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Biology and Fertility of Soils

DOI:
10.1007/s00374-016-1092-x

Published: 01/05/2016

Peer reviewed version

The mobility of nitrification inhibitors under simulated ruminant urine deposition and rainfall: a comparison between DCD and DMPP

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Abstract Urine patches within pasture soils are hotspots for N cycling and losses, where nitrification inhibitors (NI) offer a means of reducing such losses. Within urine influenced soil, more research has been conducted for dicyandiamide (DCD) than 3,4-dimethylpyrazole phosphate (DMPP). Differences in the efficacy of these NI are often ascribed to a greater mobility of DCD, which may lead to spatial separation from NH_4^+ and nitrifying microorganisms. We tested the mobility of ^{14}C-labelled DCD and DMPP relative to sheep urine derived NH_4^+ in soil columns of contrasting texture and organic matter content, following simulated rainfall. We also assessed factors influencing the vertical mobility of these NI in soils, including solubility, sorption/desorption processes and microbial degradation and uptake. Following 40 mm rainfall, without the presence of sheep urine, the distribution of both NI were similar in the soil columns, however, there was a greater retention of DCD compared to DMPP in the top 1 cm. Both NI appeared to co-locate well with urine-derived NH_4^+, and the presence of sheep urine altered the leaching profile of the NI (compared to rainfall application alone), but this effect was inhibitor and soil type dependent. A greater sorption to the soil matrix was observed for DCD in comparison to DMPP in all three studied soils, and the presence of urine generally increased desorption processes. Of the NI applied to the soil columns, 18-66% was taken up within 30 min by the microbial community. However, only small amounts (<1%) were mineralized during this period. In conclusion, due to the greater adsorption of DCD as opposed to DMPP and similarity in the degree of co-location of both NI with urine NH_4^+, the results of this study suggest that differences in microbial uptake and degradation may be more important parameters for explaining differences in the efficacy of reducing nitrification. Further work is required to determine the comparative efficacy of both NI in reducing nitrification rates under field conditions in a range of soil types and environmental conditions.

Key Words: grazed grassland; livestock ecosystems; nitrogen use efficiency; nutrient dynamics

Introduction

In pasture soils, high loadings of nitrogen (N) are deposited within ruminant urine patches and these sites are particularly vulnerable to losses of N to the environment. Typically, 20% of deposited urinary-N is leached as NO_3^-, 13% is volatilised as NH_3 and 2% is emitted as the greenhouse gas N_2O (Selbie et al. 2015). While N_2O constitutes a small agronomic loss in terms of magnitude of N, having nearly 300 times the global warming potential of CO_2 (IPCC 2007), it accounts for 46% of agricultural greenhouse gas emissions (Smith et al. 2007). The agricultural sector will need to contribute to decreasing emissions (Misselbrook et al. 2014) in order to achieve targets (80% reduction from 1990 baseline levels by 2050) set by the UK, and other governments. Reducing N loss via NO_3^- leaching and improving N use efficiency would translate to a direct economic benefit for farmers and reducing N_2O emissions from grasslands could contribute to decreasing emissions from the livestock sector.

Nitrification inhibitors (NI) are a potential mitigation strategy which can reduce losses of N from urine patches deposited to grassland soils (Di and Cameron, 2012; Ledgard et al., 2014; Luo et al., 2015). By delaying the conversion of NH_4^+ to NO_3^-, the opportunity for plant acquisition, immobilization, fixation and adsorption of NH_4^+ is increased (Di and Cameron 2007). This can potentially reduce emissions of N_2O from both nitrification and denitrification (Gilsanz et al., 2016), where nitrification has been shown to be the dominant N_2O producing process in soils with a WFPS of 35-60%, but at a higher WFPS (70%), denitrification becomes the dominant N_2O producing process (Bateman and Baggs 2005). By the same processes, NI can also reduce the amount of NO_3^- available for leaching (Di and Cameron, 2004).

Two of the most widely used NI are dicyandiamide (DCD) and dimethylpyrazole-phosphate (DMPP) (Liu et al. 2013). DCD blocks the electron transport chain in the cytochrome of ammonia monoxygenase (AMO), whereas DMPP binds indiscriminately to the membrane-bound AMO (Chaves et al. 2006; Fiencke and Bocke 2006; Benckiser et al. 2013),

delaying the first and rate-limiting step of nitrification (the oxidation of NH_4^+ to NO_2^-; Zerulla et al. 2001). In comparison to DCD, DMPP has been shown to be less phytotoxic and lower application rates are required (Wissemeier et al. 2001; Zerulla et al. 2001; Di and Cameron 2012). Both NI have demonstrable efficacy in reducing N losses from fertilizer applications (Weiske et al. 2001; Liu et al. 2013) and livestock slurry (Fangueiro et al. 2009; Pereira et al. 2010), however, DCD applications to urine patches have been more widely researched (e.g. Di and Cameron 2003; O'Callaghan et al. 2010; de Klein et al. 2011) in comparison to DMPP (e.g. Di and Cameron 2011; Di and Cameron 2012). Some authors have found a difference between the efficacy of DCD and DMPP e.g. Weiske et al. (2001) found DMPP to be reduce N_2O emissions from fertilizer by an average of 49%, whereas DCD reduced emissions by an average of 26%. Di and Cameron (2012), however, found that DCD and DMPP reduced N_2O emissions by a similar amount from cattle urine (62 and 66% reduction, respectively). The efficacy of NI in reducing N_2O emissions and NO_3^- leaching can vary widely. In a laboratory study of nine contrasting UK soils, the efficacy of DCD to inhibit NH_4^+ oxidation, net NO_3^- production and emissions of N_2O was lower in soils of high temperature, clay content and organic matter content (McGeough et al. 2016). Differences in efficacy have been attributed to a lower mobility of DMPP in comparison to DCD, due to a greater sorption of DMPP (Wissemeier et al. 2001; Zerulla et al. 2001; Di and Cameron 2012). Having a high mobility may lead to the spatial separation of NI from NH_4^+ and nitrifying microorganisms (Ruser and Schulz 2015).

Physicochemical characteristics which can influence mobility within soil include solubility and sorption/desorption processes (Carrillo-González et al. 2006). The greater the solubility of a chemical in water, the greater the potential for vertical transport. The sorption/desorption of chemicals within the soil is mainly influenced by the organic matter content of soil, where charges associated with the chemical influences the types of bond

100 established. DCD is a net neutrally charged molecule and binding takes place on the surface
101 of organic matter, through hydrogen bonding of the $–NH_2$ and =NH functional groups to
102 negative carboxyl groups of organic matter (Zhang et al. 2004; Singh et al. 2008; Shepherd et
103 al. 2012). Conversely, DMPP is positively charged and adsorption is thought to occur to the
104 mineral fraction of soils, such as clays and silts (Barth et al. 2001, 2008). As chemicals are
105 transported through soil, further biological transformations may influence the quantity of
106 substance available for vertical movement e.g. microbial uptake and mineralization. As
107 DMPP is a heterocyclic compound, it is not readily degradable, resulting in a slower
108 degradation rates in soil in comparison to DCD (Weiske et al. 2001; Chaves et al. 2006). In
109 the specific conditions of urine influenced soils, the high concentration of NH_4^+, K^+ and other
110 cations, may saturate cation exchange sites leading to further movement of NI down the soil
111 profile.

112 The objective of this study was to obtain information on how the combination of NI
113 characteristics and soil conditions can affect the mobility and co-location of NH_4^+ and NI in
114 soils, with ruminant urine as the source of NH_4^+. We investigated physicochemical (solubility
115 and sorption/desorption) and biological (microbial uptake and degradation) factors
116 influencing the vertical mobility of DCD and DMPP, in soil columns of contrasting texture
117 and organic matter following a 40 mm rainfall event, with and without the presence of sheep
118 urine. We hypothesised that 1) DCD would move further down the soil profile than DMPP
119 following simulated rainfall, due to a greater sorption of DMPP, 2) a greater co-location
120 would be observed for DMPP with urine NH_4^+, due to the lower mobility of DMPP, 3) the
121 presence of sheep urine would increase vertical movement and desorption of both NI, due to
122 saturation of soil exchange sites by ions within sheep urine and 4) a greater microbial uptake
123 and mineralization would occur for DCD in comparison to DMPP, due to a greater
124 bioavailability of DCD in comparison to DMPP.

125

126 # Materials and methods

127 ## Soil and sheep urine analysis

128 Three soil types were selected for experimentation, on the basis of contrasting texture and
129 organic matter content: a sandy loam textured Eutric Cambisol (53°24′N, 4°02′W), a sandy
130 clay loam textured Eutric Cambisol (53°14′N, 4°01′W) and a high organic matter containing
131 Sapric Histosol (52°52′N, 0°47′W). Both Eutric Cambisol samples were collected from beneath
132 moderately sheep grazed and fertilised pasture, and the Sapric Histosol was collected from a
133 eutrophic lowland peat used in intensive arable agriculture. The soil types used in this study
134 had not been previously exposed to either DCD or DMPP. A summary of soil properties is
135 presented in Table 1.

136 Soil was sampled in triplicate (0–10 cm depth), sieved (< 2 mm) in order to reduce
137 sample heterogeneity and stored at 4°C until required. Soil moisture content was determined
138 by weight difference after oven drying (105°C), and organic matter was determined on dry soil
139 by loss-on-ignition in a muffle furnace (450°C; Ball 1964). Soil C:N ratio was determined on
140 oven-dried, ground soil samples using a TruSpec® Analyzer (Leco Corp., St. Joseph, MI). The
141 cation exchange capacity (CEC) of soils was determined using an unbuffered salt extraction
142 method of Schofield (1949). Soil pH and electrical conductivity (EC) were measured using
143 standard electrodes (1: 2.5 (w/v) soil-to-distilled water). Soluble C and N were determined in
144 1:5 soil-to-0.5 M K_2SO_4 extracts using a Multi N/C 2100S Analyzer (AnalytikJena, Jena,
145 Germany), within 24 h of sample collection, according to Jones and Willett (2006). Microbial
146 C and N were determined using the chloroform fumigation-extraction method of Voroney et
147 al. (2008) (K_{EC} = 0.35 and K_{EN} = 0.5). Total available nitrate (NO_3^-), ammonium (NH_4^+) and
148 phosphate (P) were determined within 0.5 M K_2SO_4 extracts via the colorimetric procedures of
149 Miranda et al. (2001), Mulvaney (1996) and Murphy and Riley (1962), respectively. Cations

150 (Na^+, K^+ and Ca^{2+}) were determined within 1:5 (w/v) soil-to-1 M NH_4Cl extracts using a
151 Sherwood Model 410 Flame Photometer (Sherwood Scientific Ltd, Cambridge, UK).
152 Sheep urine was collected from Welsh mountain ewes fed a diet of 80% *Lolium perenne*
153 L. and 20% *Trifolium repens* L., where several urine samples from a single sheep were pooled.
154 The urine was frozen (unacidified) before use to avoid losses of N. The sheep urine had a pH
155 of 8.99 and an EC of 22 mS cm^{-1}; the urine contained a total of 2.27 g N l^{-1}, 3.00 g organic C
156 l^{-1}, 1.71 g urea N l^{-1}, 44.9 mg NH_4^+-N l^{-1}, 0.44 mg NO_3^--N l^{-1}, 0.92 mg P l^{-1}, 7.16 g K l^{-1}, 1.11
157 g Na l^{-1} and 73.3 mg Ca l^{-1}. Properties were measured directly on the urine via the methods
158 described above and urea was measured using the method of Orsonneau et al. (1992).
159
160 **Comparative mobility of ^{14}C-DCD and ^{14}C-DMPP under simulated rainfall**
161 To compare the mobility of [U]^{14}C-DCD and 5-^{14}C-DMPP (American Radiolabeled
162 Chemicals, St Louis, MO, USA) in contrasting soils under a simulated rainfall event,
163 polypropylene tubes (n = 3; 15 cm depth; 0.8 cm diameter) were repacked with sieved, field
164 moist soil (soil sampled at 0-10 cm depth) to approximate field bulk density values (9, 8 and 5
165 g for the sandy loam, sandy clay loam and Sapric Histosol). This resulted in bulk densities of
166 1.0, 0.9 and 0.4 g cm^{-3} and porosities of 0.60, 0.67 and 0.71 in the sandy loam, sandy clay loam
167 and Sapric Histosol soil columns, respectively (particle density was assumed to be 2.65 g cm^{-3}
168 in the mineral soils and 1.4 g cm^{-3} in the organic soils; Rowell 1994). The bottom of the tubes
169 contained nylon mesh, to allow for drainage of leachate and to prevent any loss of soil.
170 Nevertheless, no leachate was present following the rainfall simulations. Field relevant
171 application rates of either ^{14}C-DCD (1 g l^{-1}; 50 µl; *ca.* 1 kBq) or ^{14}C-DMPP (0.1 g l^{-1}; 50 µl;
172 *ca*. 1 kBq) were applied to the top of the column and a 40 mm rainfall event was simulated by
173 adding 2 ml of distilled water drop-wise to the soil columns, *ca*. 5 minutes after the application
174 of the NI. This rainfall event was chosen to simulate UK storm conditions which promote rapid

water movement down the soil profile. It should also be noted that these leaching rates also approximate rates of water movement down preferential flow pathways in the soil profile under lower rainfall events. Preliminary studies indicated that the wetting front generally reached, but did not exceed the soil column length (15 cm).

The soil columns were incubated for 0.5 h, at laboratory temperature, after which the tubes were cut into the following depth fractions with a scalpel: 0-1, 1-2, 2-3, 3-5, 5-7, 7-9, 9-12 and 12-15 cm. The entire cut sections (including tube, to extract soil adhered to inner edge) were extracted with 0.5 M K_2SO_4 (1:5 w/v; 0.5 h; 150 rpm). An aliquot (1.5 ml) of the soil solution was centrifuged (14 000 *g*; 5 min) and the resulting supernatant was mixed with HiSafe 3 scintillant (PerkinElmer, Llantrisant, UK) and the activity measured using a Wallac 1404 Liquid Scintillation Counter (Wallac EG&G, Milton Keynes, UK).

Effect of ruminant urine on NI mobility and co-location of NI with urine ammonium

To determine if the presence of urine influences the vertical movement of DCD or DMPP, soil columns (n = 3) were prepared and processed as above. A sheep urine deposition event was simulated *ca.* 2 minutes following application of the ^{14}C-DCD or ^{14}C-DMPP and preceding the simulated rainfall event, by applying 250 µl of sheep urine to the top of the soil column. The vertical distribution of the NI was compared to soil columns incubated without urine.

The NH_4^+ concentration of the 0.5 M K_2SO_4 extracts of each depth fraction was also determined on the urine treated soil columns, via the method described previously, to determine the co-location of ^{14}C-DCD and ^{14}C-DMPP with urine-derived NH_4^+.

Solubility of DCD and DMPP in water

To determine the water solubility of DCD and DMPP the OECD (1995) flask method was used. Briefly, 5 g of NI was added to 10 ml of water (n = 3) and incubated at 30ºC on a rotary shaker

for 24 h. One replicate was then removed and incubated at 20ºC for 24 h with occasional shaking, before centrifuging at 10 000 *g*. Samples were syringe filtered (0.2 µm) and analysed for total dissolved C, as described above, and the amount of NI dissolved in the water calculated. One of the remaining replicates was incubated for another 24 h at 30ºC and the final replicate was incubated for an additional 48 h, before incubation at 20ºC for a further 24 h and preparation of samples for analysis of dissolved C. This was conducted to ensure additional time had no effect on the amount of NI dissolved.

Sorption and desorption

Sorption isotherms were determined for ^{14}C-DCD and ^{14}C-DMPP in the three contrasting soils in the presence and absence of sheep urine. Briefly, ^{14}C-DCD or ^{14}C-DMPP was applied (50 µl; *ca*. 1 kBq) to 0.5 g (n = 3) of field moist soil, following which 2.5 ml of either 0.01 M $CaCl_2$ or sheep urine was added to the soils. A total of 8 concentrations of ^{14}C-DCD and ^{14}C-DMPP were used, ranging from 0.08-10 mg NI l^{-1}. The soil suspensions were shaken for 0.5 h at 150 *rpm* on a rotary shaker, subsequently an aliquot (1.5 ml) was centrifuged (10 000 *g*; 5 min) and the ^{14}C activity in the supernatant determined by liquid scintillation counting, as above. Sorption of NH_4^+ was also assessed as described above, using 8 concentrations ranging from 2.3-300 mg NH_4^+-N l^{-1}, in 0.01 M $CaCl_2$. The NH_4^+ concentration in the supernatant was determined as above. The partition coefficient (K_d) for the NI (with and without the presence of urine) and NH_4^+ within soil, was determined via Equation 1, where C_{ads} (µmol kg^{-1}) is the concentration adsorbed to the soil solid phase at equilibrium and C_{sol} (µmol l^{-1}) is the adsorbate concentration remaining in solution at equilibrium.

$$K_d = C_{ads} / C_{sol} \quad \text{(Eqn. 1)}$$

Desorption, with and without the presence of sheep urine, was determined by adding either ^{14}C-DCD (25 µl; *ca*. 0.5 kBq) or ^{14}C-DMPP (25 µl; *ca*. 0.5 kBq) at two concentrations

(1 and 10 mg l^{-1}) to 0.2 g of soil (n = 3). Four successive washes of the soil were conducted by adding either 1 ml of 0.01 M $CaCl_2$ or sheep urine and by conducting a final wash with 0.5 M K_2SO_4. Samples were shaken for 0.5 h at 150 *rpm* on a rotary shaker between the additions of fresh wash solution. At the end of each wash period the samples were centrifuged (10 000 *g*; 5 min) and the supernatant removed prior to adding fresh 0.01 M $CaCl_2$ or sheep urine. The activity in the supernatant was determined as described previously, where the activity residing in the entrained solution trapped within the soil matrix was accounted for. After the final wash, soils were dried (105 °C; 24 h) and ground before combustion in an OX400 biological oxidizer (RJ Harvey, Hillsdale, NJ, USA), where evolved $^{14}CO_2$ was captured in Oxysolve C-400 (Zinsser analytic, Frankfurt, Germany) to quantify ^{14}C remaining bound to soils following washes.

Substrate mineralization and microbial uptake

Mineralization of ^{14}C-DCD and ^{14}C-DMPP in the three soils was determined to quantify the degradation of the NI during the course of the incubation. 0.3 ml of ^{14}C-DCD or ^{14}C-DMPP (*ca.* 0.5 kBq ml^{-1}; 0.1 and 1 g l^{-1}) were added to 1 cm^3 of soil (n = 3), contained in 10 cm^3 glass vessels. Evolved $^{14}CO_2$ was captured by flowing (*ca*. 100 ml min^{-1}) moist air over the soil surface, with the outflow passing through two consecutive 0.1 M NaOH traps (capture efficiency > 95%; Hill et al. 2007). Traps were changed after 0.05, 0.12, 0.25, 0.5, 1, 2, 4 and 8 h, and the activity in the solution determined by liquid scintillation counting as above.

To enable calculation of the ^{14}C-NI pool taken up by soil microbes, ^{14}C-DCD or ^{14}C-DMPP (0.3 ml; *ca.* 0.5 kBq; 0.1 and 1 g l^{-1}) was pipetted evenly onto the soil surface (1 g; n = 3) and extractions using ice-cold 0.5 M K_2SO_4 (1:5 w/v) were conducted at 0.05, 0.12, 0.25, 0.5 and 1 h following addition of the substrate. The soils were shaken (150 rpm; 0.5 h) and subsequently centrifuged (10 000 *g*, 10 min). The ^{14}C in the resulting supernatant was

250 determined via liquid scintillation counting as described above. Uptake of the substrate by soil
251 microbes was calculated by deducting the 0.5 M K_2SO_4 extractable pool from the starting ^{14}C
252 pool. This is assuming the extraction procedure removed all exchangeable ^{14}C-NI and the
253 remainder was taken up into the microbial biomass.
254
255 **Statistical analysis**
256 To compare the vertical mobility of ^{14}C-DCD and ^{14}C-DMPP under rainfall, to determine how
257 urine influences the vertical mobility of the NI and to compare the co-location of the NI with
258 that of urine-NH_4^+, a one-way ANOVA with Tukey's post-hoc test was used to compare each
259 section depth, following testing for normality (Ryan-Joiner test) and homogeneity of variance
260 (Levene's test). The same analysis was conducted on the slope of the linear sorption isotherms,
261 following log transformation of data. A one-way ANOVA was also conducted for desorption
262 after the fourth wash in either urine or $CaCl_2$, for mineralization at the 8 h time point and
263 microbial uptake after 1 h. All statistical analyses were performed in Minitab 17.1.0 (Minitab
264 Inc., State College, PA).
265
266 # Results
267 **Comparative vertical mobility of NI following simulated rainfall**
268 The distribution of extractable DCD-^{14}C and DMPP-^{14}C following simulated rainfall was
269 generally similar within each soil type (Fig. 1). However, a greater retention of ^{14}C-DCD was
270 observed in comparison to ^{14}C-DMPP in the top 0-1 cm depth fraction of the sandy loam (Fig.
271 1a) and sandy clay loam columns (Fig. 1b). The total amount of DCD-^{14}C extracted from the
272 columns with 0.5 M K_2SO_4 after 0.5 h was 66.6 ± 1.29, 63.9 ± 0.65 and 38.8 ± 0.53% of that
273 applied to the sandy loam, sandy clay loam and the Sapric Histosol, respectively. In
274 comparison, the percentage of DMPP-^{14}C extracted from the columns was 79.4 ± 2.14, 72.5 ±

1.42 and 39.1 ± 1.39% of that applied to the sandy loam, sandy clay loam and Sapric Histosol, respectively.

The presence of sheep urine reduced ($p < 0.01$) the quantity of extractable DCD-^{14}C and DMPP-^{14}C in the top 1 cm of the sandy loam columns (Fig. 1a and d), increased ($p < 0.01$) the amount of extractable DCD-^{14}C in the bottom 12-15 cm depth fraction and had no effect ($p > 0.05$) on the extractable amount of DCD-^{14}C and DMPP-^{14}C in each remaining depth fraction. The presence of sheep urine did not influence the extractable amount of DCD-^{14}C or DMPP-^{14}C in any studied depth fraction of the sandy clay loam columns (Fig. 1b and e). The presence of urine had no effect ($p > 0.05$) on the amount of extractable DCD-^{14}C in each depth fraction of the Sapric Histosol (Fig. 1c and f). However, it decreased ($p < 0.001$) the extractable amount of DMPP-^{14}C from the top 1 cm and increased ($p < 0.001$) the extractable amount in the 12-15 cm depth fraction.

The percentage of applied DCD-^{14}C extracted from the soil columns with applied sheep urine plus rainfall was 79.6 ± 6.58, 72.9 ± 1.92 and 43.6 ± 0.73% of the added label applied to the sandy loam, the sandy clay loam and Sapric Histosol, respectively. The total amount of DMPP-^{14}C extracted from the soil columns under the same conditions was 79.4 ± 0.77, 71.5 ± 3.54 and 47.9 ± 0.01% in the sandy loam, sandy clay loam and Sapric Histosol, respectively. In conclusion, urine increased the total amount of DCD extracted from the soils, but had no effect on DMPP.

Co-location of NI with urine-derived ammonium

In general, the distribution of both DCD-^{14}C and DMPP-^{14}C within the soil profile coincided well with the urine-derived NH_4^+ (Fig. 2). A greater ($p < 0.001$) percentage of total column extractable DCD-^{14}C in comparison to NH_4^+ was found in the top 1 cm in all three soil types (Fig. 2a, b, and c), indicating a retention of DCD at the soil surface. A greater ($p < 0.001$)

300 percentage of total extractable NH_4^+ in comparison to DMPP-^{14}C was found in the 9-12 cm
301 depth fraction of the sandy loam columns (Fig. 2d). Greater ($p < 0.001$) amounts of total
302 extractable NH_4^+ in comparison to DCD-^{14}C were also found in the 9-12 cm depth fraction of
303 the sandy clay loam columns (Fig. 2e), indicating some dis-location of NI with NH_4^+ at depth.
304 No differences were observed at any depth fraction for DMPP-^{14}C and urine-NH_4^+ in the sandy
305 clay loam or the Sapric Histosol columns (Fig. 2e and f, respectively), indicating similar
306 vertical distributions under conditions of mass flow.
307
308 **Solubility of DCD and DMPP in water**
309 The solubility of DMPP at 20°C was significantly higher ($p < 0.001$) at 125 ± 2.4 g l^{-1} in
310 comparison to that of DCD at 73.2 ± 2.0 g l^{-1}. An increasing trend was not observed in the
311 replicates maintained for 48 and 72 h, indicating that saturation of the NI within the matrix had
312 occurred after 24 h.
313
314 **Sorption**
315 Sorption isotherms for DCD-^{14}C (Fig. 3a, c and e), DMPP-^{14}C (Fig. 3b, d and f) and NH_4^+ (Fig.
316 4) were linear, where all R^2 values were greater than 0.95. The gradient of the linear sorption
317 isotherms were steeper ($p < 0.001$) in the Sapric Histosol compared to the other soil types for
318 both DCD-^{14}C, DMPP-^{14}C and NH_4^+ indicating greater amounts of sorption in this soil type.
319 In comparison to DMPP-^{14}C, greater sorption occurred for DCD-^{14}C in the Sapric Histosol in
320 both matrices (0.01 M $CaCl_2$ and urine). However, no differences were observed between
321 DCD-^{14}C and DMPP-^{14}C sorption in the other two soil types ($p > 0.05$). In the Sapric Histosol
322 the gradient of the DMPP-^{14}C sorption isotherm was steeper ($p < 0.001$) in the 0.01 M $CaCl_2$
323 matrix as opposed to the urine matrix. The calculated soil-to-solution partition coefficients (K_d;
324 Table 2) followed the trend sandy loam < sandy clay loam < Sapric Histosol for DCD-^{14}C,

DMPP-^{14}C and NH_4^+; for the NI this trend was observed at both concentrations and within both matrices.

Desorption

Generally, the presence of urine increased total desorption (Fig. 5) of both DCD-^{14}C and DMPP-^{14}C numerically (although not statistically) at the fourth consecutive wash, in all soil types. The presence of urine increased desorption of DMPP-^{14}C in the Sapric Histosol at 1 mg l^{-1} ($p < 0.001$; Fig. 5k) and 10 mg l^{-1} ($p < 0.05$; Fig. 5l). Interestingly, the same trend was not observed for DCD-^{14}C in the same soil type (Fig. 5i and j). Desorption of DCD-^{14}C in the $CaCl_2$ matrix was greater ($p < 0.05$) in the sandy loam soil (Fig. 5a and b) compared to the Sapric Histosol (Fig. 5i and j), but desorption was no greater ($p > 0.05$) in the sandy clay loam soil (Fig 5e and f). In the urine matrix desorption of DCD-^{14}C was lower ($p < 0.01$) in the Sapric Histosol (Fig. 5i and j) compared to the sandy loam (Fig. 5a and b) and sandy clay loam (Fig. 5e and f) textured Eutric Cambisol at both studied concentrations. For both studied concentrations of DMPP-^{14}C in the $CaCl_2$ matrix, desorption was lower ($p < 0.01$) in the Sapric Histosol (Fig. 5k and l) in comparison to either the sandy loam (Fig. 5c and d) or the sandy clay loam (Fig. 5g and h) Eutric Cambisol. In the urine matrix, however, no differences in desorption of DMPP-^{14}C was observed at either concentration between the soil types.

When comparing between the applied ^{14}C-NI in the $CaCl_2$ matrix, there was a greater desorption ($p < 0.05$) of DMPP-^{14}C (Fig. 5e and f) in comparison to DCD-^{14}C (Fig. 5g and h) in the sandy clay loam. The same trend was true for the urine matrix, except no differences were observed between DCD-^{14}C and DMPP-^{14}C at 1 mg compound l^{-1}. In the urine matrix, greater ($p < 0.001$) amounts of DMPP-^{14}C (Fig. 5k and l) desorbed in comparison to DCD-^{14}C (Fig. 5i and j) in the Sapric Histosol, at both studied concentrations. The final wash conducted with 0.5 M K_2SO_4 typically only increased desorption by minor amounts (ranging from 1.4 to

6.0%), indicating that the previous washes had removed the majority of the extractable NI-^{14}C from the soils. The mass balance for the total recovered DCD-^{14}C and DMPP-^{14}C following biological oxidation was 101 ± 0.63% and 101 ± 0.95% respectively, for all soil types, applied concentrations and matrices.

Mineralization

The results of the mineralization assay confirmed that only minor degradation of the ^{14}C-NI would have occurred under the conditions and duration (0.5 h) of the column study. Mineralization of ^{14}C-DCD within all three soil types ranged from 0.10 to 0.35% of added ^{14}C label, over 0.5 h. For ^{14}C-DMPP the amount degraded over the period of the column incubation was even lower, ranging from 0.03 to 0.16% of the added ^{14}C label. After 8 h, the amount of ^{14}C-DCD mineralized was still low, ranging from 0.41 to 1.67% of the ^{14}C-label applied in the three soil types and at both studied concentrations; the amount of ^{14}C-DMPP mineralized after 8 h ranged from 0.05 to 0.25% of the applied label. Greater amounts ($p < 0.01$; 0.84 ± 0.15 and 1.53 ± 0.30% more at 0.1 and 1 g NI l^{-1}, respectively) of ^{14}C-DCD mineralized in the Sapric Histosol in comparison to ^{14}C-DMPP, at both studied concentrations. The same pattern was also seen for the sandy clay loam textured Eutric Cambisol at 0.1 g NI l^{-1}, where 0.83 ± 0.12% more ^{14}C-DCD was mineralized in comparison to ^{14}C-DMPP. No differences were observed in the amount of ^{14}C-DMPP mineralized between all soil types at either studied concentration. For ^{14}C-DCD at 0.1 g l^{-1}, 1.17 ± 0.21% and 0.69 ± 0.18% more^{14}C-DCD mineralized in the Sapric Histosol in comparison to either the sandy loam or sandy clay loam textured Eutric Cambisol, respectively. No differences ($p > 0.05$) were observed in the amount of ^{14}C-DCD mineralized between the different soils at the higher studied concentration.

Microbial uptake

After 1 h in the sandy loam textured Eutric Cambisol, no difference ($p < 0.05$) was observed between the amount of DCD-^{14}C or DMPP-^{14}C acquired by the soil microbes, which ranged between 20 and 23% of that applied, at both studied concentrations. The same trend was observed in the sandy clay loam textured Eutric Cambisol, where uptake ranged from 18 to 28% of that applied. In the Sapric Histosol, greater amounts ($p < 0.001$) of DCD-^{14}C (66 ± 0.36%) was acquired by soil microbes in comparison to DMPP-^{14}C (51 ± 2.67%) at the higher application rate, however, no differences ($p > 0.05$) between DCD-^{14}C (56 ± 0.32%) and DMPP-^{14}C (61 ± 1.01%) were observed at the lower concentration. The microbial uptake was two to three-fold greater ($p < 0.001$) in the Sapric Histosol compared to the mineral soils for both NI and at both studied concentrations. The results of the microbial uptake study correspond well with that of the soil column studies, indicating that the deficit in the amount of ^{14}C-NI recovered from the soil columns is that which was immobilised into microbial biomass and degraded within the soils.

Discussion

Our first hypothesis was that DCD would be more mobile and translocate further down the soil profile than DMPP, due to the positive charge and rapid sorption of DMPP to soil colloids (Azam et al. 2001). The results of the column study investigating the vertical movement of DCD and DMPP over 0-15 cm under a 40 mm rainfall event, revealed that the mobility of both NI were similar, and DCD did not appear to be more mobile than DMPP. A greater sorption for DCD in comparison to DMPP was found in the organic and mineral soils, contradicting our hypothesis that a greater adsorption of DMPP would occur. A greater sorption was found in the Sapric Histosol compared to the mineral soils for both NI, suggesting negatively charged domains within organic matter are important for adsorption processes. However, if the results of Fig.5 are expressed on a per g of organic C basis, the results between the soils are more

400 similar, suggesting a partition phenomenon rather than charged-based sorption. Compounds
401 possessing a greater octanol-water partition coefficient than others will show slower sorption;
402 the octanol-water partition coefficient (Log P) of DCD and DMPP is predicted to be -1.03 and
403 0.92 for DCD and DMPP, respectively (Chemicalize.org, 2016). In addition, DCD is
404 hydrophilic (Turowski and Deshmukh 2004), which in combination with the low value for the
405 octanol-water partition coefficient, suggests strong absorption and permeation into organic
406 matter. Protection and occlusion of NI from nitrifiers and other microbes due to sorption, may
407 reduce the effectiveness of NI, at least in the short term. Sorption may also protect against some
408 microbial degradation, and if remobilisation of NI occurs, it may prolong the inhibitory effect
409 (Barth et al. 2001).

410 Under some circumstances DMPP has been found to be more effective than DCD at
411 inhibiting nitrification and reducing N_2O losses (Weiske et al. 2001; Chaves et al. 2006;
412 Irigoyen et al. 2006) and this difference in efficacy is often attributed to the lower mobility of
413 DMPP in comparison to DCD and hence a greater spatial separation of DCD with NH_4^+.
414 Nevertheless, the inhibition of the oxidation of NH_4^+ to NO_3^- only occurs when the nitrifying
415 population have taken up the NI. This study only examined the mobility of NI and NH_4^+,
416 however, a consideration of the distribution of nitrifying microorganisms and their acquisition
417 of the NI is an important aspect for future research. Our second hypothesis was that DMPP
418 would co-locate with urine-NH_4^+ more than DCD. In our study, both NI appeared to coincide
419 well with urine-derived NH_4^+, with only few incidences of the percentage of extractable NH_4^+
420 being higher than the extractable NI-^{14}C label at depth. Nevertheless, this study only focused
421 on the short-term coincidence of NI with urine-NH_4^+, and further generation of urine-NH_4^+
422 would occur post urea hydrolysis. Being a neutral compound, urea may also be susceptible to
423 vertical transport (Dawar et al., 2011). However, urea hydrolysis is normally complete within
424 ca. 2 days, reducing the time available for vertical movement. Comparing the soil-to-solution

partition coefficients of the NI and NH_4^+, it appears that the K_d of NH_4^+ and DCD are more similar than that of DMPP and NH_4^+ in the sandy loam and the sandy clay loam soils, but large differences were found for both NI compared to NH_4^+ in the Sapric Histosol. It may be possible to use NI and NH_4^+ K_d values from differing soil types in order to assess which (if any) inhibitor may be more effective, where similar K_d values may result in a greater co-location of the two chemicals. Further work is required to assess whether this would be a useful proxy for reducing N_2O emissions and improving NI use in order to maximise efficacy.

The third objective was to determine if the presence of sheep urine resulted in a greater vertical movement of both NI. Without the presence of urine (rainfall only), a retention of DCD was observed in the top 1 cm of the sandy loam and sandy clay loam textured soils, and a retention of both NI was found in the top 1 cm of the Sapric Histosol. A retention of NI at the surface may be beneficial in that nitrification decreases with pasture soil depth (Young et al. 2002), which may result in a greater coincidence of NI with nitrifiers, nevertheless, the use of sieved soils in this study would have altered any natural depth distribution of nitrifiers in the incubated soil profiles. The addition of sheep urine to the soil columns had a mixed effect on the depth distribution of the NI, depending on the soil type and inhibitor. Relative to simulated rainfall alone, the presence of sheep urine resulted in a greater amount of extractable DCD-^{14}C at the bottom of the sandy loam soil columns, and reduced the extractable amount of both DCD-^{14}C and DMPP-^{14}C in the top 1 cm of the sandy loam soil columns, however, no differences in depth distribution of either NI were observed following sheep urine and rainfall application to the sandy clay loam soil. The presence of sheep urine resulted in lower amounts of DMPP-^{14}C at the top 1 cm and greater amounts of extractable DMPP-^{14}C at 12-15 cm in the Sapric Histosol, but no such trend was observed for DCD-^{14}C. To consider the reasons behind these results, a consideration of the soil, NI and urine properties are required.

449 The soils used in this study were all of a similar pH (Table 1), however the addition of
450 urine would have altered the soil pH and made conditions in the soil columns more alkaline.
451 As DCD is amphipathic, sorption has been shown to be pH dependent, where increases in pH
452 above pH 5 lead to increased sorption (Zhang et al. 2004). This may partially explain why the
453 vertical distribution of DCD-^{14}C was similar whether urine was present or not in the Sapric
454 Histosol. In the solubility assay, DMPP was found to be over 1.5 times more soluble in water
455 than DCD. However, a saturated solution (125 g l^{-1}) of DMPP is acidic (ca. pH 3), whereas
456 dissolving DCD results in a near neutral pH. The solubility of DMPP at pH 7, is reported to be
457 46 g l^{-1} (Zerulla et al. 2001) - considering this value results in DCD having a greater solubility
458 than DMPP. Thus, DMPP solubility may vary widely as a function of soil pH and buffer
459 capacity, but whether this influences the mobility relative to DCD is unclear. As the soils used
460 in this study had a similar pH, NI solubility would not have varied much due to the effect of
461 soil type.

462 The sandy loam and sandy clay loam soils had a similar CEC and organic matter content
463 (Table 1), however, both parameters were greater in the Sapric Histosol. The NI sorption and
464 partitioning into organic matter, and the availability of cation exchange sites may have all
465 contributed to the differences in the distribution of the NI, with and without the presence of
466 urine. The low CEC in the mineral soils and saturation of these exchange sites with cations
467 present within the urine, may explain the higher amounts of DCD-^{14}C and DMPP-^{14}C at the
468 bottom of the sandy loam soil columns, compared to the rainfall only treatment. This trend did
469 not hold true, however, for the sandy clay loam columns despite the similarity in soil properties.

470 The results of our desorption assays revealed that even after one wash with 0.01 M
471 $CaCl_2$, a large proportion of DCD and DMPP was remobilised into solution. This supports the
472 theory that remobilisation of these NI may occur e.g. following urine deposition and/or heavy
473 rainfall events. In the case of ruminant urine events this effect may be important, as urine is

generally deposited at varying times following NI application to pasture. While the presence of urine generally increased desorption, the effect was strongest for DMPP-^{14}C in the Sapric Histosol, and weakest for DCD-^{14}C in the same soil type. This again shows a clear contrast in the binding mechanisms and behaviour of these two NI. In the Sapric Histosol, the vertical movement of DMPP was enhanced due to the presence of urine. This trend was not observed for DCD, which may be due to differences in the physico-chemical properties of these NI. The partitioning and adsorption of DCD in the organic soil may have prevented its vertical movement due to urine addition.

The short-term microbial mineralization of DCD was faster in comparison to DMPP in all soil types, which supports our fourth hypothesis. This is consistent with results of other studies, where DMPP has been found to have a longer residence time in comparison to DCD (Chaves et al. 2006). DCD degrades to CO_2 and NH_4^+ via guanilyc urea, guanidine, and urea (Amberger 1986; Kelliher et al. 2008). The first stage of DCD degradation can occur on the surface of metal oxides, which catalyse the reaction of DCD and water to guanylurea (Hallinger et al. 1990). Biological degradation of DCD by common soil microorganisms has also been demonstrated, where DCD is supplied as the sole N source in pure culture (Hauser and Haselwandter 1990; Schwarzer et al. 1998). As DMPP is a heterocyclic compound, it is not readily degradable (Chaves et al. 2006), although information on the mechanism and degradation pathways of this inhibitor are still lacking. Results from this study indicate that the microbial community were better able to degrade DCD in comparison to DMPP, where DMPP degrading bacteria may take longer time periods to establish in comparison to DCD. DMPP has also been found to have a longer effect on the abundance of ammonium oxidizing bacteria in comparison to DCD (Kuo et al. 2015) and there is evidence that DMPP has an inhibitory effect on both ammonium oxidizing bacteria and archaea (Florio et al. 2014).

In this study even after 1 h, the microbial uptake of both inhibitors accounted for a large proportion of that applied. Approximately 20% was taken up by soil microbes in the mineral soils and > 50% of that applied was taken up by soil microbes in the Sapric Histosol, which was likely to be a function of the greater microbial biomass in this soil. To be effective, the NI would need to be acquired by the target microbial biomass (ammonium oxidizing bacteria and archaea). Immobilisation into non-target microbial biomass could, therefore, equate to a fairly large removal mechanism for these NI and this requires further investigation. No difference was observed between DCD and DMPP in the proportion acquired by soil microbes in the mineral soils. However, uptake was greater for DCD compared to DMPP in the Sapric Histosol at the higher studied concentration. This suggests a slight preference of, or bioavailability of DCD to the soil microbial community in the short-term.

The results of this study should be considered with care, as repacked soils were used and there were no preferential flow pathways as would occur under field conditions, which would potentially enhance the vertical movement of either NI. The soils were also sieved, which removed any natural variation of soil properties which can occur with depth. The use of soil columns could have also promoted vertical movement of NI or urine, by restricting lateral diffusion. This is in contrast to the study by Azam et al (2001), where shallow petri dishes were used and NI and NH_4^+ were applied in the centre. This approach may have promoted lateral movement of solutes. Further work should attempt to establish the comparative efficacy of both NI, their uptake by nitrifying and non-nitrifying microorganisms and co-location with NH_4^+ over longer time scales and under field conditions.

Conclusions

A similar distribution of DCD and DMPP was observed up to a depth of 15 cm following a simulated rainfall event in one organic and two mineral soils. The presence of sheep urine did

not influence the depth distribution of DCD following rainfall, but enhanced the movement of DMPP down the profile, especially in the organic soil. A greater sorption was found for DCD in comparison to DMPP in the soil types studied here and the presence of urine generally increased desorption of both NI. The results of our study suggest that the efficacy of NI are influenced more by differences in microbial uptake and degradation rates than by differences in sorption and desorption rates to the soil matrix.

Acknowledgements KAM would like to thank the School of Environment, Natural Resources and Geography, Bangor University, for sponsoring this study. This work was supported by the UK Natural Environment Research Council, under grant award (NE/M015351/1).

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718 **Figure Legends**

719 **Figure 1**. The percentage of extractable DCD-^{14}C or DMPP-^{14}C at depth fractions of sandy
720 loam (a and d), sandy clay loam (b and e) and Sapric Histosol (c and f) columns,
721 following a simulated 40 mm rainfall event (a, b and c) and a sheep urine deposition
722 plus a 40 mm rainfall event (d, e and f). Soil type labels apply to each column and
723 legends apply to each row of panels. Bars represent means (n = 3) and error bars
724 represent SEM.

725 **Figure 2**. The percentage of extractable DCD-^{14}C (a, b and c) or DMPP-^{14}C (d, e and f)
726 compared to NH_4^+ at differing soil depth fractions following a simualted urine
727 deposition plus 40 mm rainfall event applied to sandy loam (a and d), sandy clay
728 loam (b and d) and Sapric Histosol (c and f) columns. Soil type labels apply to each
729 column and legends apply to each row of panels. Bars represent means (n = 3) and
730 error bars denote SEM.

731 **Figure 3**. Linear sorption isotherms for ^{14}C-DCD (a, c and e) and ^{14}C-DMPP (b, d and f) in
732 either a 0.01 M $CaCl_2$ or sheep urine matrix, in a sandy loam (a and b), sandy clay
733 loam (c and d) and Sapric Histosol (e and f). Symbols represents means (n = 3), bi-
734 directional error bars represent SEM for sorption and equilibrium solution
735 concentrations, legends apply to each column of panels and soil type labels apply to
736 each row of panels.

737 **Figure 4**. Linear sorption isotherms of NH_4^+ in 0.01 M $CaCl_2$ matrix, in three soils (sandy loam
738 and sandy clay loam textured Eutric Cambisol and a Sapric Histosol). Symbols
739 represents means (n = 3) and bi-directional error bars represent SEM for sorption
740 and equilibrium solution concentrations.

741 **Figure 5**. Cumulative desorption of ^{14}C-DCD and ^{14}C-DMPP in a sandy loam (a, b, c and d),
742 sandy clay loam (e, f, g and h) and a Sapric Histosol (i, j, k and l) at 1 mg DCD l^{-1}

743 (a, e and i), 10 mg DCD l^{-1} (b, f and j), 1 mg DMPP l^{-1} (c, g and k) and 10 mg DMPP
744 l^{-1} (d, h and l) in either a 0.01 M $CaCl_2$ or sheep urine matrix. Text in the top row of
745 panels applies to each respective column of panels and legend applies to all panels.
746 Symbols represents means (n = 3) and error bars represent SEM for sorption and
747 equilibrium solution concentrations.