ in either winter or spring (Figure 3b). There were no significant differences in δNH_4^+-N concentration between the PT or NP system in winter. In spring, the δNH_4^+-N

concentration was significantly higher in the absence of plants (NP) in the surface 0-8 cm of the profile, though only very low concentrations remained.

In both seasons, the δNO_3^--N contents of all layers were significantly greater in the absence of plants (NP) compared to where plants were present (PT) (Figure 3c). In the plant system, negligible concentrations of NO_3^--N remained in the soil at the end of either season. The NO_3^--N concentration was greatest in the surface soil layer and decreased with depth in the surface 10 cm.

The δ soil pH was significantly less in the presence of plants (PT) compared to the NP system in all soil layers in both winter and spring (Figure 3d), with the exception of the 10-15 cm layer in winter and the 15-20 cm layer in spring. The mean differences in the δ soil pH between PT and NP systems was 0.56 and 0.33 pH units for winter and spring, respectively.

Effect of confinement

Because of the grazing by sheep at the end of the spring period, only the winter data are reported. There were no significant differences in soil pH, exchangeable potassium and mineral N concentrations where treatments were confined to PVC tubes in the ground (PT) compared to where treatments were applied to an unconfined system (PU) (Table 2). Soil of the unconfined system had a significantly higher moisture content (by approximately 0.01-0.04 % g g^{-1}) than soil confined in tubes in all layers.

Insert Table 2 here

Plant dry matter yield and N yield in plant tops were greater at the site of application in the PT than the PU system (Table 3). Plant growth and N uptake outside of the initial site of urine application did not differ from those of the no-urine control of the PT system.

However, significant differences relative to the control were observed for both plant growth and N yield in concentric rings outside the initial site of urine application in the unconfined system (PU). Approximately 70 % of the ^{15}N recovered from plant tops came from the site of application of synthetic urine where plants were not confined by tubes. Plant material from Ring 1 and Ring 2 (the concentric rings outside of the point of urine application) accounted for approximately 23 and 6 % of recovered ^{15}N respectively. In the PT system, 98 % of ^{15}N recovered by plants came from within the cores.

Insert Table 3 here

Effect of season on net acidification

During the winter experimental period, urine application resulted in net acidification, compared to the control, of 0.23 and 0.24 units in the 2-4 and 4-6 cm layers respectively (Figure 4). In spring, significant net acidification occurred between 2 and 8 cm with the maximum being over 0.4 pH in the 2-4 cm interval. Urine application in both seasons created acidic subsurface layers with significantly more net acidification occurring in spring than in winter in the 0-2 and 2-4 cm layers (Figure 4).

Insert Figure 4

Discussion

The changes in soil pH resulting from the application of simulated urine solution are the result of N movement and transformations that occur through time in vertically stratified locations within the profile (Condon *et al*. 2004).

The rapid application of treatment solution favoured movement of urea through macropores causing a redistribution of urea through the soil following application. This result is consistent with Williams and Haynes (1994) who observed that bromide labelled urine moved to a depth of 15 cm following sheep urine application to a silt loam. The mass

of urea moving down from the surface 0-2 cm was greatest in winter when soil moisture content at the time of application was near field capacity. Downward movement of simulated urine was less in spring when the soil was drier and the portion of the water applied was retained in the surface 2 cm bringing it to field capacity. The lower recovery (90 ± 2.2 %) of applied urea in winter may be due to more initial leaching loss in the wetter winter soil relative to spring (97 ± 6.6 % recovery).

Following the initial infiltration of urea into the soil, hydrolysis rapidly occurs. As a result, the soil pH at W1 and S1 increased by 1.2 and 0.8 pH units in the surface 10 cm for winter and spring, respectively. The smaller pH increase in spring may be a result of greater net immobilisation and plant uptake (as ammonium) in spring due to the warmer temperatures compared to winter (Figure 1). This is supported by there being less profile NH_4^+-N present at S1 compared to W1 (Figure 2c). Plant utilisation of applied N was greater at S1 (7 ± 1.1 %) than W1 (3 ± 0.5 %) and can be assumed to have been taken up as NH_4^+-N as nitrification is negligible whilst volatilisation is occurring following urine addition (Condon *et al*. 2004). Either plant uptake or net immobilisation will add acid to the soil in response to utilisation of ammonium (Helyar and Porter 1989).

Ammonia volatilisation losses were similar for winter and spring so any acid addition due to ammonia loss would be expected to be the same in both seasons. Ammonia volatilisation was relatively low compared with other work with similar moisture and temperature conditions. Sherlock and Goh (1984) and Condon *et al.* (2004) using synthetic urine (urea) solutions and Black *et al.* (1985) using urea fertiliser reported losses of approximately 20-30 % using the same measurement technique. The lower volatilisation reported here may be associated with the K^+ added in the synthetic urine in this work. Rappaport and Axley (1984) used a sandy loam soil (pH 6) in a laboratory experiment to show that increasing the addition of KCl relative to urea addition caused a decrease in NH_3

volatilisation possibly due to a decrease in pH by cation exchange of added K^+ with native soil H^+

Once volatilisation had decreased to negligible rates (W1 and S1), the accumulation of NH_4^+-N formed by hydrolysis of urea generally reflected the initial distribution of urea in winter but in spring NH_4^+-N accumulated below the 0-2 cm layer where urea was initially present. The NH_4^+-N distribution in spring may be due to either leaching of urea subsequent to the S0 sampling but before hydrolysis or leaching of NH_4^+-N post hydrolysis. The former is supported by the observed pH increase in the top 10 cm at S1 suggesting hydrolysis must have occurred in layers below the initial site of urea at S0. Even so, while NH_4^+-N is not normally leached, conditions in this experiment favoured movement. The soil was sandy with a low cation exchange capacity (CEC). The concentration of NH_4^+-N at the second sampling in each season (W1 and S1) was much higher than that which normally occurs in soil and the presence of K^+ in the simulated urine would also favour leaching. Exchangeable K^+ and NH_4^+-N behave similarly during cation exchange (Phillips *et al.* 1988) and the combined effect of NH_4^+-N and K^+ on leaching of cations in urine patches has been demonstrated by Condon *et al*. (2005). It follows that the downward movement of NH_4^+-N at concentrations in excess of the CEC would have occurred in both seasons but would be expected to be greater in winter when the soil is wetter. This movement of NH_4^+-N post urea hydrolysis spatially separates the alkaline reaction from subsequent acidifying transformations thereby creating acidic subsurface layers.

By W2 and S2 the NH_4^+-N concentration in each layer had decreased significantly from the previous sampling as a result of nitrification and plant uptake resulting in decreased pH forming an acidic subsurface layer of -0.24 to -0.43 pH units in the 2-6 cm depth for the

winter and spring experiments, respectively (Figure 2e). The production of NO_3^- as evidence of nitrification is not possible in the presence of plants as they may utilise NO_3^- that is formed. There were no differences between seasons for plant utilisation of added N. The presence of small concentrations of NH_4^+-N remaining in the 10-20 cm layers is consistent with slower rates of nitrification and plant uptake (Figure 2c). This was especially evident in spring where greater than 5 % of applied N was present as NH_4^+-N. It is known that the rate of nitrification will decrease with depth (Young *et al*. 1995; Purnomo *et al.* 2000) and with decreasing soil water potential (Sabey 1969). In spring, gravimetric moisture content of the lower profile was approximately 0.06 (g g^{-1}) later in the experimental period owing to greater drying by increased plant water use where urine was applied (Figure 2 a). This dry soil would be likely to also limit microbial activity at this depth. Bronson *et al*. (1999) also reported evidence that low soil moisture can limit nitrification in urine patches. In their study, 28 % of applied urine-^{15}N remained in a sandy soil 28 days after application due to a lack of soil moisture.

At the completion of the experiments almost all of the N added in the simulated urine solution had been utilised with the exception of trace quantities of NO_3^--N in spring. There were no differences between seasons in total plant utilisation of N at the end of the experiments however the total recovery of applied N decreased with time in both seasons. The decrease in recovery through time is consistent with the observations of Black *et al*. (1985) and is likely to relate to increased accumulation of N in the root biomass and net biological immobilisation. The net effect of the completed N transformations following addition of simulated urine in winter or spring was the formation of acidic subsurface layers (Figure 2e). As the mineral N had been expended, the acidity recorded at the completion of the experiments would remain to influence soil process and plant production.

Comparison of these results with urine treated profiles in the absence of plants demonstrates the influence of plant uptake on soil moisture, mineral N concentration and the resultant pH profile at the completion of the experiment. In general, the absence of plants allowed the soil to remain wetter (Figure 3a), potentially allowing more downward movement of NH_4^+-N. The lack of alkalinity added to the soil during NO_3^--N utilisation by plants ensures that pH changes due to nitrification resulted in greater net acidification and to greater depths than when plants were present (Figure 3d). Simulated urine application to soil in the absence of plants represents urine return during grazing on patchy pasture of low ground cover, a harvested crop stubble or a dormant pasture. If subsequent movement of NO_3^--N from the surface 10 cm took place the greater acidification of subsurface layers would not be reversed.

Acidic subsurface layers formed regardless of season of application in this study. The magnitudes of acidification reported here were for an application rate of 448 kg N ha^{-1}. This is less than half the average rate of 1,089 kg N ha^{-1} recently reported for sheep by Selbie *et al.* (2015). Hence subsurface acidification could be considerably greater than those reported here when the N application rates are higher.

We have shown for the first time that acidic layers can develop within the top 10 cm of soil in pastures in the field following urine return. The formation is related to stratification of the biological processes within the N cycle. Previous glasshouse studies of this topic which similarly showed the development of acidic subsurface layers following urine application have been reported by Black (1992) and Condon *et al.* (2004; 2005). The extent of acidification was significantly greater in spring than winter as the biological processes involved are influenced by environmental conditions. Soil moisture influences the rate of infiltration and movement of urinary constituents which ultimately alter the depth and magnitude of acidification. Other studies by our group have reported development of these

497 layers of acidification under one season of cropping (Evans *et al.* 1998) and crop/pasture
498 rotations without grazing (Paul *et al*., 2001). Other studies have observed the natural
499 occurrence of these acidic subsurface layers (Conyers *et al.*, 1996, 1997; Pinkerton and
500 Simpson, 1986; Young *et al.*, 2002). Paul *et al.* (2003) reported that these acidic subsurface
501 layers occurred in crop and pasture soils in most countries and under forest and as well as
502 bare soil.

503 Acidic subsurafce layers directly influence plant growth (Pinkerton and Simpson,
504 1986), legume nodulation (Burns *et al*. 2017), as well as the rates of nitrification (Young *et*
505 *al.* (2002) and nitrogen mineralisation (Purnomo *et al.* 2000; Paul *et al.* 2001). The presence
506 of acidic subsurface layers is often not identified by commercial soil testing as sampling
507 occurs in 10 cm depth increments. The mixing of soil from a 10 cm interval sample will
508 often mask the presence of the acidic subsurface layer, finer (5 cm) sampling increments are
509 required. Once identified, options to neutralise the acid layers or minimise their development
510 are limited in pasture systems. Limestone, applied at commonly used rates that aim to bring
511 the pH_{Ca} to 5.0-5.2 has been shown to have limited ability to move down the profile below
512 the depth of placement (Scott *et al.* 1997). This can exaggerate the stratification of soil pH
513 as the amelioration is confined to the surface layer (Conyers *et al*. 2003). Neutralisation of
514 acidity formed from urine application requires incorporation of lime to a depth of at least 10
515 cm, a practice not practical in perennial pasture systems. Amelioration without incorporation
516 requires soil pH to be maintained above pH_{Ca} 5.5 with lime over several decades to allow
517 alkali to move deeper into the soil (Li *et al*. 2019).

518 Our observations demonstrating the development of acidic subsurface layers under
519 annual pasture following the application of synthetic urine were not an artefact of the soil
520 being confined within a plastic tube during experimentation. There was no significant
521 difference in the development of acidic layers during winter in soil within tubes or in soils

that were unconfined. This occurred despite 29 % of recovered ^{15}N being present in plants sampled in the rings outside the site of urine application in an unconfined system. Additionally, urine treated soil at the site of application was drier in the unconfined system compared with soil within the PVC cores. These two observations suggest that roots from plants outside the site of application were able to take up N and water in the unconfined system. In a similar experiment in New Zealand, Buckthought *et al.* (2016) found that plants outside the zone of the urine N application recovered 33 % of applied ^{15}N, which is similar to our 29 %. They did show some movement of ^{15}N out of the application zone one day after treatments were applied and this was attributed to lateral flow of urine in macropores, as we suggested for movement of synthetic urine to a depth of 20 cm in our study. The ^{15}N uptake from the equivalent of our outer ring was attributed to root uptake under the site of application and transfer to plant tops in the outer zone. Importantly, the pH changes associated with the N uptake occurred at the site of the N uptake, that is, within the site of application. The acidity measured within the 20 cm deep cores of our study was not different when plants were confined compared to where they were not. Therefore, the use of PVC cores to enable detailed study of N transformations did not influence the outcome of the study.

Much of the published research relating to urine patches focuses on the consequences of N losses as they impact on the N economy of pastures and the environment due the NO_3^--N leaching and losses of N gases through denitrification (Selbie *et al.* 2015). Our work has focused on the chronic consequences of stock urine return on the formation of soil acidification. Stock urine return to pastures has been shown to result in the formation of acidic subsurface layers, the severity of which is influenced by environmental conditions, largely soil moisture, and the presence of actively growing plants. The most acidic layer formed at depths of 2-6 cm following a single application of simulated sheep urine. Greater

acidity resulted from urine application in spring than in winter. The cumulative effect of stock urine return is long term acidification within the surface 10 cm of soil which will restrict the agronomic performance of pasture and the production system.

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Conflicts of interest: The authors declare no conflicts of interest.

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Table 3. Ryegrass dry matter yield (DMY) (g m^{-2}) and nitrogen yield in plant tops (g N m^{-2}) following application of simulated urine to confined tubes (PT) or unconfined (PU) in winter. Data are means from site of application (Core) or concentric rings (Ring 1 and 2) around the core at the completion of the experiment (W3). Within DMY and N yield individually, means marked with different letter are significantly different ($p<0.05$) in rows (treatments within site) or columns (site within treatment).

692

693

694 Table 1 Soil properties at the commencement of the experiment.

695

Depth (cm)	pH (0.01 M $CaCl_2$)	Organic C (% C)	Colwell P (mg kg^{-1})	CEC (cmolc kg^{-1})
0-2	5.8	1.5	25	5.84
2-4	5.7	1.6	29	6.28
4-6	5.7	1.3	31	6.09
6-8	5.7	1.2	35	5.91
8-10	5.8	1.1	20	5.17
10-15	5.7	0.5	12	3.81
15-20	5.6	0.4	10	3.62

696 697 698 699 700 701 702 703 704

705

706 Table 2
707

708 Soil properties at the completion of the winter experiment (W3) following application of simulated urine to soil within PVC cores (PT) or to soil
709 without cores in an unconfined system (PU). Values in bold within a layer indicate statistical differences ($p<0.05$).

710

	Soil pH (1 M KCl)		Water content (g g^{-1})		NH_4^+-N (mg $layer^{-1}$)		NO_3^--N (mg $layer^{-1}$)		K (cmolc kg^{-1})	
Depth	PT	PU	PT	PU	PT	PU	PT	PU	PT	PU
0-2	5.40	5.40	**0.12**	**0.16**	0.5	0.4	0.0	0.0	0.4	0.5
2-4	5.30	5.25	**0.13**	**0.17**	0.5	0.2	0.0	0.0	0.3	0.3
4-6	5.33	5.28	**0.13**	**0.16**	0.5	0.2	0.0	0.0	0.2	0.3
6-8	5.41	5.37	**0.13**	**0.15**	0.3	0.4	0.0	0.0	0.3	0.3
8-10	5.38	5.43	**0.11**	**0.13**	0.4	0.1	0.0	0.0	0.3	0.3
10-15	5.25	5.37	**0.10**	**0.11**	0.4	0.0	0.0	0.0	0.3	0.2
15-20	5.25	5.16	**0.10**	**0.11**	0.0	0.1	0.0	0.0	0.3	0.2

711

Table 3

Ryegrass dry matter yield (DMY) (g m^{-2}) and nitrogen yield in plant tops (g N m^{-2}) following application of simulated urine to confined tubes (PT) or unconfined (PU) in winter. Data are means from site of application (Core) or concentric rings (Ring 1 and 2) around the core at the completion of the experiment (W3). Within DMY and N yield individually, means marked with different letter are significantly different ($p<0.05$) in rows (treatments within site) or columns (site within treatment).

	PT				PU			
	DMY (g m^{-2})		N yield (g N m^{-2})		DMY (g m^{-2})		N yield (g N m^{-2})	
Site	Control	Urine	Control	Urine	Control	Urine	Control	Urine
Core	513 a	1733 b	6.4 a	31.5 b	413 a	1122 d	5.4 a	19.9 c
Ring 1	320 c	326 c	4.4 a	5.2 a	389c	708 b	5.0 a	10.6 d
Ring 2					388 c	577 b	4.8 a	8.1 b

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Figure 1. Rainfall and temperature during the experimental period. Bars, rainfall (mm day^{-1}); soil temperature at a depth of 1 cm, solid line; soil temperature at 9 cm dashed line

Figure 2. Change in soil properties (Δ), between urine and control, for each sampling (●0, ○1, ■2 and □3) of winter (W) and spring (S) experiments, treatments applied to PVC cores in the field growing ryegrass pasture; (a) gravimetric moisture content (g g^{-1}), (b) mass of urea-N per layer, (c) NH_4^+-N mass per layer (d) NO_3^--N mass per layer and (e) soil pH (1 M KCl), in each layer at each sampling time.
Bars indicate standard error in (b), (c), (d) and lsd's at $p<0.05$ in (a) and (e), ns = no significant difference

Figure 3. Change in soil properties relative to the start of the experiments (δ), between urine and control, at the end of experimental period (winter W and spring S) for treatments with: ● no plants in tubes (NP), ○ plants in tubes (PT); (a) the gravimetric moisture content (g g^{-1}), (b) in NH_4^+-N mass in each layer, (c) in NO_3^--N mass in each layer, and (d) soil pH (1 M KCl).
Bars indicate lsd's at $p<0.05$. ns = no significant difference

Figure 4. Net acidification (δ soil pH (1 M KCl)) at the end of the winter (●) and spring (○) periods in the presence of plants. Bars indicate lsd's at $p<0.05$ between season. ns = no significant difference

Figure 1

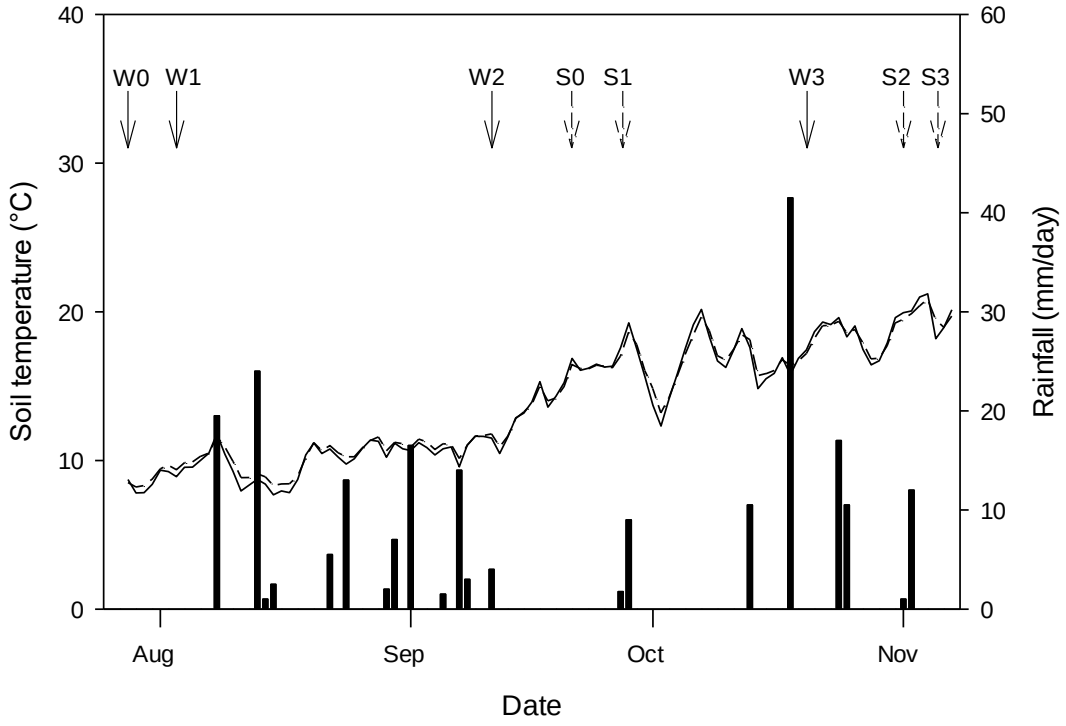

W0
W1
W2
S0
S1
W3
S2
S3
Soil temperature (°C)
Rainfall (mm/day)
Aug
Sep
Oct
Nov
Date
0
10
20
30
40
0
10
20
30
40
50
60

Figure 2

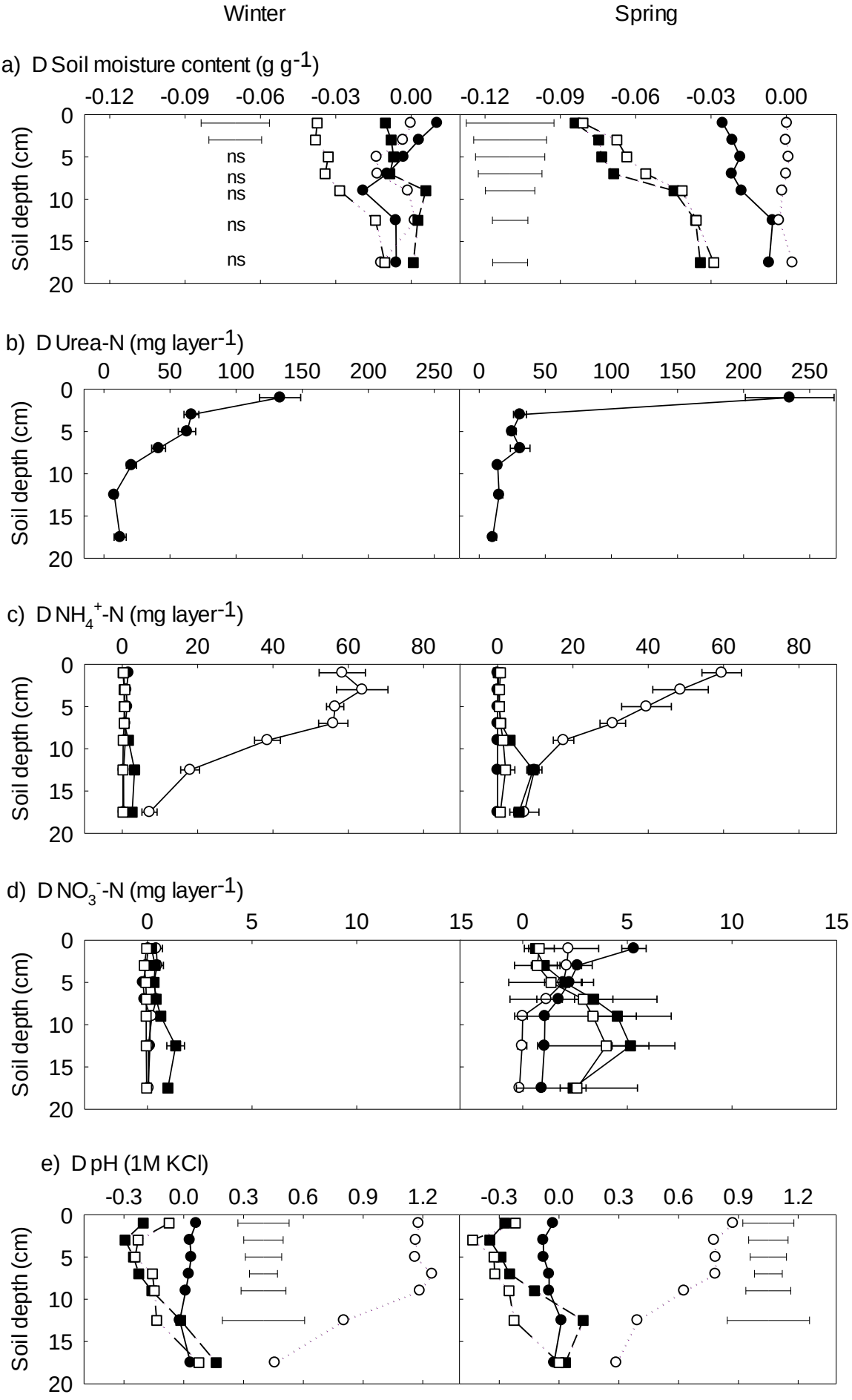

Winter
Spring
a) D Soil moisture content (g g-1)
-0.12 -0.09 -0.06 -0.03 0.00
ns
ns
ns
ns
ns
Soil depth (cm)
0 5 10 15 20
b) D Urea-N (mg layer-1)
0 50 100 150 200 250
c) D NH4+-N (mg layer-1)
0 20 40 60 80
d) D NO3--N (mg layer-1)
0 5 10 15
e) D pH (1M KCl)
-0.3 0.0 0.3 0.6 0.9 1.2

Figure 3

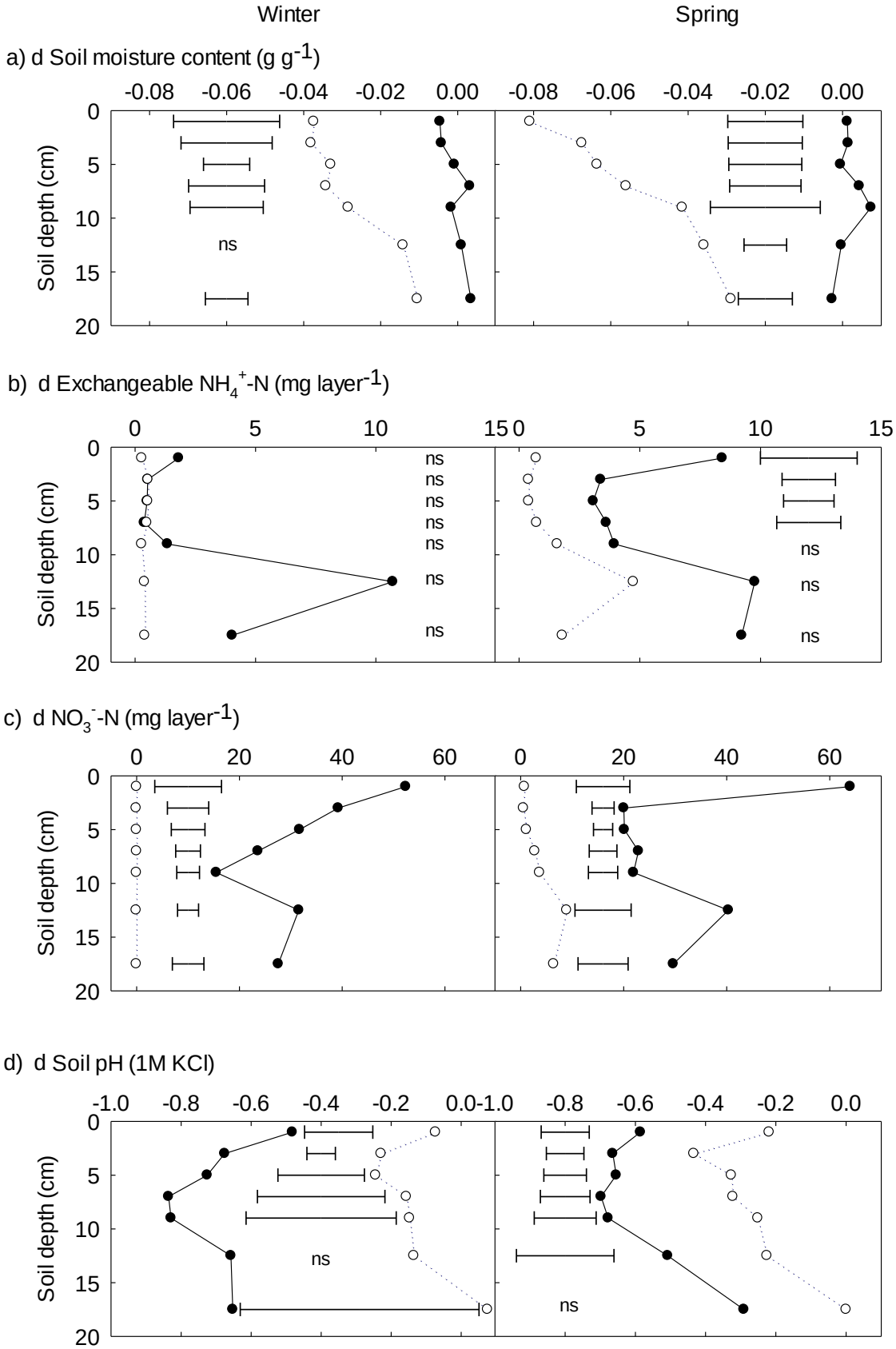
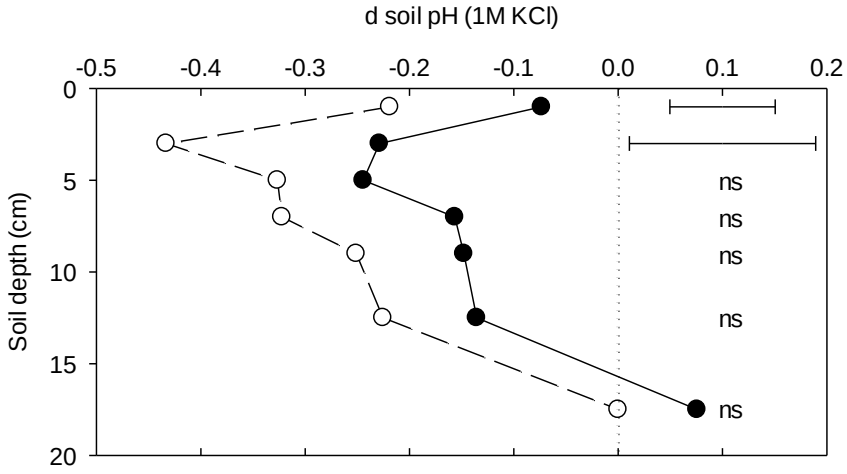

Winter
Spring
a) d Soil moisture content (g g-1)
-0.08 -0.06 -0.04 -0.02 0.00
Soil depth (cm)
0 5 10 15 20
ns
b) d Exchangeable NH4+-N (mg layer-1)
0 5 10 15
ns
c) d NO3--N (mg layer-1)
0 20 40 60
d) d Soil pH (1M KCl)
-1.0 -0.8 -0.6 -0.4 -0.2 0.0
0.0 -0.2 -0.4 -0.6 -0.8 -1.0

Figure 4


d soil pH (1M KCl)
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
0
5
10
15
20
Soil depth (cm)
ns
ns
ns
ns
ns