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Measurement of grass uptake of the urease inhibitor NBPT and of the nitrification inhibitor dicyandiamide co-applied with granular urea

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Abstract

Grass uptake and phytoaccumulation factors of N-(n-butyl) thiophosphoric triamide (NBPT) and dicyandiamide (DCD) were quantified. Following the application of urea fertiliser treated with the inhibitors in Irish grassland, grass samples were collected at 5, 10, 15, 20 and 30 day time intervals following five application cycles. Uptake of NBPT by grass was below the limit of quantitation of the analytical method (0.010 mg NBPT kg^{-1}). Dicyandiamide concentrations in grass ranged from 0.004 to 28 mg kg^{-1} with the highest concentrations measured on days 5 and 10. A reducing trend in concentration was found after day 15. The DCD phytoaccumulation factor was ranged from 0.004% to 1.1% showing that DCD can be taken up by grass at low levels when co-applied with granular urea. In contrast NBPT was not detected indicating that grass uptake is unlikely when co-applied with granular urea fertiliser. The contrasting results are likely due to very different longevity of DCD and NBPT along with the much lower rate of NBPT which is used compared with DCD.

Keywords: N-(n-butyl) thiophosphoric triamide, Dicyandiamide, phytoaccumulation factor, residue, Ireland

Introduction

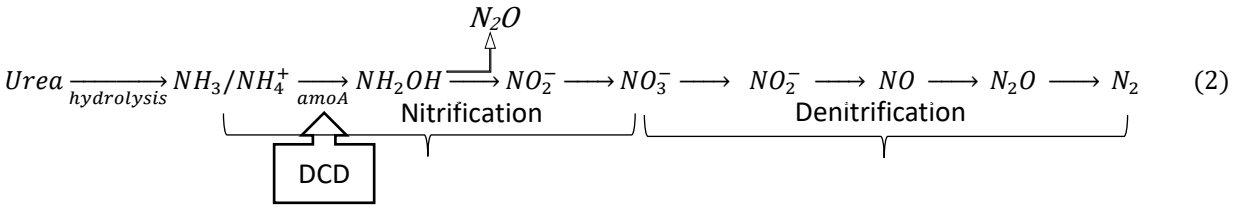
In the last decades, dairy consumption has grown tremendously in the most developed nations resulting dairy industry becoming more prominent and intensive (Alvarez et al. 2010; Winsten et al. 2010; O'Brien et al. 2018). Dairy products are highly nutritious and their inclusion in the human diet provides essential amino acids (EAA) and micronutrients otherwise lacking in typical plant-based foods (Haug et al. 2007). Worldwide, dairy products consume an average of 87 kg per person annually, with 82.4% of the world's milk produced by dairy cows (Britt et al. 2018). It is currently estimated that 10% of the global milk production originates from grazing based systems (Joubran et al. 2021). Grass from grazing pasture is the basis for dairy production systems in regions with temperate climates, such as in Ireland, Europe, the United States, New Zealand and parts of Australia. Consumers typically perceive grass-fed or pasture-based farming positively regarding cow welfare and environmental sustainability (Armbrecht et al. 2019; Shortall et al. 2019). Grass growth often responds positively to increased availability of soil nitrogen (N), which is the critical limiting nutrient. Therefore, application of inorganic N fertilisers is regularly used to optimise pasture production. However, plant utilisation of fertiliser N can frequently be less than 50% (Anas et al. 2020). Nitrogen applied in excess of the pasture's agronomic requirements may lead to increased N losses to the environment through nitrate leaching, ammonia volatilisation, surface runoff and gaseous nitrous oxide (N_2O) emissions to the atmosphere (Garwood et al. 1986; Good et al. 2011; Forrestal et al. 2017; Krol et al. 2020; Rahman et al. 2021a). When urea is applied to the soil, it rapidly hydrolyzes to ammonium carbonate. Urea hydrolysis, catalysed by urease enzymes, is a fast process in soil and involves proton consumption, increasing the soil pH in the surrounding area of urea granules (Cantarella et al. 2018). The urea hydrolysis produces ammonium (NH_4^+)/ammonia (NH_3) and CO_2. The simplified equation is as follows:

$$CO(NH_2)_2 + 2H^+ + 2H_2O \xrightarrow{urease} 2NH_4^+/NH_3 + H_2O + CO_2 \qquad (1)$$

NBPT

As urea hydrolysis consumes protons (H^+), the soil pH increases, driving the equilibrium between NH_4^+ and NH_3 toward the formation of the gaseous form (Cantarella et al. 2018).

After urea hydrolysis, NO_3^- and gaseous N_2O are produced through the microbial nitrification (the oxidation of NH_3/NH_4^+ to NO_2^- and subsequently NO_3^- by aerobic ammonia-oxidizing bacteria) and denitrification (the reduction process of NO_3^- to N_2 mediated by facultative anaerobic bacteria) process (Bouwman et al. 2003; Butterbach-Bahl et al. 2013) in the soil which is shown below by an equation.



Research has focused on developing a range of technologies to mitigate N losses. Inhibitor technology, including urease and nitrification inhibitors, are useful tools for reducing environmental losses from nitrogen-based fertilisers. The urease inhibitor, N-(nbutyl) thiophosphoric triamide (NBPT), has proven to be one of the most cost-effective tools in mitigating ammonia volatilization losses from urea (Fischer et al. 2016). Treating urea with the urease inhibitor NBPT, a structural analog of urea, has the active ingredient N-(n-butyl) thiophosphoric triamide (NBPT). The oxygen analog of NBPT (NBPTo) inhibits the hydrolytic action of soil urease, which catalyses the hydrolysis of urea to ammonium carbonate, by blocking the active site of the urease enzyme (Engel et al. 2015). Therefore, the lower rate of hydrolysis prevents steep pH increases around the fertiliser granule as well as the formation of high concentrations of NH_3 in the topsoil for a period of 1-2 weeks, allowing a longer time for urea interaction with the soil and rainfall events occurring during this period move the urea downwards, thus reducing the NH_3 volatilization loss potential from urea (Forrestal et al. 2017; Lanigan et al. 2018; Cantarella et al. 2018).

The nitrification inhibitors e.g. dicyandiamide (DCD) slow down the first step of the nitrification - the conversion of NH_4^+-N to hydroxylamine (NH_2OH) in soil (Amberger et al. 1989; Di and Cameron 2002; Singh et al. 2008; Di et al. 2009; Guo et al. 2013). The mode of action of DCD is 1) to restrict the first step of nitrification by deactivating the ammonia monooxygenase enzyme (amoA) of *Nitrosomonas* and/or *Nitrosospira* (the genera of nitrifying bacteria responsible for the oxidation of ammonium (NH_4^+) to nitrate (NO_3^-)) and 2) also behaving as metal chelator by removing copper, which is a co-factor of amoA, thus avoiding the oxidation of NH_4^+ and decreasing the NO_3^--N content in the soil (Di and Cameron 2002; Singh et al. 2008; Ruser and Schulz 2015; Rahman et al. 2021b). Thus, DCD possibly limits NO_3^- leaching and N_2O emissions from soil. DCD has been proven to reduce nitrate leaching and N_2O emissions from fertilisers by up to 80% in temperate conditions (Dennis et al. 2012; Cameron et al. 2014; Harty et al. 2016; Roche et al. 2016). Globally there is concern over chemical residues in food products and an increasing need to ensure that chemicals used in agricultural production do not impact food safety. This was highlighted when residues of the nitrification inhibitor DCD was observed in milk products from New Zealand after oral administration to lactating cows (Welten et al. 2016). A laboratory study by Pal et al. (2016) found that 2.7 to 5.2% of applied DCD was absorbed by pasture plants via foliage/roots after 37 days. Marsden et al. (2015) also observed slower DCD degradation rates within wheat plants that can be a direct route for DCD to enter the food chain. A recent review by Ray et al. (2020) suggested

a comprehensive risk assessment for effective evaluation of potential risks associated with individual inhibitor technologies.

To the best of our knowledge, no field-based studies have been carried out to investigate grass uptake and phytoaccumulation factors of NBPT or of DCD when co-applied with urea fertilisers. The present study evaluates the uptake of NBPT and DCD by grass and to quantify the phytoaccumulation factors of these inhibitors when co-applied with urea fertiliser.

Materials and method

During 2021, grass uptake of the urease inhibitor NBPT and the nitrification inhibitor DCD applied to separate plots with urea fertiliser was evaluated in field trials at the Teagasc, Environment Research Centre, Johnstown Castle, Co. Wexford, Ireland (52°18′N, 6°30′W). The dominant grass species in the sward was perennial ryegrass (*Lolium perenne* L.). The experimental plots were arranged in a randomised complete block design with five replicates per treatment. The experimental unit is an individual plot with a plot size of 10 m x 2 m. Fertilisers were applied at a rate of 250 kg N ha^{-1} yr^{-1}, in seven split applications throughout the year for all the treatments (Table 1). The treatments were: urea, urea fertiliser treated with the urease inhibitor N-(n-butyl) thiophosphoric triamide (urea+NBPT) and urea with the nitrification inhibitor dicyandiamide (urea+DCD). These fertilisers had been applied to the same plots every year since 2015. The source of the urease inhibitor NBPT was Agrotain, which was coated onto urea granules at 465 mg kg^{-1} NBPT (on a urea weight basis) giving a NBPT application rate of 0.25 kg NBPT ha^{-1} yr^{-1} based on 250 kg N ha^{-1} applied (Table 1). For the urea+DCD granules, the DCD was incorporated into the urea melt as part of the manufacturing process, at a rate of 1.6% on a urea weight basis, giving a DCD rate of c. 9 kg DCD ha^{-1} yr^{-1} (Table 1).

Table 1 Fertiliser N application rates (kg N ha^{-1}) and date of split application for each treatment.

Split application fertiliser	N application r kg N $split^{-1}$	N application dat 2021	Harvesting dates, 2021					Dose (on a urea weight bas (kg ha^{-1})	
			Day 5	Day 10	Day 15	Day 20	Day 30	NBPT	DCD
Split 1	30	28/01	-	-	-	-	25/03	0.03	1.04
Split 2	30	25/03	-	-	-	-	21/04	0.03	1.04
Split 3	50	23/04	28/04	04/05	10/05	14/05	26/05	0.05	1.74
Split 4	40	28/05	02/06	07/06	14/06	21/06	29/06	0.04	1.39
Split 5	40	02/07	07/07	12/07	19/07	23/07	03/08	0.04	1.39
Split 6	30	06/08	12/08	16/08	21/08	26/08	06/09	0.03	1.04
Split 7	30	11/09	15/09	20/09	25/09	01/10	11/10	0.03	1.04
Total	250							0.25	8.68
Total N application= 250 kg ha^{-1} $year^{-1}$			Grass samples were collected beginning after the 3rd application						
Total sampling unit = 3 treatments (urea, urea+NBPT, urea+DCD) x 5 replicates x 5 sampling days (day 5,10,15,20,30) x 5 splits of fertili applications = **375**									

Grass sampling and preparation for analysis

Grass samples were collected at intervals of 5, 10, 15, 20 and 30 days following fertiliser application. A quadrat of 0.5 m^2 was placed on the selected area and the grass was cut down to 5 cm using a handheld electronic shears (Art. 8885, Gardena Accu Shears, Gardena, Ulm, Germany) and transferred into a labelled ziplock bag. Grass samples were immediately transferred into a cooler box with ice packs. The total grass fresh weight per sampled area was recorded to determine grass dry matter yield and a subsample was collected to determine N uptake and dry matter (DM) content. Fresh subsamples of grass were weighed and dried at 70 $^{\circ}$C before reaching constant weight. Once dried, grass dry weight was recorded to calculate dry matter (DM).

Dry matter (%) = (Dry grass sample/fresh grass sample) x 100.

Grass dry matter yield was calculated as:

Grass dry matter yield (kg ha^{-1}) = (Green grass weight x dry matter (%) x plot size)/100.

Following grass sampling, a 10 g subsample of fresh grass was cut into small pieces, sealed in ziplock bags and stored at -80^{o}C for further analysis.

Laboratory procedure

Approximately 10 g of frozen fresh grass samples were ground using a blender in the presence of dry ice (solid CO_2) (Rogers and Bendich 1989), mixed at a ratio of 3:1 (w/w) to facilitate fine grinding while keeping the sample frozen. Ground grass samples were placed into 100 ml plastic containers and with a lid drilled with holes in a refrigerator at 2-4 °C for ~24 hrs until all the dry ice was vaporized. The samples were placed (approximately 5 g) in freezer bags (Ziplock) and all the air was removed before closing. The samples were stored at -80 °C and transported in sealed containers packed with dry ice to the analytical laboratory to be tested for NBPT or DCD content.

Grass Sample analysis

The methodology was established at the Teagasc Food Research Centre Ashtown (TFRCA) for the analysis of NBPT and DCD using LC-MS/MS technique.

All the reagents utilized in this study were of analytical grade. Ultra-pure Water (Millipore 18.2 MΩ.cm) was generated in-house using a Milli-Q water purification system from Merck-Millipore (Cork, Ireland). Methanol 215 and Acetonitrile 200 for UV were purchased from ROMIL Ltd. (Cambridge, UK). Formic acid (98–100%), dicyandiamide (DCD) 99% purity and N-(n-butyl) thiophosphoric triamide (NBPT) 98% purity were supplied by Sigma-Aldrich (Arklow, Ireland). Ammonium carbonate salt - certified for HPLC - was bought from Fisher Scientific (Blanchardstown,

Ireland), while N-(n-butyl) phosphoric triamide (NBPTo) which is an oxon analog of NBPT was sourced from Santa-Cruz biotechnology (Heidelberg, Germany). The chemicals and reagents were stored in line with the manufacturers' recommendations.

Standard Preparations

NBPT and NBPTo standards were prepared in line with the method recommended by Nkwonta et al. (2021). In brief, primary stock solutions of both compounds were prepared at concentrations of 1 mg mL^{-1} in MeOH. Aliquots of these stock solutions were placed in amber vials and stored at -30 °C. Working mixed calibration standard solutions of both NBPT and NBPTo were prepared on the day of analysis by diluting secondary stock solutions of 10 µg mL^{-1} (which were prepared the same day) to give 20, 40, 100, 200, 500, 1000, 2500, and 5000 ng mL. These were also stored at -30 °C and utilized within one week. DCD standard and the internal standard stock solutions were prepared at concentrations of 1 mg mL^{-1}in MeOH. Aliquots were placed in amber vials and stored at -30 °C (up to six months) while working standard calibration concentrations of 100, 250, 400, 550, 700, 850, 1000, and 1150 ng mL^{-1} were prepared on the day of analysis in MeCN by diluting a secondary stock solution of 10 µg mL^{-1}. Internal standard working solution was also prepared on the same day in MeCN to a concentration of 850 ng mL^{-1}.

Calibration

To quantify the target analytes in grass samples, a 8-point matrix-matched calibration was prepared. For NBPT and NBPTo, 1 g of blank homogenized grass samples were fortified with 250 µL of the mixed solvent standard calibrants to give final concentrations of 0.005, 0.010, 0.025, 0.050, 0.125, 0.250, 0.625 and 1.250 mg kg^{-1}; while those of DCD were fortified with 400 µL of the calibrants to give final concentrations of 0.004, 0.100, 0.160, 0.220, 0.280, 0.340, 0.400 and 0.460 mg kg^{-1}. As NBPT and NBPTo are prone to degradation, the pH of the calibration solutions was maintained at pH 9.0 by addition of 100 µL of ammonium carbonate buffer (1 M, pH 9.4) to the samples before extraction.

Preparation and extraction

Homogenised grass samples stored at -80 °C were thawed at room temperature (19 – 21 °C) for 20 – 30 minutes. The samples were then weighed (1.00 ± 0.01 g) into 50 mL polypropylene centrifuge tubes. Blank grass samples were used to weigh out eight matrix-matched calibration samples, two negative controls and four recovery control samples (2 high and 2 low levels) and extracted at the same time with test samples for NBPTo, NBPT and DCD analysis.

A modified quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction method was adopted (Anastassiades et al. 2003). In the case of NBPT and NBPTo extraction: all samples were spiked with 100 µL of

ammonium carbonate buffer and the calibration samples fortified with 250 µL of calibrants. The samples were left to equilibrate in a fume hood for 10 min. A ceramic homogenising pellet was placed in each tube, MeCN, 4 mL, was added using a dispenser and the samples were shaken for 1 min in a Merris minimix shaker (Galway, Ireland). The tubes were then centrifuged at 6000 ×*g* for 5 min at 4 °C. The supernatants were transferred, using a Pasteur pipette, into 15 mL tubes containing 900 mg $MgSO_4$, 150 mg primary secondary amines (PSA) sorbent, C_{18} sorbent, and 50 mg graphitized carbon black (GCB) for clean-up and chlorophyll removal. The tubes were shaken vigorously and centrifuged at 6000 × g for 5 min at 4 °C and the clear supernatants, 400 µL, were filtered using Whatman uniprep HPLC vials (0.22 µm pore size). For the recovery control samples, 375.5 µL, were placed in the uniprep HPLC vials using a positive displacement pipette and two of the samples fortified with 24.5 µL of level 2 calibrant (40 ng mL^{-1}) and another two with level 5 (500 ng mL^{-1}), respectively. NBPT and NBPTo (below the limit of quantitation) of 0.010 mg kg^{-1} was achieved by the method developed for this manuscript. For the DCD extraction, the eight calibration blank samples were spiked with 400 µL of solvent standard calibrants (100 to 1150 ng mL^{-1}) while internal standard, 200 µL, was added to all samples to be analysed. The samples were left to equilibrate for 5 – 10 min in a fume hood. Ceramic homogeniser pellets were placed in each tube and 4 mL of MeCN were added using a dispenser. The tubes were tightly closed and vigorously shaken using a mini mix shaker for 1 min. They were then centrifuged at 6000 g for 5 min at 4 °C and the supernatant transferred into 15 mL tube containing 900 mg $MgSO_4$, 150 mg PSA/C_{18} sorbent and 50 mg GCB for clean up. The tubes were also vigorously shaken for 1 min using a mini mix shaker and centrifuged at 6000 ×g for 5 min at 4 °C. The resulting supernatant, 400 uL, was filtered using a Whatman uniprep single use HPLC filter vials before analysis with LC-MS/MS instrument. For the recovery control samples, 362 µL, were placed in the uniprep HPLC vials using a positive displacement pipette and two of the samples fortified with 38 µL of level 2 calibrant (250 ng mL^{-1}) and another two with level 5 (700 ng mL^{-1}), respectively. The range of average recoveries was 80 – 110% for both compounds. The % CV or % RSD was 6% and 8%, respectively, which were within the acceptable limits of 15%.

UHPLC-MS/MS analysis

The method reported by Nkwonta et al. (2021) was adopted for the simultaneous analysis of NBPT and NBPTo except that an Agilent Infinity II UHPLC system (Santa Clara, CA, USA) coupled to AB SCIEX 6500$^+$ QTrap triple quadrupole mass spectrometer (Framingham, MA, USA) was utilised. In the case of DCD analysis, chromatographic separation was also performed on Agilent Infinity II UHPLC instrument equipped with HSS C_{18} column (2.1 × 100 mm, 1.8 µm particle size) attached to a VanGuard precolumn (2.1 × 5 mm) with the same packing material, all from Waters Corporation (Milford, MA, USA). The autosampler compartment and the column temperatures were maintained at 10 and 35 °C, respectively. A binary solvent gradient separation was employed, comprised of 0.1% formic acid in H_2O

(Mobile phase A), and MeCN: H_2O (90:10, v/v—Mobile phase B), pumped at a flow rate of 0.6 mL min^{-1}. The gradient started with 97% B at initial time (0.00 min) and decreased linearly to 90% B (0.00 – 0.50 min); and 60% B (0.50 – 1.50 min); subsequently, there were linear increases at 1.50 – 2.00 min (90% B) and 2.00 – 4.00 (97% B) for column clean-up and equilibration. Injection volumn was 5 µL and the total run time was 4 min with DCD eluting at 0.81 min (Retention time). The eluent was diverted to waste between (a) 0 and 0.5 min and (b) 1.2 to 4.0 min to minimise source contamination. Needle wash solvent was MeOH:H_2O (90:10, v/v). Precursor and product ions for DCD analytes and the internal standards were determined by manual tuning through a teed infusion with mobile phase in the AB SCIEX 6500^+ QTrap triple quadrupole mass spectrometer (Warrington, UK) equipped with electrospray ionisation probe (ESI). Generic source parameters were initially utilised during tuning and then optimised by assessing different value ranges for each parameter. The values that gave optimum sensitivity after replicate injections, were chosen. The instrument was controlled by Analyst software (version 1.7) and operated in the ESI positive mode with ion spray voltage set at 3500 V. The optimised source parameters were 20 psi for curtain gas, 350 °C for source temperature, and 60 psi for both ion source gas 1 and 2 (GS1 and GS2). The collision gas pressure (CAD) was set at "High" within the available options of the instrument. The declustering potentials (DP), collision energies (CE), cell exit potential (CXP), were optimised for individual transitions. Each parameter was ramped up across a range of values and the optimum values were selected based on intensity (Table 2). Data processing was performed using MultiQuant (version 3.0.3) software.

Table 2 Mass spectrometer conditions for the MRM of 4 mass pair in ESI^+ mode

Analyte	Measured ic	Precursor (m/z)	Product (*m*	DP (V)	EP (V)	CE (V)	CXP (V)
DCD	$[M + H]^+$	85.0	68.0	45.28	10	24.48	7.03
	$[M + H]^+$	85.0	43.0	45.28	10	18.25	5.70
DCD_$^{15}N_4$	$[M + H]^+$	89.0	45.0	31.09	10	18.67	7.64
	$[M + H]^+$	89.0	70.9	31.09	10	27.53	11.77

Data Calculations

On each sampling occasion, NBPT and DCD concentrations (mg kg^{-1}) were converted to grass uptake (mg ha^{-1}) by multiplying with dry matter yield (kg ha^{-1}) of the sampling plots. These calculations were performed for each replicate of a sampling occasion and grass uptake was presented as kg ha^{-1}. Phytoaccumulation factors (%) for NBPT or DCD were calculated by the following equation by Dowdy et al. 1996 and Rose et al. 2012

$$\text{Phytoaccumulation factors (\%)} = ((\text{NBPT or DCD residue uptake} - \text{Untreated Control})/ (\text{NBPTor DCD application rate})) \times 100$$

The cumulative phytoaccumulation factor at day 30 was calculated as the mass of DCD uptake at day 30 multiplied by five harvests expressed as a percentage of applied DCD.

Statistical Analysis

Statistical analysis was performed on the data using the PROC GLIMMIX procedure of SAS 9.4 (2002-2010; SAS institute INC., Cary, NC, USA). Analysis of variance (ANOVA) with the general linear model (GLM) procedure was used to examine the difference between DCD uptake and dry matter yield for urea, urea+NBPT and urea+DCD fertilisers in different time points (5, 10, 15, 20, 30) of the specific harvesting cycle. Where differences were identified, the F-protected least significant difference (LSD) test was used to determine the significance of the differences at the 95% level ($P < 0.05$). Simple regression was also undertaken to check the correlation between DCD concentration and different time points. The dataset's independence, normality and homogeneity of variance were examined and data were log-transformed if necessary. The equality of error variance of PROC GLM procedure was used to test homogeneity of variance and PROC UNIVARIATE test was used to test normality.

Results

Grass dry matter yield, grass NBPT concentration and uptake

Grass samples were collected from five harvesting cycles from the urea+NBPT applied plots at different time intervals (5, 10, 15, 20, 30 day). Grass dry matter yield was significantly affected by different harvesting times (Table 3). Grass dry matter yield showed an increasing trend with harvesting time. Day 30 showed a significantly higher dry matter yield for all the treatments for all the harvesting cycles. The highest dry matter yield (1536 ± 157 kg ha^{-1}) was recorded from the urea+NBPT fertiliser harvested in June which was also higher compared to urea+DCD treatments for all harvesting cycles (Table 4).

The concentration of NBPT or NBPTo was below the method LOQ (0.010 mg kg^{-1}) in all grass samples (Table 3). The urea treatment was considered as control which also showed that the concentration of NBPT or NBPTo were below the method LOQ. Therefore, NBPT uptake by grass was also below the method LOQ (0.001 mg kg^{-1}) in all samples.

298 **Table 3** Grass dry matter yield, NBPT residue concentration over time for different harvesting cycles. Data are mean ±
299 standard error, (n=5).

Harvesting time	N application kg N ha^{-1}	NBPT Application Rate (kg ha^{-1})	Day	Urea+NBPT		Grass NBI uptake (g ha^{-1})
				DM yield (kg ha^{-1})	NBPT/NBPTo concentration (mg kg^{-1})	
Harvesting in May (26/05)	50	0.05	5	151^{d} ± 7	<LOQ	0
			10	762^{bc} ± 45	<LOQ	0
			15	611^{c} ± 70	<LOQ	0
			20	853^{b} ± 67	<LOQ	0
			30	1299^{a} ± 112	<LOQ	0
Harvesting in June (29/06)	40	0.04	5	156^{d} ± 12	<LOQ	0
			10	378^{c} ± 68	<LOQ	0
			15	1010^{b} ± 48	<LOQ	0
			20	1196^{b} ± 117	<LOQ	0
			30	1536^{a} ± 157	<LOQ	0
Harvesting in August (03/08)	40	0.04	5	56^{c} ± 5	<LOQ	0
			10	140^{c} ± 13	<LOQ	0
			15	438^{b} ± 43	<LOQ	0
			20	543^{b} ± 41	<LOQ	0
			30	893^{a} ± 78	<LOQ	0
Harvesting in September (06/09	30	0.03	5	321^{c} ± 19	<LOQ	0
			10	362^{c} ± 34	<LOQ	0
			15	444^{c} ± 7	<LOQ	0
			20	655^{b} ± 45	<LOQ	0
			30	897^{a} ± 57	<LOQ	0
Harvesting in October (11/10)	30	0.03	5	121^{d} ± 16	<LOQ	0
			10	260^{c} ± 26	<LOQ	0
			15	382^{b} ± 40	<LOQ	0
			20	602^{a} ± 48	<LOQ	0
			30	679^{a} ± 58	<LOQ	0

< LOQ = NBPT concentrations were below the limit of quantitation (0.010 mg kg^{-1})

300

301

302 **Grass dry matter yield, DCD concentration, uptake and phytoaccumulation factor**

303 Grass dry matter yield was significantly ($p \leq 0.05$) affected by different harvesting times and the increasing trend was
304 observed with harvesting time (Table 4). Day 30 showed the highest dry matter yield (1507 kg ha^{-1}) among all the
305 harvesting cycles.

306 DCD concentration in the grass samples ranged from 0.004 to 28 mg DCD kg^{-1} grass (mean, 6.8 ± 0.06 mg kg^{-1}, n
307 = 125) and changed significantly ($P \leq 0.05$) over the five harvesting days (5, 10, 15, 20, 30) for each harvesting cycle
308 (Table 4).

Table 4 Grass dry matter yield, DCD residue concentration, grass DCD uptake and DCD phytoaccumulation factors over time for different harvesting cycles. Data are mean ± standard error, (n=5).

Harvesting time	N Application rate (kg N ha^{-1})	DCD Application Rate (kg ha^{-1})	Day	Urea+DCD		Grass DCD uptake (kg ha^{-1})	DCD biotransfer factor (%)
				DM yield (kg ha^{-1})	Residue concentration (mg kg^{-1} grass)		
Harvesting in May (26/05)	50	1.74	5	$140^{c} \pm 9$	$0.51^{c} \pm 0.04$	0.0001	0.004
			10	$416^{b} \pm 10$	$5.19^{b} \pm 5.00$	0.0022	0.124
			15	$573^{b} \pm 55$	$9.96^{a} \pm 2.90$	0.0057	0.328
			20	$897^{a} \pm 18$	$6.05^{b} \pm 1.23$	0.0054	0.312
			30	$874^{a} \pm 123$	$0.06^{c} \pm 0.01$	0.0001	0.003
Harvesting in June (29/06)	40	1.39	5	$134^{d} \pm 12$	$10.24^{a} \pm 0.44$	0.0014	0.099
			10	$317^{d} \pm 57$	$10.96^{a} \pm 0.97$	0.0035	0.250
			15	$745^{c} \pm 72$	$6.72^{b} \pm 0.98$	0.0050	0.360
			20	$1114^{b} \pm 84$	$13.6^{a} \pm 5.74$	0.0152	1.090
			30	$1507^{a} \pm 48$	$1.1^{c} \pm 0.63$	0.0017	0.119
Harvesting in August (03/08)	40	1.39	5	$80^{c} \pm 6$	$19.78^{a} \pm 0.77$	0.0016	0.114
			10	$212^{c} \pm 14$	$6.33^{b} \pm 0.62$	0.0013	0.097
			15	$551^{b} \pm 75$	$2.77^{c} \pm 0.80$	0.0019	0.110
			20	$507^{b} \pm 31$	$1.9^{c} \pm 0.61$	0.0013	0.069
			30	$829^{a} \pm 132$	$0.31^{d} \pm 0.10$	0.0003	0.018
Harvesting in September (06/09)	30	1.04	5	$165^{d} \pm 22$	$15.89^{a} \pm 1.24$	0.0026	0.252
			10	$359^{c} \pm 33$	$8.89^{b} \pm 0.56$	0.0032	0.307
			15	$450^{c} \pm 31$	$0.89^{c} \pm 0.21$	0.0004	0.039
			20	$684^{b} \pm 63$	$0.19^{c} \pm 0.05$	0.0001	0.012
			30	$925^{a} \pm 15$	$0.08^{c} \pm 0.03$	0.0001	0.007
Harvesting in October (11/10)	30	1.04	5	$203^{d} \pm 37$	$15.77^{a} \pm 4.29$	0.0032	0.308
			10	$308^{cd} \pm 35$	$15.37^{a} \pm 2.22$	0.0047	0.455
			15	$418^{bc} \pm 41$	$13.45^{a} \pm 2.10$	0.0056	0.541
			20	$542^{b} \pm 56$	$0.98^{b} \pm 0.14$	0.0005	0.051
			30	$836^{a} \pm 37$	$0.08^{b} \pm 0.02$	0.0001	0.006

309 The average DCD concentration showed a decreasing trend over time which were 12, 9, 7, 5 and 0.3 mg kg^{-1} grass on
310 day 5, 10, 15, 20, 30, respectively. The highest DCD concentration (20 mg kg^{-1}) was recorded on day 5 during the month
311 of August following application of 1.39 kg DCD ha^{-1} with urea and a cumulative application of 4.5 kg DCD ha^{-1} to that
312 point in the growing season. The relationship between DCD concentration and the harvesting days for each cycle is
313 shown in Appendix Fig. 1 and Fig. 2. In general, a declining trend was observed for DCD concentration over time and
314 a significant negative relationship between DCD concentration and harvest day was found in the harvesting cycles in
315 August, September and October.

316 The average amount of DCD that was taken up by grass ranged between 0.1 to 15.2 g DCD ha^{-1} over the total
317 experimental period (5 harvesting cycles). DCD was taken up by grass and in general, these amounts decreased over
318 time with maximum amounts of 0.015 kg ha^{-1} (equivalent to 1.1% of the total DCD applied) at day 20 during harvesting
319 cycle 2 when 1.39 kg DCD ha^{-1} was applied with urea during the month of June. Grass harvesting in August, September
320 and October showed that the DCD uptake was higher on days 5 and 10 and a reduced trend was shown after day 15. The
321 average DCD uptake were 1.8, 3.0, 3.7, 4.5 and 0.5 g ha^{-1} for day 5, 10, 15, 20 and 30, respectively. The DCD
322 phytoaccumulation factor ranged from 0.004% to as high as 1.1% of the applied DCD on one occasion over the five
323 harvesting days (5, 10, 15, 20 and 30) from each harvesting cycle (Table 4). The average phytoaccumulation factors
324 were 0.16, 0.25, 0.28, 0.31 and 0.03 at day 5, 10, 15, 20 and 30 respectively. A cumulative of 5.1% of the total applied
325 DCD residue was taken up by grass over the experimental period.
326

327 **Discussion**

328 **NBPT uptake by grass**

329 NBPT concentrations were below the method LOQ (0.010 mg kg^{-1}) for all the urea+NBPT treated plots in this study.
330 The lack of NBPT/NBPTo measured in grass suggests that its uptake by grass is not likely when co-applied with granular
331 urea fertiliser. This is consistent with the rapid degradation of NBPT and NBPTo reported by Engel et al. (2015). The
332 findings of the present study are also supported by the recent study by Nkwonta et al. (2021) in which 516 milk samples
333 were analysed from cows grazing land applied with urea+NBPT fertiliser. It was found that NBPT and NBPTo were
334 also below the method LOQ of 0.0020 mg kg^{-1} in the milk samples. NBPT degrades over time when applied with urea
335 in the soil (Byrne et al. 2020). Structurally, NBPT is an amphiphilic molecule with both a polar phosphoramide region
336 and a lipophilic butyl chain allowing it to interact with both polar and non-polar phases (Pro et al. 2014). As NBPT is
337 considered a surface active material which may limit the shelf life of treated urea fertilizer (Pro et al. 2014; Ruser et al.
338 2015; Byrne et al. 2020). Engel et al. (2015) concluded that NBPT and NBPTo degraded in acidic to slightly alkaline

soils (pH 5.1–7.6) due to its chemical hydrolysis and microbial action would also dominant in alkaline environments (pH 8.2). There is a positive relationship to the increasing numerical pH value and NBPT half-life. NBPT is reported to have a half-life of 1.6 days in soils at pH 5, 9.8 days at pH 5.5 and 42 days at pH 6 (Byrne et al. 2020). The European Chemicals Agency (ECHA) indicated that half-life of NBPT is 26–30 days at 12–25 °C in soil. In the present study, the average soil pH of the study site was 5.6 and the soil temperature was ranged from 9 to 24.6°C (average 16°C) (in Appendix Table 1 and Fig. 1). In the present study grass samples were collected over five harvesting cycles after application of 465 mg kg^{-1} NBPT (0.0485% or 0.25 kg NBPT ha^{-1} y^{-1}) coated with urea (on a urea weight basis) from different intervals (5, 10, 15, 20, 30 days) and found no NBPT/NBPTo residue in the grass, which suggests a minimal risk exists of NBPT residue entry into the food chain. NBPT is normally present at very low levels (0.038–0.064 wt % of urea) in the final fertilizer formulations and it's inclusion level is regulated in Europe. Cantarella et al. (2018) demonstrated that low concentrations of NBPT are effective in inhibiting urea hydrolysis and can therefore suppress ammonia loss (Manunza et al. 1999). Our findings suggest that NBPT can be used as urease inhibitor with granular urea fertiliser in temperate grassland conditions with uptake by grass unlikely.

DCD entry into the food chain

DCD uptake by grass must be considered as a feasible route for intake by animals. This study demonstrates, for the first time that the average amount of DCD that was taken up by grass ranged between 0.1 to 15.2 g DCD ha^{-1} over the total experimental period (5 harvesting cycles) after application of 6.6 kg ha^{-1} DCD with urea granules to a grassland pasture. The present study also found that the DCD phytoaccumulation factor was ranged from 0.004% to 1.1% after the five harvesting cycle in a temperate grassland. In a laboratory study by Pal et al. (2016) found that 2.7 to 5.2% of applied DCD was absorbed after application of 10 kg DCD ha^{-1} which did not change with time after 37 days of the experimental period. However, in the present study, DCD concentration showed a decreasing trend over time (day 5>10>15>20>30) for three of the five harvesting cycles. The concentration was higher (≤ 20 mg kg^{-1}) on day five and decreased over time and remained detectable (≤ 0.08 mg kg^{-1}) in the grass samples up to 30 days. DCD is reported to have a half-life of an average of 37 days according to McGeough et al. (2016) depending on the temperature. The half-life of DCD reduces with increasing temperature. Di and Cameron (2004) reported that the half-life reduced from 90 days at 8 ºC to 0 days at 20 ºC. The average soil temperature of the experimental plot was 16 °C (ranged from 9 - 24.6 °C) (in Appendix S Table 1; S Figure 1), which demonstrates the lower DCD concentration at day 30 for all the cycles. Cahalan et al. (2015) found a significant effect of soil type on DCD degradation which was attributed to variations in soil organic matter content and associated microbiological activity. Vilsmeier (1991) found 37.4% and 0.3% of applied DCD in the straw

and grains of spring wheat at harvest, respectively, until 17 weeks after application. Vilsmeier (1991) suggested that DCD is highly soluble in water, it can be absorbed by the plant roots via mass flow and translocated above ground where it can accumulate and crystallize at the hydathodes (secretory tissues located at the leaf tips that secrete water), a phenomenon that is apparently due to plant cells being unable to metabolize DCD.

A few other studies have shown that the uptake of DCD can occur in different crops with variable uptake and application rates depending on the precipitation and differing soil type. Wünsch and Amberger (1989) found that DCD was translocated to the stem and leaves within 24 h of application in grapevines and remained detectable in the aboveground biomass up to 15 days after fertiliser application. Marsden et al. (2015) observed DCD uptake by wheat plants. Teske and Matzel (1985) reported that DCD was taken up by rye and maize plants at rates of 40 –74% when 30 kg DCD ha^{-1} was applied with cattle manure. In line with previous findings on different crops, the present study also found that DCD is taken up by grass and demonstrates a risk of DCD residues being consumed by grazing animals.

The present study collected the grass samples from five harvesting cycles after application of 1.6% DCD (6.6 kg DCD ha^{-1} y^{-1}) coated with urea (on a urea weight basis) from different intervals (5, 10, 15, 20, 30 days) and found that an average 0.0042% to 1.1%of DCD residue uptake by grass which should be considered as feasible routes for contamination of milk products. As DCD is a relatively weak inhibitor, is normally present at high levels (15–30 kg DCD ha^{-1}) in the final fertiliser formulations to achieve equivalent suppression of nitrogen loss. Further risk assessment of the potential for DCD to transfer to food products is needed to account for the range of processing steps between grass consumption and milk/meat consumption.

Concluding remarks

The results showed that the uptake of NBPT by grass was below 0.010 mg kg^{-1} (limit of quantitation achieved by the method developed). DCD concentration in the grass samples ranged from 0.004 to 28 mg DCD kg^{-1} grass and DCD phytoaccumulation factor was ranged from an average 0.0042% to 1.1% after the five harvesting cycle in a temperate grassland. Therefore, the findings of the present study suggest that DCD uptake by grass should be considered as a feasible route for animal intake of DCD and requires further study to minimise its uptake by grass.

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Author Contributions

Niharika Rahman designed the research, conducted field research, performed statistical analyses; drafted the paper and had primary responsibility for the final content. Chikere G. Nkwonta designed the research and conducted laboratory analysis. Patrick Forrestal designed the research and supervised. Martin Danaher, Karl G. Richards, Sean A Hogan, Enda Cummins, Macdara O' Neill, Aishwarya Ray, Maria P. Byrne wrote, discussed, reviewed and approved the final manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate: Not applicable.

Consent to publish: Not applicable.

Competing interests: The authors declare no competing interests.

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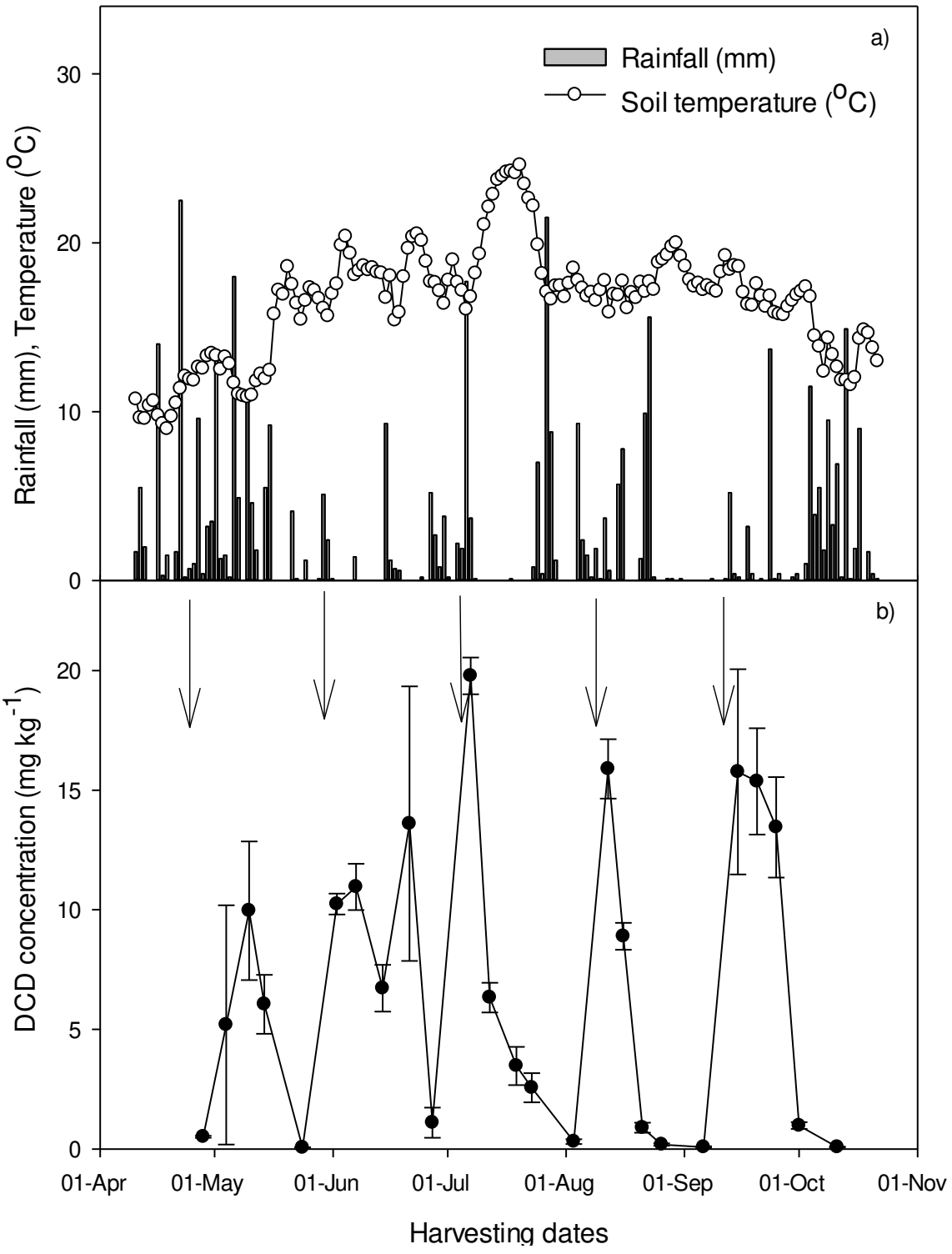
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Appendix

Table 1 Soil characteristics (0–10 cm) of the experimental site.

Soil properties	Urea	Urea+NBPT	Urea+DCD
pH	5.68	5.6	5.52
Organic matter (%)	6.78	6.92	6.90
*available P (mg kg^{-1})	4.90	4.92	5.13
*available K (mg kg^{-1})	114.58	109.46	99.56
*available Mg (mg kg^{-1})	90.10	98.94	98.22

*grass available nutrients were determined using Morgan's extract (Morgan, 1941).



578

579 **Fig. 1**a) Average soil (5 cm depth) temperature and rainfall over the sampling period. b) Grass DCD concentrations in

580 different time points. Data are mean ± standard error. Arrows indicate application dates of fertilisers.

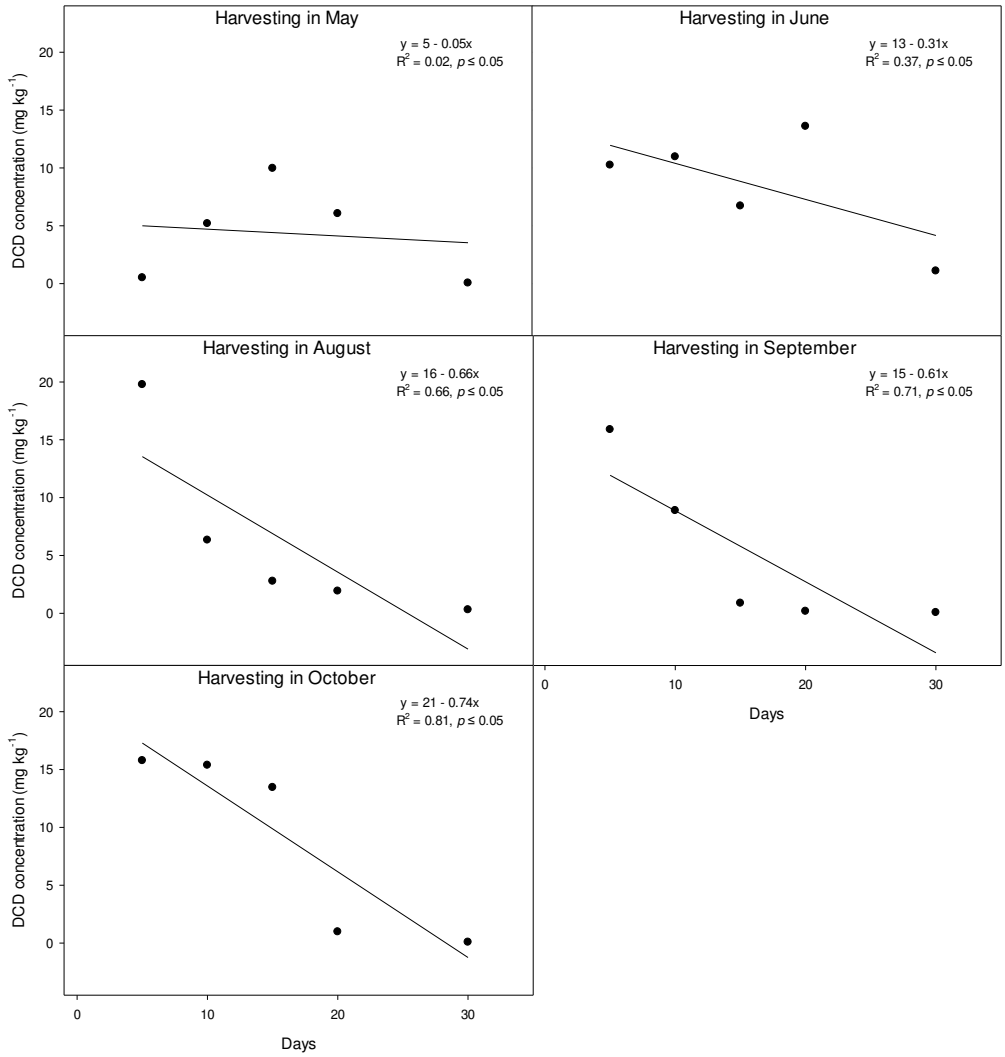


Fig. 2 Relationship between the DCD concentrations (mg kg^{-1}) with different harvesting days.