ROUTES OF DICYANDIAMIDE UPTAKE IN PASTURE PLANTS: A PRELIMINARY GLASSHOUSE STUDY

Pranoy Pal*, Andrew M. S. McMillan, Surinder Saggar

Landcare Research, Ecosystems and Global Change team, Palmerston North, New Zealand
**Corresponding author: palp@landcareresearch.co.nz*

Abstract
The nitrification inhibitor, dicyandiamide (DCD) can mitigate nitrate leaching and nitrous oxide emissions in New Zealand pastures and was commercially available to farmers (as spray suspension or granular formulations) until January 2013, when its use was suspended due to detection of traces of DCD in exported milk. DCD in the milk must have entered into the ruminant's body via ingested pasture and/or soil adhering to the pasture. The question is: did the DCD originate solely from the leaf surface or was it absorbed into leaf tissues? Alternatively, was the DCD taken up by the roots and translocated to the shoots? We investigated these routes of DCD into the plant by separately examining leaf uptake and root uptake in two glasshouse experiments. In experiment 1, DCD (at 10 kg ha^{-1}) was sprayed onto the foliage of ryegrass/clover growing on an intact soil core, of which 41–64% was intercepted by the foliage. Surface residues of DCD were quantified periodically by thorough rinsing of the foliage. The surface DCD residues decreased ($P < 0.005$) over 21 days. The foliar uptake (absorbed DCD) quantified by analysing the DCD content of a blended extract of the rinsed plant material ranged between 2.7 and 5.2% of the DCD applied and did not change over time. Experiment 2 quantified the root uptake of DCD in two soils of contrasting drainage by analysing the blended extract of the foliage for DCD over 37 days. The DCD uptake in the foliage was between 2.6 and 6.3%, which increased over time ($P < 0.001$) in both the soils. During the second harvest (97 days after DCD application), 1.2–2.8% of the DCD was detected in the foliage but no DCD was found in both the soil and roots. There was little pasture growth during the study period. This preliminary study raises several questions: is the DCD protected from decomposition in both the pasture shoot and root or in the rhizosphere for continuous uptake? Are these results reproducible and can these estimates be extrapolated to field conditions? Will similar levels of DCD be taken up under lower interception by the foliage/soil?

Introduction

Dicyandiamide (DCD) is a nitrification inhibitor (NI) that has been proven to reduce nitrate (NO_3^-) leaching (Francis 1995; Malcolm et al. 2015; MfE 2014) and nitrous oxide (N_2O) emissions (Cameron et al. 2014; De Klein et al. 2014; Kim et al. 2014; Ledgard et al. 2014; MfE 2014), and increase pasture yield (Carey et al. 2012) in New Zealand pastures. Based on nationwide trials, the New Zealand National Greenhouse Gas Inventory has incorporated the mitigating effects of using DCD assuming an average reduction of 67% in direct N_2O emissions and 53% in NO_3^- leaching from excretal N when DCD is applied (Clough et al. 2008; MfE 2014).

Dicyandiamide (2-cyanoguanidine; $C_2H_4N_4$; 66.6% N) is a non-volatile, strongly alkaline, water-soluble, white crystalline compound and chemically and physically stable which allows it to be most effectively formulated with N fertilisers. Dicyandiamide, like other NIs (such as nitrapyrin, thiourea, mercaptobenzothiazole, DMPP), deactivates the active site of the

ammonia monooxygenase enzyme, which is the key enzyme responsible for the first, rate-limiting step of the nitrification process – the conversion of NH_4^+-N to hydroxylamine (NH_2OH) (Amberger 1989; Di and Cameron 2002; Singh et al. 2008).The deactivation of this enzyme slows the production of NO_3^--N in the soil.

The efficacy of DCD depends on soil pH, soil structure, humidity, temperature (Bronson et al. 1989; Rajbanshi et al. 1992a; Rajbanshi et al. 1992b), soil moisture and organic matter content, fertiliser management and the rate of inorganic and microbial degradation (Schwarzer et al. 1998). The recommendations for DCD application to New Zealand pasture included: an application rate of 10 kg ha^{-1} in 800 L water, twice per year in late autumn and late winter within three days of the excreta or fertiliser-N being applied using a fine particle suspension (Clough et al. 2008; Di and Cameron 2005) or 10 kg ha^{-1} in case of the granular formulation (Monaghan et al. 2009). It is also suggested that at least 10 mm of rain/irrigation must fall following DCD application, before animals are introduced to the pasture or crop. This is to ensure that all DCD is washed off the plant leaves before grazing to avoid its consumption by dairy cattle and also to enhance its interaction with nitrifying microflora within the soil.

DCD was commercially available to New Zealand farmers until January 2013, when its use was suspended due to detection of traces of DCD in exported milk. The contamination incident has highlighted the need to understand the pathway by which DCD entered the dairy cow – was a part of the leaf-residing DCD absorbed into leaf tissues between the time of application and its expected wash-off due to irrigation/rainfall? Alternatively (and possibly in addition to the above mechanism), was the portion of applied DCD that was deposited on the soil surface taken up by the roots and translocated to the shoots?

Solutes, gases or nutrients can be absorbed through the leaves via leaf cuticle and stomata of plants (Eichert and Fernández 2012) and this mechanism is commonly used as a means of achieving nutrient uptake when fertilising golf courses (Stiegler et al. 2011; Stiegler et al. 2013) and horticultural crops (Bi and Scagel 2008; Bondada et al. 2001; Dong et al. 2002; Garnica et al. 2009). Among these studies, urea is the most commonly studied foliar fertiliser because it is a relatively small molecule [$CO(NH_2)_2$; 60 g mol^{-1}] and could be taken up by plants potentially on a mass flow basis via foliage. Because of the similarity between DCD and urea in terms of molecular weight (84 g mol^{-1}) and structure, we suspected that DCD could also be taken up in pasture plants. Vilsmeier (1991) conducted a greenhouse study using spring wheat (*Triticum aestivum* L.) grown in pots and applied ^{15}N-labelled ammonium sulphate and DCD in a 9:1 ratio. The author found 37.4% and 0.3% of the applied DCD in the straw and grains at harvest, respectively, 17 weeks after application. Root uptake of DCD has not been investigated in pasture species in New Zealand soils at the relatively lower, recommended application rates.

We conducted two glasshouse studies to differentiate and quantify the foliar and root uptake of DCD in pasture plants at various time intervals following its application in two contrasting soil types. We hypothesised that DCD would be taken up by both foliar and root uptake pathways.

Materials and methods

Experiment 1 – Determining the foliar uptake of DCD

This experiment was conducted under controlled conditions with constant temperature (15 ± 1^{o}C), stable humidity (90.7 ± 8.1% relative humidity) and a regular cycle of 16 h of an artificial light source (photosynthetically-active radiation, PAR, ranging from 280 to 330

μmol m^{-2} s^{-1}) followed by 8 h of dark. The temperature of 15°C simulated average autumn temperatures during May–June in pasture soils of New Zealand. Intact soil samples (10 cm diameter, 10 cm height) were collected using stainless steel (SS) cores from a permanent ryegrass–clover pasture managed for sheep grazing at Massey University Research Dairy Farm 1, Palmerston North, New Zealand. The sampled soil was an alluvial, well-drained, Manawatu sandy loam, (weathered fluvial recent soil (Hewitt 1998)) with total carbon 31 g kg^{-1} soil. The SS cores were placed on a 12 cm diameter glass saucer where 25 mL of deionised (DI) water was applied at two day intervals. Prior to spraying DCD, the shoots from all cores were cut to 5 cm height to simulate grazing. On the day of treatment application, DCD was sprayed on to the foliage of each core (n = 6 and a control that was sprayed with DI water) at 0.630 mL per core equivalent to 10 kg DCD ha^{-1} in 800 L water. Shoots were harvested at 7 h after the spray and on days 1, 2, 5, 8, 15, and 21 after DCD application. Immediately following each sampling, the shoot biomass was weighed and then extracted twice with water (described below), filtered, and analysed to determine the surface residues of DCD. The same shoots were then pulverised using a mortar and pestle, extracted, filtered, and analysed as described below.

Experiment 2 – Determining the root uptake of DCD

This experiment was conducted under the same conditions as Experiment 1. Using SS cores, intact soil samples of two contrasting soil types were collected from two permanent ryegrass–clover pastures in Palmerston North, New Zealand. The soils differed in their soil organic matter (SOM) contents and drainage. This soil was selected because it has been reported that the DCD efficacy may differ with varying SOM contents (Singh et al. 2008; Zhang et al. 2004). One of the soils in this current study was the Manawatu soil described above, while the other soil was a poorly drained Tokomaru soil (total carbon, 36 g kg^{-1} soil) and is classified as an Argillic-fragic Perch-gley Pallic soil (Hewitt 1998).

On the day of treatment application, the lower half of each collected soil core was removed from the core liners, sieved to 4 mm, treated with DCD (0.63 mL DCD mixed thoroughly with the soil) and repacked into the core. The upper (undisturbed) and lower (repacked) soil fractions were separated by a SS mesh (4 mm) so that the intact fraction remained on the top where new pasture can grow. The SS cores sat on a 12 cm diameter glass saucer where 25 mL of DI water was applied at two day intervals. Immediately following DCD application, the shoots from all cores were cut to 5 cm height to simulate grazing. Shoots were harvested at 9, 15, 22, 30, and 37 days after treatment application and DCD concentrations were determined as above. On the day of treatment application additional soil cores were prepared in a similar fashion and analysed for DCD immediately after its application to determine the proportion of DCD potentially available for plant uptake or adsorbed onto the SOM. After day 37, a modified Hoagland's solution (Hoagland and Arnon 1950) was applied to each core every alternate day until day 97, when the cores were destroyed and the roots, soil, and shoots were extracted with DI water (in a 1:40 ratio), filtered, and analysed for DCD as above. On day 97, for each soil type, five plant extracts from randomly selected cores were analysed for DCD concentrations to get an estimate if DCD could still be detected after approximately three months of application.

Sample extraction

On the days of sampling, the shoots were extracted with water (1:40, fresh shoot: deionised water) on an end-over-end shaker for 20 min. The extract was filtered through Whatman No. 42 filter paper. The filtrate (30 mL) was stored in designated containers until further analysis using high performance liquid chromatography (HPLC). After recording the amount of the

first extract adhering to the leaf surface, the same shoots were subjected to a second extraction and then analysed as above to assess if it could wash off further surface residues. Following the second water extraction, the shoots were pulverised using a mortar and pestle, extracted and filtered as above and analysed using method described below to determine the DCD that might have absorbed into the leaf tissues.

Quantification of the surface residues of DCD

Each 5 mL of filtrate obtained from the two extractions of surface residues above was acidified with 0.2 mL of 0.66 M H_2SO_4 and allowed to stand for 30 min before centrifuging (10 000 rpm for 15 min) to remove precipitated material and optimise the pH to the HPLC conditions. The concentration of DCD in the acidified supernatant was determined on a Waters 2695 HPLC (Waters Co., Milford, MA, USA) using a cation-H guard column (30 × 4.6 mm internal diameter; ID) with a 0.025 M H_2SO_4 mobile phase at a flow rate of 0.6 mL min^{-1} and a 210 nm ultraviolet (UV) spectrophotmetric detector. The limit of detection of DCD with the above method was 0.05 mg DCD L^{-1} (Kim et al. 2012). These analyses were performed at Massey University, Palmerston North, New Zealand.

Quantification of the absorbed residues of DCD

The modified method of Schwarzer and Haselwandter (1996) was adopted where the pulverised extract was purified by eluting it through a Waters Sep-Pak™ cartridge and analysed on a Shimadzu HPLC (Shimadzu Co., Kyoto, Japan) fitted with a Bio-Rad Aminex® organic acid column HPX-87H (300 × 7.80 mm ID) and the DCD peak detected using an UV detector at 220 nm. These samples were analysed by AgResearch, Hamilton, New Zealand.

Statistical analyses

For both experiments, on each sampling occasion, DCD concentrations from the untreated controls, if any, were deducted from the treatments and then converted to amounts (mg m^{-2}). These calculations were performed for each replicate of a sampling occasion. Analysis of variance (ANOVA) on the DCD amounts (for surface residues in Experiment 1 and foliar residues for Experiments 1 and 2) was performed using sampling day as a treatment. For both experiments, an ANOVA was also performed on the fresh, aboveground biomass per core. For Experiment 2, the DCD amounts were tested in a two-way ANOVA using soil type and sampling day as the treatments. All ANOVAs were tested using the statistical software GenStat© version 14.2 (GenStat 2011). All treatment differences were tested using Tukey's test (95% confidence interval). All data presented here are mean ± standard deviation (SD).

Results and discussion

Foliar uptake of DCD

Foliar interception of DCD spray

Of the total amount of DCD (991.08 mg m^{-2}) sprayed, 520 ± 85 mg DCD m^{-2} (mean ± sd; n = 6) was recovered in the first extraction at 7 h following application. The second extraction was able to recover a further 4.4% of the DCD applied on the leaf surface. A total of 56.9 ± 9.1% of the applied DCD was intercepted on the leaves (Fig. 1). This value is in the range of values reported in other studies, e.g. Kim et al. (2012) in a field study, applied DCD at 10 kg ha^{-1} to clover-ryegrass pasture and reported interception rates of 4.3 to 39.8% while De Klein et al. (2014) found soil DCD concentrations in the range of 3 to 7 kg ha^{-1} immediately after application indicating approximately 50% of the applied DCD was intercepted by the foliage. The surface residues of DCD in the current study decreased significantly (P < 0.005) over time except for a slight increase on day 5 (Fig. 1) and 36.5 ± 9.5% of the applied DCD could

be recovered at the end of the experimental period. This is in accordance with the study of Kim et al. (2012) who reported that DCD persisted on the plant canopy for < 6 to 16 days that was mainly dependent on rainfall and pasture height.

Aboveground biomass on the day of treatment application weighed 749.9 ± 219.9 g m^{-2}. Pasture biomass in the cores remained constant during the experimental period, except elevated values (778.3 ± 226.5 g m^{-2}) observed on day 2. Thus there was little pasture growth during the study period.

Foliar absorption of DCD

The total amount of DCD in the plant leaves that resulted from foliar uptake ranged from 13 to 90 mg DCD m^{-2} (mean, 36 ± 18 mg m^{-2}, n = 42) and did not change significantly (P = 0.295) over the 21 day experimental period. These values translate to 2.7 to 5.2% of the DCD applied (Fig. 1). This current study demonstrates for the first time that a significant proportion of DCD can be taken up via foliage by ryegrass-clover plants, supporting our hypothesis. Vilsmeier (1991) suggested that plant cells are unable to metabolise the DCD once absorbed. This might be the reason for why the foliar-absorbed DCD did not degrade over time in the current study.

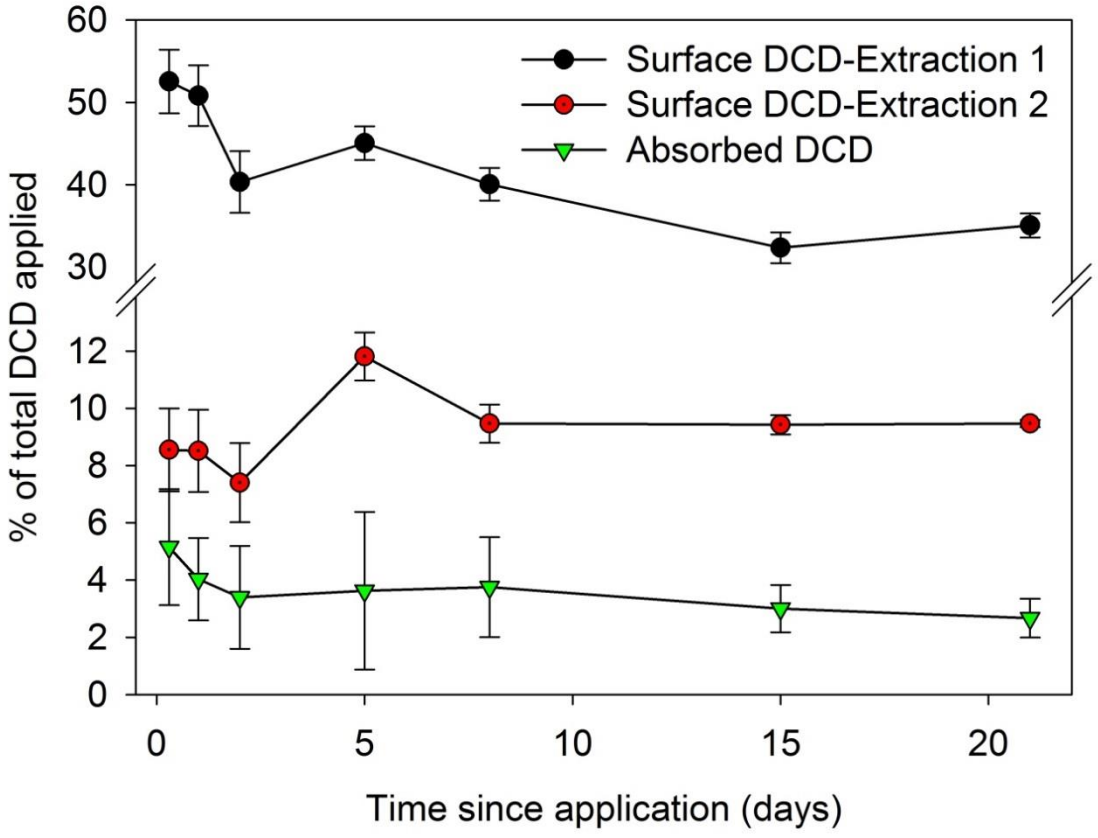

Figure 1. Proportions of DCD on the leaf surface and within the leaf tissues via foliar uptake over time.

Root uptake of DCD

DCD was taken up by the roots and translocated to the shoots and these amounts increased significantly (P < 0.001) over time with maximum amounts of 63 ± 29 mg m^{-2} (equivalent to

6.3% of the total DCD applied) on day 37 in the Tokomaru soil. The uptake rates did not differ between the two soil types ($P > 0.05$) and uptake rates ranged between 2.6 and 6.3% of the applied DCD on day 37 (Fig. 2). These uptake rates are much lower than that of Vilsmeier (1991) who observed about 38% uptake in spring wheat. Vilsmeier (1991) suggested that DCD can be absorbed by the plant roots via mass-flow and translocated aboveground where it is accumulated and may even be found crystallised at the hydathodes (secretory tissues located at the leaf tips that secrete water) and this phenomena is apparently due to plant cells being unable to metabolise DCD. The author suggested that the relatively higher uptake rate was probably due higher transpiration rates, higher DCD application rates, and relatively slower DCD decomposition in the soil-sand potting mixture conducted under controlled conditions.

Destructive analysis of randomly selected cores after 97 days of DCD application showed no residues in the roots and soil. However, 0.20−3.62% and 1.96−3.72% of the applied DCD was detected in the shoots of the Manawatu and Tokomaru soils, respectively. These uptake rates via roots further support Vilsmeier (1991), who suggested that once the DCD is taken up within the plant system, it is not prone to degradation as it would be when in soil because of the plant's inability to metabolise the absorbed DCD. Our study also shows that the DCD residues that are washed off from the leaves but remain on the top soil are subject to root uptake (indicating that granular formulations would also be a source of plant-absorbed DCD).

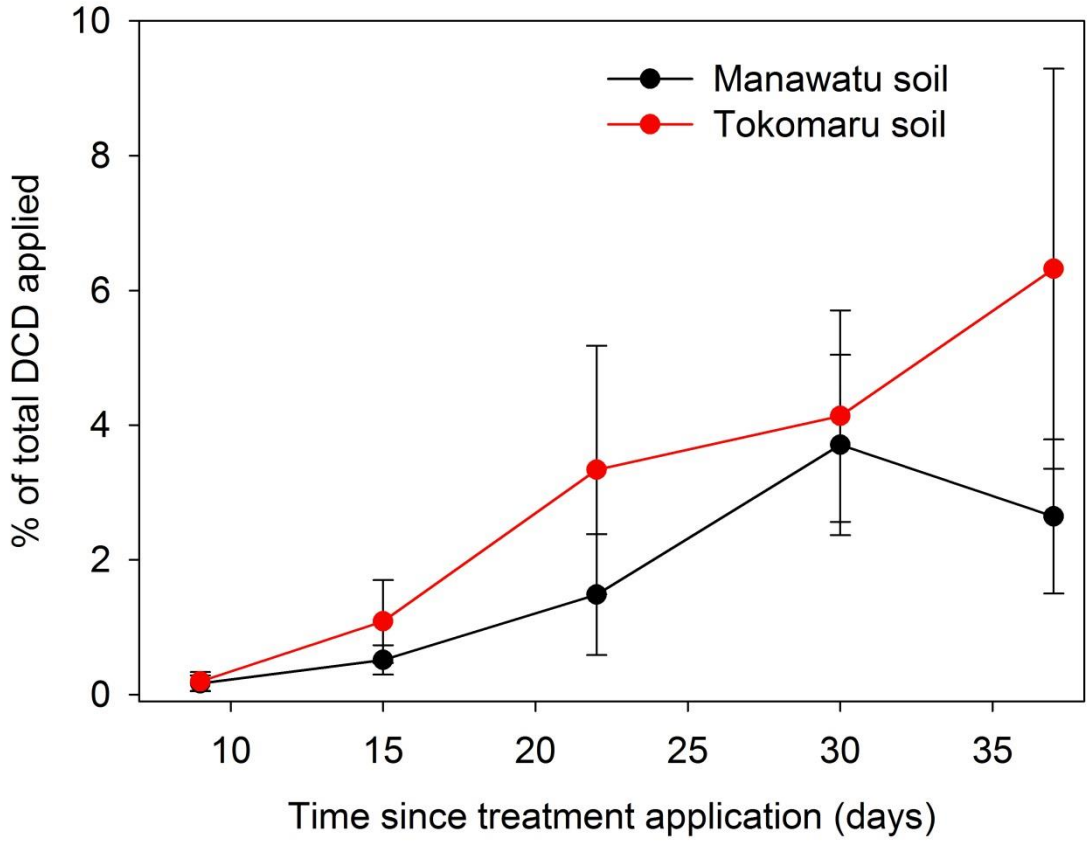

Figure 2. Fraction of applied DCD found in the shoots of clover-ryegrass plants via root uptake in two different soil types over time.

Conclusion

We investigated the uptake (absorption) of DCD that was either sprayed to foliage or incorporated into the soil in two separate glasshouse experiments and demonstrated for the first time that a significant proportion of DCD can be taken up by ryegrass-clover pastures via both foliar uptake (2.7−5.2% of the applied within 21 days of application) and root uptake (2.6−6.3% of the applied within 37 days of application) pathways. We observed that once the DCD is taken up by either of the foliar or root uptake pathways, it is not prone to degradation as it would be when in soil because of the plants' inability to metabolise the absorbed DCD. Foliar and root uptake must be considered as feasible routes for contamination of milk products.

Acknowledgements

This research was supported by Core funding for Crown research institutes from the Ministry of Business, Innovation and Employment's Science and Innovation Group. Thanks to Sarah Stephens (Royal Society of New Zealand Science Teacher Fellow) for assistance in sample processing; Anne Austin for proofreading; Dr Donna Giltrap for scientific critique; Thilak Palmada and Peter Berben for soil core collection; Peter Bishop (Massey University) for DCD analyses of surface residues; and Martin Kear (AgResearch, Hamilton) for DCD analyses of the pulverised extracts.

References

Amberger A (1989) Research on dicyandiamide as a nitrification inhibitor and future outlook. Communications in Soil Science and Plant Analysis 20:1933−1955

Bi G, Scagel CF (2008) Nitrogen uptake and mobilization by *Hydrangea* leaves from foliar-sprayed urea in fall depend on plant nitrogen status. Hortscience 47:2151–2154

Bondada BR, Syvertsen JP, Albrigo LG (2001) Urea nitrogen uptake by citrus leaves. Hortscience 36:1061–1065

Bronson KF, Touchton JT, Hauck RD (1989) Decomposition rate of dicyandiamide and nitrification inhibition. Communications in Soil Science and Plant Analysis 20:2067–2078

Cameron KC, Di HJ, Moir JL (2014) Dicyandiamide (DCD) effect on nitrous oxide emissions, nitrate leaching and pasture yield in Canterbury, New Zealand. New Zealand Journal of Agricultural Research 57:251–270

Carey PL, Jiang S, Roberts AH (2012) Pasture dry matter responses to the use of a nitrification inhibitor: a national series of New Zealand farm trials. New Zealand Journal of Agricultural Research 55:63–72

Clough TJ, Kelliher FM, Clark H, van der Weerden TJ (2008) Incorporation of the Nitrification Inhibitor DCD into New Zealand's 2009 National Inventory. Report prepared for the Ministry of Agriculture and Forestry by Lincoln University and AgResearch. Ministry of Agriculture and Forestry, Wellington

De Klein CAM, Letica SA, Macfie PI (2014) Evaluating the effects of dicyandiamide (DCD) on nitrogen cycling and dry matter production in a 3-year trial on a dairy pasture in South Otago, New Zealand. New Zealand Journal of Agricultural Research 57:316−331

Di HJ, Cameron KC (2002) The use of a nitrification inhibitor, dicyandiamide (DCD), to reduce nitrate leaching from cow urine patches in a grazed dairy pasture under irrigation. Soil Use and Management 18:395–403

Di HJ, Cameron KC (2005) Reducing environmental impacts of agriculture by using a fine particle suspension nitrification inhibitor to decrease nitrate leaching from grazed pastures. Agriculture, Ecosystems and Environment 109:202–212

Dong S, Cheng L, Scagel CF, Fuchigami LH (2002) Nitrogen absorption, translocation and distribution from urea applied in autumn to leaves of young potted apple (*Malus domestica*) trees. Tree Physiology 22:1305–1310

Eichert T, Fernández V (2012) Uptake and release of elements by leaves and other aerial plant parts. In: Marschner P (ed) Marschner's Mineral Nutrition of Higher Plants. 3 edn. Elsevier, Oxford, UK, p 71−84

Francis GS (1995) Management practices for minimising nitrate leaching after ploughing temporary leguminous pastures in Canterbury, New Zealand. Journal of Contaminant Hydrology 20:313–327

Garnica M, Houdusse F, Yvin JC, Garcia-Mina JM (2009) Nitrate modifies urea root uptake and assimilation in wheat seedlings. Journal of the Science of Food and Agriculture 89:55−62

Hewitt AE (1998) New Zealand Soil Classification. Landcare Research Science Series. 2 edn. Manaki Whenua Press, Lincoln, New Zealand

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. California Agricultural Experimental Station:Circular 347

Kim D-G, Giltrap D, Saggar S, Hanly JA (2014) Field studies assessing the effect of dicyandiamide (DCD) on N transformations, pasture yields, N_2O emissions and N-leaching in the Manawatu region. New Zealand Journal of Agricultural Research 57:271–293 doi:10.1080/00288233.2013.855244

Kim D-G, Giltrap D, Saggar S, Thilak P, Berben P, Drysdale D (2012) Fate of the nitrification inhibitor dicyandiamide (DCD) sprayed on a grazed pasture. Soil Research 50:337–347

Ledgard SF, Luo J, Sprosen MS, Wyatt JB, Balvert SF, Lindsey SB (2014) Effects of the nitrification inhibitor dicyandiamide (DCD) on pasture production, nitrous oxide emissions and nitrate leaching in Waikato, New Zealand. New Zealand Journal of Agricultural Research 57:294–315

Malcolm BJ, Cameron KC, Edwards GR, Di HJ (2015) Nitrogen leaching losses from lysimeters containing winter kale: the effects of urinary N rate and DCD application. New Zealand Journal of Agricultural Research 58:13-25 doi:10.1080/00288233.2014.961644

MfE (2014) New Zealand's Greenhouse Gas Inventory 1990–2012. Ministry for the Environment, Wellington, New Zealand. Available at: http://www.mfe.govt.nz/publications/climate/greenhouse-gas-inventory-2014/greenhouse-gas-inventory-2014-year.pdf

Monaghan RM, Smith LC, Ledgard SF (2009) The effectiveness of a granular formulation of dicyandiamide (DCD) in limiting nitrate leaching from a grazed dairy pasture. New Zealand Journal of Agricultural Research 52:145−159 doi:10.1080/00288230909510499

Rajbanshi SS, Benckiser G, Ottow JCG (1992a) Effects of concentration, incubation temperature, and repeated applications on degradation kinetics of dicyandiamide (DCD) in model experiments with a silt loam soil. Biology and Fertility of Soils 13:61–64

Rajbanshi SS, Benckiser G, Ottow JCG (1992b) Mineralisation kinetics and utilization as a N source of dicyandiamide (DCD) in soil. Naturwissenschaften 79:26–27

Schwarzer C, Auer B, Klima J, Haselwandter K (1998) Physiological and electron microscopical investigations of syntropic dicyandiamide degradation by soil bacteria. Soil Biology and Biochemistry 30:385–391

Schwarzer C, Haselwandter K (1996) Rapid quantification of the nitrification inhibitor dicyandiamide in soil samples, nutrient media and cell-free extracts. Journal of Chromatography A 732:390–393

Singh J, Saggar S, Giltrap D, Bolan NS (2008) Decomposition of dicyandiamide (DCD) in three contrasting soils and its effect on nitrous oxide emission, soil respiratory activity, and microbial biomass - an incubation study. Australian Journal of Soil Research 46:517–525

Stiegler JC, Richardson MD, Karcher DE, Roberts TL, Norman RJ (2011) Field-based measurement of ammonia volatilisation following foliar applications of urea to putting green turf. Crop Science 51:1767–1773 doi:10.2135/cropsci2010.09.0507

Stiegler JC, Richardson MD, Karcher DE, Roberts TL, Norman RJ (2013) Foliar absorption of various inorganic and organic nitrogen sources by creeping bentgrass. Crop Science 53:1148–1152 doi:10.2135/cropsci2012.08.0511

Vilsmeier K (1991) Fate of ammonium-N in pot studies as affected by DCD addition Fertiliser Research 29:187–189

Zhang HJ, Wu ZJ, Zhou QX (2004) Dicyandiamide sorption-desorption behaviour on soils and peat humus. Pedosphere 14:395–399