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Marsden, K.A.; Scowen, M.; Hill, P.W.; Jones, D.L.; Chadwick, D.R.

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# Plant acquisition and metabolism of the synthetic nitrification inhibitor dicyandiamide and naturally-occurring guanidine from agricultural soils

Karina A. Marsden · Matthew Scowen · Paul W. Hill ·  
Davey L. Jones · David R. Chadwick

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## Abstract

**Background and aims** There is increasing interest and use of nitrification inhibitors (NI) in agroecosystems, yet little is known of their fate *in planta*. Residues of the organic, N-rich NI, dicyandiamide (DCD), have been found in milk products following commercial application to pasture. We investigated whether plant acquisition and metabolism of DCD were consistent with plant-mediated transmission from soil to agricultural food products.

**Methods** Uptake rates, translocation to the shoot, degradation of the label within wheat tissue and availability within two soils of DCD and the structurally similar naturally occurring N-rich molecule, guanidine, were measured using  $^{14}\text{C}$  labelling.

**Results** Under sterile conditions, over 2 h wheat took up (34 and 14  $\mu\text{mol g}^{-1}$  root DW  $\text{h}^{-1}$  at 1 mM: DCD and guanidine, respectively), translocated (7–15 and 19–22 %) and metabolised (0.4 and 0.9 % of uptake) DCD- and guanidine- $^{14}\text{C}$ . Both molecules were also acquired from soil by wheat despite concurrent soil sorption and microbial uptake.

**Conclusions** Both DCD and guanidine can be acquired and metabolised by graminaceous plants. Although probably not a significant route of N acquisition, plant

uptake provides a direct route of DCD entry into the food chain.

**Keywords** Bioavailability · Dicyandiamide (DCD) · Guanidine · Mineralization · Nitrification inhibitor · Nitrogen cycle

## Introduction

Within agricultural soils, nitrification represents one of the dominant nitrogen (N) flow pathways and is responsible for generating  $\text{NO}_3^-$  which can be lost to the wider environment via leaching and denitrification (Zerulla et al. 2001). To reduce these N losses, effective management strategies are required to improve N use efficiency (NUE) within most agroecosystems. One potential solution to the problem is the application of synthetic or natural nitrification inhibitors (NI) to the soil to slow the conversion of  $\text{NH}_4^+$  to  $\text{NO}_3^-$  (Subbarao et al. 2012; Abalos et al. 2014).

Among the many identified NI, synthetic dicyandiamide (DCD;  $\text{C}_2\text{H}_4\text{N}_4$ ) is one of the most widely researched and one of the few used at a commercial scale (O'Callaghan et al. 2010; Liu et al. 2013). DCD has been investigated in a wide range of arable and livestock-based agroecosystems, where applications of DCD (10–30  $\text{kg ha}^{-1}$ ) have been shown to be effective in reducing nitrous oxide ( $\text{N}_2\text{O}$ ) emissions following spreading of either N fertilisers (Weiske et al. 2001; Di and Cameron 2006; Cui et al. 2011), livestock slurry (Hatch et al. 2005) or ruminant urine (Di and Cameron

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K. A. Marsden (✉) · M. Scowen · P. W. Hill · D. L. Jones ·  
D. R. Chadwick  
School of Environment, Natural Resources and Geography,  
Bangor University, Bangor, Gwynedd LL57 2UW, UK  
e-mail: afp06c@bangor.ac.uk

2006; Dai et al. 2013; Barneze et al. 2015). DCD application to soil has also been shown to reduce  $\text{NO}_3^-$  leaching after the application of inorganic N fertilisers and from livestock urine patches (Di et al. 2009; Cui et al. 2011). A recent meta-analysis investigating the effect of soil-applied NI (including DCD), indicated that on average they result in an 8 % increase in crop yield and a 13 % increase in NUE (Abalos et al. 2014).

A range of application routes for DCD have been investigated including introduction to urine patches by oral administration to cattle (O'Connor et al. 2013; Welten et al. 2013), infusion of DCD into the rumen or abomasum of sheep (Ledgard et al. 2008), incorporation into fertiliser granules or addition of DCD in a biodegradable hydrogel to slow its release in soil (Minet et al. 2013). However, the simplest, least controversial and consequently most widespread route is direct application to soil. Although direct application to soil is both practical and has demonstrable efficacy, this method leaves DCD susceptible to degradation by soil microbes, which may reduce persistence and increases in NUE. There is also the potential for removal from soil due to uptake by plant roots.

In 2012, low level residues of DCD were found in New Zealand dairy products from which plant interception and uptake of DCD with subsequent transfer to ruminant milk have been hypothesised to be key vectors in its contamination (Kim et al. 2012; Chen et al. 2014). Although DCD is not currently known to pose a significant risk to human health, the discovery of DCD in milk has led to a voluntary suspension of sale and use of DCD in New Zealand until international acceptable limits for its presence in milk products can be agreed (Ministry for Primary Industries 2013).

Whether plants are able to take up DCD is currently uncertain. Similarly, whether (if taken up) DCD persists in plant tissues or is rapidly degraded is unknown. Historically DCD has been suggested for use as an organic fertiliser, although as opposed to intact plant uptake of this molecule, prior degradation to  $\text{NH}_4^+$  (and subsequent nitrification to  $\text{NO}_3^-$ ) was the presumed route of N supply to plants (Reddy 1964). Limited evidence from some >100 year old investigations (Hutchinson and Miller 1912) show increased soil residence of DCD in the absence of plants, and reported phytotoxicity and yield reduction at high rates of DCD addition ( $25 \text{ kg ha}^{-1}$ ) to clover, suggest that plant uptake does take place (Amberger 1986; Di and Cameron 2004; Macadam et al. 2003; Kelliher et al. 2008). The

occurrence of the structurally similar molecule guanidine ( $\text{CH}_5\text{N}_3$ ; Fig. 1) and derivatives in a wide range of organisms including soil, plants and associated microbes may also suggest the existence of capacity for acquisition and assimilation of DCD by plants (Kato et al. 1986; Prescott and John 1996; Schulten and Schnitzer 1998; Kawano and Hwang 2010; G uthner et al. 2014).

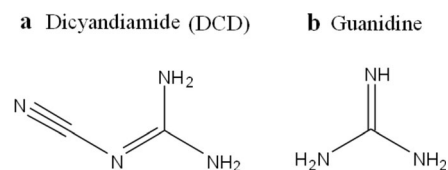
This investigation was predicated on the need to determine whether 1) the capacity of plants to take up DCD through their roots and translocate it to shoots is great enough, and 2) that the rate of subsequent degradation *in planta* is slow enough, to make plants a realistic route for the transmission of DCD from soil to agricultural food products. It also presents an opportunity to investigate earlier speculation on the potential of guanidine and similar compounds to be added to the rapidly growing list of routes via which plants have the capacity to obtain N from soil (Hutchinson and Miller 1912; Lewis 1936; Bollard 1966; Warren 2014).

We aimed to test the following hypotheses: 1) graminaceous plants have the capacity to take up both DCD and guanidine with their roots; 2) both DCD and guanidine can be metabolised by plants; 3) plants can take up both DCD and guanidine from soil; 4) the magnitude of competing substrate removal processes from soil (microbial uptake, mineralization and sorption) will be regulated by soil type.

## Materials and methods

### Soil properties

Two contrasting UK agricultural soils were used in this study (Table 1). The first was a mineral sandy loam textured Eutric Cambisol collected from a sheep-grazed fertilised grassland in North Wales ( $53^\circ 14' \text{N}$ ,  $4^\circ 01' \text{W}$ ), while the second was an organic Sapric Histosol collected from an intensive arable production



**Fig. 1** Molecular structure of the nitrification inhibitor, dicyandiamide (DCD; Panel **A**), and its naturally occurring analogue guanidine (Panel **B**), which are used in this study

**Table 1** Soil properties of Eutric Cambisol and Sapric Histosol used in soil rhizosphere microcosms. Values represent means $\pm$ SEM,  $n=4$ , letters indicate significant differences between the two soils and results are reported on a dry soil weight basis

Soil Property	Eutric Cambisol	Sapric Histosol
Moisture Content (%)	25.0 $\pm$ 0.27 a	61.2 $\pm$ 0.09 b
Organic Matter (%)	7.70 $\pm$ 1.41 a	77.2 $\pm$ 0.47 b
Cation Exchange Capacity (meq 100 g <sup>-1</sup> )	14.8 $\pm$ 0.68* a	80.8 $\pm$ 0.80* b
pH	6.67 $\pm$ 0.14 a	6.37 $\pm$ 0.06 a
Electrical Conductivity ( $\mu$ S cm <sup>-1</sup> )	50.1 $\pm$ 1.85 a	103 $\pm$ 9.56 b
Total Carbon (g kg <sup>-1</sup> )	23.8 $\pm$ 1.50 a	391 $\pm$ 1.15 b
Total Nitrogen (g kg <sup>-1</sup> )	2.92 $\pm$ 0.07 a	26.5 $\pm$ 0.07 b
Total Organic Carbon (mg C kg <sup>-1</sup> )	88.2 $\pm$ 6.29 a	959 $\pm$ 79.1 b
Total Organic Nitrogen (mg N kg <sup>-1</sup> )	10.6 $\pm$ 3.70 a	37.8 $\pm$ 5.35 b
Microbial C (g kg <sup>-1</sup> )	1.58 $\pm$ 0.05 a	4.41 $\pm$ 0.20 b
Microbial N (g kg <sup>-1</sup> )	0.33 $\pm$ 0.03 a	0.96 $\pm$ 0.08 b
NH <sub>4</sub> <sup>+</sup> (mg N kg <sup>-1</sup> )	4.29 $\pm$ 0.32 a	5.90 $\pm$ 0.69 a
NO <sub>3</sub> <sup>-</sup> (mg N kg <sup>-1</sup> )	4.34 $\pm$ 0.20 a	27.8 $\pm$ 2.45 b
PO <sub>4</sub> <sup>3-</sup> (mg P kg <sup>-1</sup> )	9.98 $\pm$ 0.39 a	32.0 $\pm$ 6.68 b
K <sup>+</sup> (meq kg <sup>-1</sup> )	12.3 $\pm$ 2.36 a	8.46 $\pm$ 2.72 a
Na <sup>+</sup> (meq kg <sup>-1</sup> )	0.95 $\pm$ 0.05 a	5.08 $\pm$ 0.37 b
Ca <sup>2+</sup> (meq kg <sup>-1</sup> )	71.5 $\pm$ 11.0 a	619 $\pm$ 12.5 b

\*  $n=3$

area in East Anglia (52°52'N, 0°47'W). At each site, four independent replicate soil samples (0–10 cm) were collected, sieved to pass 2 mm, and stored at 4 °C in gas permeable polythene bags until the start of the experiment. Soil moisture content was determined by oven drying (105 °C, 24 h), and soil organic matter content by loss-on-ignition (450 °C, 16 h; Ball 1964). Total soil C and N content was determined using a CHN2000 Analyzer (Leco Corp., St. Joseph, MI). Soil pH and electrical conductivity (EC) were measured using standard electrodes in 1:2.5 (w/v) soil: distilled water extracts. Available C and N was determined using a 1:5 (w/v) soil-to-0.5 M K<sub>2</sub>SO<sub>4</sub> extract within 24 h of sample collection, according to Jones and Willett (2006). Total dissolved C and N in the extracts were determined with a Multi N/C 2100S (AnalytikJena, Jena, Germany). Microbial biomass C and N were determined by CHCl<sub>3</sub> fumigation-extraction according to Voroney et al. (2008) using K<sub>EC</sub> and K<sub>EN</sub> correction factors of 0.35 and 0.50, respectively. K<sub>2</sub>SO<sub>4</sub>-extractable P, NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> were determined using the colorimetric methods

of Murphy and Riley (1962), Miranda et al. (2001) and Mulvaney (1996), respectively. Exchangeable cations were measured within 1:5 w/v soil-to-1 M NH<sub>4</sub>Cl extracts using a model 410 flame photometer (Sherwood Scientific Ltd., Cambridge, UK).

#### Plant uptake and translocation of DCD and guanidine under sterile conditions

DCD and guanidine uptake rates were determined under sterile conditions to determine if they were taken up intact (i.e., without prior microbial cleavage). Wheat seeds (*Triticum aestivum* var. Granary) were surface sterilised with 14 % (v/v) NaClO and 80 % ethanol and grown aseptically according to Hill et al. (2011). Briefly, surface sterilised seeds were germinated on agar containing 50 % Murishage and Skoog basal medium, to screen for microbial contamination; after which they were transferred aseptically to Phytatrays (Sigma Aldrich, Gillingham, UK) containing sterile perlite and 50 % Murishage and Skoog basal medium, supplemented with 10 mg l<sup>-1</sup> Na-metasilicate. Wheat plants were grown at 20 °C, with a 16 h photoperiod and light intensity (PAR) of 500  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, until they had reached the third leaf stage. The roots of individual intact wheat plants ( $n=4$ ) were placed in 12 ml of sterile (0.2  $\mu$ m-filtered) solution containing either <sup>14</sup>C-DCD or <sup>14</sup>C-guanidine (ca. 1 kBq ml<sup>-1</sup>; American Radiolabeled Chemicals, St Louis, MO, USA), for a period of 2 h. A solution concentration of 1 mM DCD was chosen to reflect the DCD concentration found within soil solution in response to typical field application rates (10 kg ha<sup>-1</sup>) and two lower concentrations of 0.01 and 0.1 mM, were chosen to represent subsequent dilution of field applied DCD by diffusive and mass flow processes. Subsequently, the plant roots were thoroughly rinsed in 0.01 M CaCl<sub>2</sub>, followed by deionised water and the roots and shoots oven dried (80 °C, 24 h). To quantify the <sup>14</sup>C content of the plants, the roots and shoots were separately combusted in an OX400 biological oxidizer (RJ Harvey, Hillsdale, NJ, USA), the <sup>14</sup>CO<sub>2</sub> captured in Oxosol scintillant (National Diagnostics, Atlanta, GA, USA) and <sup>14</sup>C measured using a Wallac 1404 Liquid Scintillation Counter (Wallac EG&G, Milton Keynes, UK). To visualise the location of <sup>14</sup>C-DCD and <sup>14</sup>C-guanidine in the root and shoot tissues, the <sup>14</sup>C distribution within intact plants was imaged using a Cyclone Plus phosphor-imaging system (PerkinElmer, Waltham, MA, USA) using an exposure time of 1 h.

## Plant metabolism of DCD and guanidine

To determine whether DCD or guanidine could be mineralized within the plant, sterile wheat seeds were prepared as described previously. The roots of intact plants were then placed in sterile (0.2  $\mu\text{m}$ -filtered) solutions containing either  $^{14}\text{C}$ -DCD or  $^{14}\text{C}$ -guanidine (4 ml; 1 kBq  $\text{ml}^{-1}$ ; 0.01 mM). The plants were then placed in sterile 250  $\text{cm}^3$  polypropylene vessels through which moist air was passed at a rate of *ca.* 600  $\text{ml min}^{-1}$ . The outflow was bubbled through Oxosol scintillant to capture any respired  $^{14}\text{CO}_2$ . The Oxosol was changed after 1, 5, 10, 20, 40, 60, 90 and 120 min and captured  $^{14}\text{C}$  measured as described above.

## Plant uptake of DCD and guanidine from soil

To determine the uptake rates of DCD and guanidine from soil, wheat seeds ( $n=4$ ) were individually sown into Eutric Cambisol or Sapric Histosol rhizosphere microcosms (240 mm long; internal diameter 8 mm) as described in Owen and Jones (2001). Each microcosm contained approximately 12 g FW soil. Plants were grown under the same conditions used for the sterile uptake study (Section 2.2), until they had reached the third leaf stage. At this point, solutions of  $^{14}\text{C}$ -DCD or  $^{14}\text{C}$ -guanidine (*ca.* 1 kBq  $\text{ml}^{-1}$ ; 0.01, 0.1 and 1 mM) were injected directly into the rhizosphere soil. A total of 4 injections (0.25 ml each) using 1 ml polypropylene syringes and 18 gauge needles at four depths (3, 9, 15 and 21 cm) were made, to facilitate an even distribution of solution within the microcosms. After 2 h, plants were removed from the microcosms and washed thoroughly in 0.01 mM  $\text{CaCl}_2$ , followed by distilled water. After drying (80  $^\circ\text{C}$ , 24 h), the  $^{14}\text{C}$  content of the root and shoot material was determined as described above. To estimate the quantity of root in contact with the injected solution, blue ink was injected as above into another set of microcosms ( $n=4$ ). The sections of root exposed to the ink were removed, washed, dried (80  $^\circ\text{C}$ ) and weighed.

This experiment was repeated under similar conditions, utilising unlabelled DCD (1 mM), in order to establish whether the intact DCD molecule could be detected in wheat shoot extracts via HPLC. After injection of the substrate and washing of the root material, the shoot was separated from the root. The shoots of four wheat plants were bulked ( $n=3$ ) in order to increase the likelihood of ascertaining a measurable peak on the

HPLC, and ground in 2 ml of DMSO in a borosilicate Griffiths tube; 1 ml of the DMSO extract was then evaporated to dryness under vacuum in a rotary evaporator. The pellet was resuspended in 0.5 ml of HPLC grade water, centrifuged (10 000g), and analysed for DCD using an adapted method of Turowski and Deshmukh (2004) on a Varian ProStar HPLC, with UV detection at 215 nm. The column used was a Luna 5u SCX (250 $\times$ 4.6 mm; 5  $\mu\text{m}$ ; 100  $\text{Å}$ ), DCD eluted after *ca.* four minutes in this system. Chromatograms were compared to standards and the control plants (no injected DCD).

## DCD and guanidine mineralization within soils

DCD and guanidine mineralization were determined, over the same time course as the rhizosphere uptake study (Section 2.5), by measuring the rate of  $^{14}\text{CO}_2$  evolution after the addition of 0.2 ml of  $^{14}\text{C}$ -DCD or  $^{14}\text{C}$ -guanidine (*ca.* 5 kBq  $\text{ml}^{-1}$ ; 0.01, 0.1 and 1 mM) to 1  $\text{cm}^3$  of each soil ( $n=4$ ). Soils were contained in a 10  $\text{cm}^3$  sealed glass vessel, with moist air flowing (*ca.* 100  $\text{ml min}^{-1}$ ) over the soil surface. Evolved  $^{14}\text{CO}_2$  was captured by passing the outflow through two consecutive 0.1 M NaOH traps (capture efficiency >95 %; Hill et al. 2007). Traps were changed after 1, 5, 10, 20, 40, 60, 90 and 120 min, and the activity in the solution determined by liquid scintillation counting after mixing with HiSafe 3 scintillant (PerkinElmer, Llantrisant, UK).

## DCD and guanidine microbial uptake and sorption within soils

The amount of substrate remaining in soil solution (i.e., that remaining after microbial uptake and abiotic removal processes) was determined according to the centrifugal-drainage procedure of Hill et al. (2008). Briefly,  $^{14}\text{C}$ -DCD or  $^{14}\text{C}$ -guanidine (0.2 ml; *ca.* 5 kBq  $\text{ml}^{-1}$ ; 0.01, 0.1 or 1 mM) was pipetted evenly onto the soil surface. After 1, 5, 10, 20, 40, 60, 90 and 120 min, soil solution was recovered by centrifugation (4000g, 1 min, 20  $^\circ\text{C}$ ) and the amount of  $^{14}\text{C}$ -DCD or  $^{14}\text{C}$ -guanidine in the recovered soil solution determined by liquid scintillation counting as described above.

The amount of DCD or guanidine sorbed to the solid phase and present in soil solution was determined by performing 0.5 M  $\text{K}_2\text{SO}_4$  extracts over time. Briefly,  $^{14}\text{C}$ -DCD or  $^{14}\text{C}$ -guanidine was mixed with soil and



incubated as described above. After 5, 10, 20, 40, 60, 90 or 120 min the soil was extracted with ice-cold 0.5 M  $K_2SO_4$  (1:5 w/v) by shaking ( $150 \text{ rev min}^{-1}$ ) for 20 min. After shaking, the extracts were centrifuged ( $10,000g$ , 10 min) and  $^{14}C$  in the supernatant determined by liquid scintillation counting. The amount of substrate sorbed to the soils was calculated by deducting the soil solution pool from the  $K_2SO_4$  extractable pool; the amount of substrate taken up by soil microbes was determined by deducting the soil solution pool and the sorbed pool from the total substrate added.

### Statistical analysis

All measurements were performed in quadruplicate unless otherwise stated. Log-transformation of data, followed by a one-way ANOVA with Fisher's LSD post-hoc test, was used to compare the following: wheat root uptake rates of DCD and guanidine under sterile conditions, the percentage of substrate translocation to wheat shoot biomass under sterile conditions, wheat root uptake rates from soil rhizosphere microcosms (after correcting for root weight exposed to injected solutions), percentage of substrate translocation to shoot biomass from wheat grown in soil, microbial uptake and sorption of the substrate at the 2 h timepoint. Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance assumptions (Levene's test) prior to conducting the ANOVA. Microbial mineralization data were analysed by one-way ANOVA as above, except data were not transformed. An independent samples *t*-test was used to compare plant metabolism of assimilated compounds at the 2 h time point, after testing the normality and homogeneity of variance assumptions. All statistical analyses were performed in IBM SPSS Statistics 20.0.

### Results

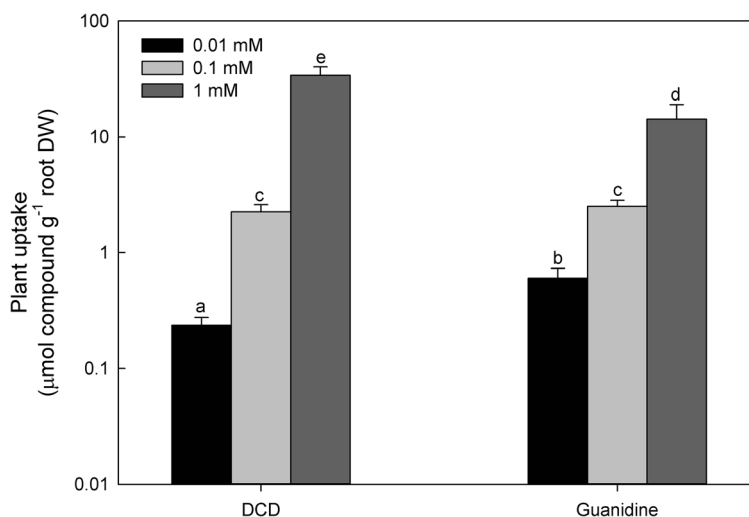
DCD and guanidine uptake and tissue localization in sterile wheat plants

Rates of uptake of  $^{14}C$ -DCD and  $^{14}C$ -guanidine by wheat roots increased ( $p < 0.001$ ) with increasing concentration (from 0.01 to 1 mM) in sterile solution (Table 2; Fig. 2). Phosphorimaging revealed a fairly

**Table 2** Summary of results for wheat uptake rates and tissue localization of  $^{14}C$ -DCD and  $^{14}C$ -guanidine (0.01, 0.1 and 1 mM) under sterile conditions and from Eutric Cambisol and Sapric Histosol rhizosphere microcosms following a 2 h chase period. Values represent means  $\pm$  SEM ( $n=4$ )

	Experimental condition			$^{14}C$ -DCD			$^{14}C$ -Guanidine						
		Sterile		Eutric Cambisol		Sapric Histosol		Sterile		Eutric Cambisol		Sapric Histosol	
			0.01 mM	0.1 mM	1 mM	0.01 mM	0.1 mM	1 mM	0.01 mM	0.1 mM	1 mM	0.01 mM	0.1 mM
Uptake rate ( $\mu\text{mol g}^{-1} \text{ root DW h}^{-1}$ )	Sterile	0.23 $\pm$ 0.04	2.25 $\pm$ 0.35	34.0 $\pm$ 6.29	0.60 $\pm$ 0.13	2.51 $\pm$ 0.32	14.3 $\pm$ 4.72	0.60 $\pm$ 0.13	2.51 $\pm$ 0.32	14.3 $\pm$ 4.72	0.60 $\pm$ 0.13	2.51 $\pm$ 0.32	14.3 $\pm$ 4.72
Uptake rate ( $\text{nmol g}^{-1} \text{ root DW h}^{-1}$ )	Eutric Cambisol	3.02 $\pm$ 0.92	30.9 $\pm$ 6.17	353 $\pm$ 94.3	7.13 $\pm$ 1.88	49.8 $\pm$ 13.9	328 $\pm$ 66.0	7.13 $\pm$ 1.88	49.8 $\pm$ 13.9	328 $\pm$ 66.0	7.13 $\pm$ 1.88	49.8 $\pm$ 13.9	328 $\pm$ 66.0
Uptake rate ( $\text{nmol g}^{-1} \text{ root DW h}^{-1}$ )	Sapric Histosol	2.18 $\pm$ 0.57	21.1 $\pm$ 1.72	174 $\pm$ 13.4	5.25 $\pm$ 0.77	47.2 $\pm$ 14.7	211 $\pm$ 39.2	5.25 $\pm$ 0.77	47.2 $\pm$ 14.7	211 $\pm$ 39.2	5.25 $\pm$ 0.77	47.2 $\pm$ 14.7	211 $\pm$ 39.2
Shoot translocation (% of acquired label)	Sterile	7.46 $\pm$ 1.18	7.76 $\pm$ 0.50	15.0 $\pm$ 4.05	22.4 $\pm$ 3.96	19.0 $\pm$ 6.58	22.0 $\pm$ 3.18	22.4 $\pm$ 3.96	19.0 $\pm$ 6.58	22.0 $\pm$ 3.18	22.4 $\pm$ 3.96	19.0 $\pm$ 6.58	22.0 $\pm$ 3.18
Shoot translocation (% of acquired label)	Eutric Cambisol	9.13 $\pm$ 2.28	5.42 $\pm$ 0.51	7.37 $\pm$ 2.36	0.71 $\pm$ 0.25	1.58 $\pm$ 0.40	2.09 $\pm$ 0.48	0.71 $\pm$ 0.25	1.58 $\pm$ 0.40	2.09 $\pm$ 0.48	0.71 $\pm$ 0.25	1.58 $\pm$ 0.40	2.09 $\pm$ 0.48
Shoot translocation (% of acquired label)	Sapric Histosol	7.80 $\pm$ 1.72	7.55 $\pm$ 1.48	7.16 $\pm$ 1.54	0.94 $\pm$ 0.13	1.10 $\pm$ 0.64	2.24 $\pm$ 0.20	0.94 $\pm$ 0.13	1.10 $\pm$ 0.64	2.24 $\pm$ 0.20	0.94 $\pm$ 0.13	1.10 $\pm$ 0.64	2.24 $\pm$ 0.20

**Fig. 2** Rates of  $^{14}\text{C}$ -dicyandiamide (DCD) and  $^{14}\text{C}$ -guanidine uptake by wheat (*Triticum aestivum*) roots grown in sterile hydroponic culture at three concentrations (0.01, 0.1 and 1 mM). Values represent means $\pm$ SEM ( $n=4$ ) and different letters indicate significant differences between means of compounds and concentrations (Fisher's LSD;  $p<0.05$ )



even distribution of the  $^{14}\text{C}$  label added as DCD within the shoot biomass (Table 2; Fig. 3), with the majority remaining in the roots. Phosphorimaging of  $^{14}\text{C}$  added as guanidine in the wheat plant showed an even distribution throughout the root system, but it was present predominantly in the lower regions of the shoot (Table 2; Fig. 3). Wheat roots acquired 2.37 % less ( $p<0.01$ ) of the  $^{14}\text{C}$  label added as DCD in comparison to guanidine at a concentration of 0.01 mM. No differences ( $p>0.05$ ) were observed at 0.1 mM, while 1.71 % more ( $p<0.01$ ) DCD was acquired in comparison to guanidine at 1 mM.

#### Plant mineralization of assimilated DCD and guanidine

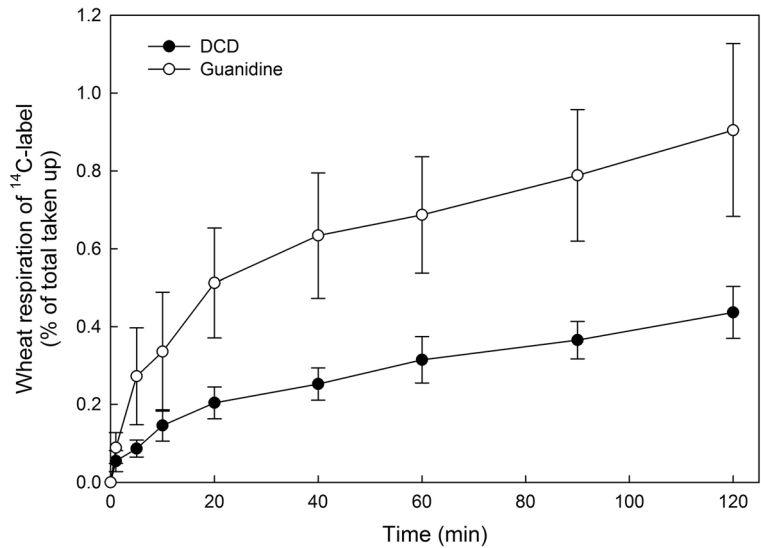
Of the added  $^{14}\text{C}$ -DCD and  $^{14}\text{C}$ -guanidine taken up by the plant, only small amounts were mineralized to  $^{14}\text{CO}_2$  during the experiment. After 2 h,  $0.44\pm 0.07$  % of the acquired  $^{14}\text{C}$ -DCD and  $0.90\pm 0.22$  % of acquired  $^{14}\text{C}$ -guanidine had been metabolised to  $^{14}\text{CO}_2$  within the plant with no differences observed between the two substrates ( $p>0.05$ ). For both compounds, respiration rates tended to be faster in the first 20 min of the incubation (Fig. 4).



**Fig. 3** Phosphorimages showing the distribution of  $^{14}\text{C}$ -dicyandiamide (DCD; Panel A) and  $^{14}\text{C}$ -guanidine (Panel B) within sterile wheat plants. The plants were exposed to each substrate (0.01 mM) for 2 h prior to imaging. The dark colour in

the top images represents the distribution of  $^{14}\text{C}$ -label within the plant tissue while, the images underneath are the corresponding photographs of the same plants

**Fig. 4** Cumulative mineralization of  $^{14}\text{C}$ -dicyandiamide (DCD) or  $^{14}\text{C}$ -guanidine in sterile wheat plants. Plants were exogenously supplied with each substrate (0.01 mM) for the whole 2 h monitoring period. Values represent mean  $\pm$  SEM ( $n=4$ )



DCD and guanidine uptake from soil rhizosphere microcosms

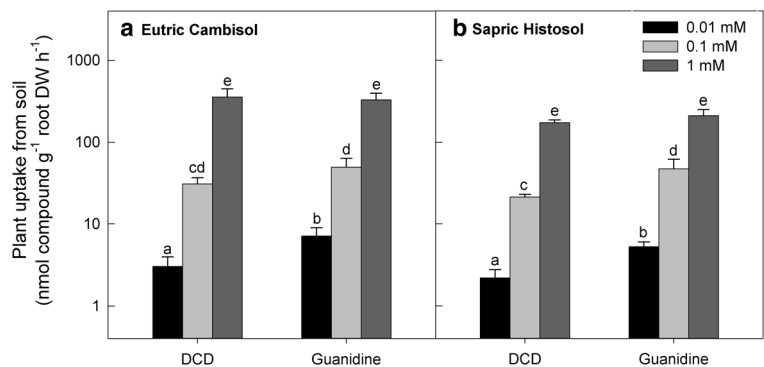
Wheat plants took up DCD- $^{14}\text{C}$  and guanidine- $^{14}\text{C}$  when grown in soil-filled microcosms (Fig. 5; Table 2), however, the rates were lower than those grown under sterile conditions. To evaluate the quantity of roots which were exposed to the injected  $^{14}\text{C}$  labelled substrates a blue ink tracer was injected into the microcosms. From this, we estimated that  $41 \pm 8$  and  $32 \pm 5$  % of the total root biomass was exposed to the injected  $^{14}\text{C}$ -substrates in the Eutric Cambisol and Sapric Histisol soil, respectively.

Increasing the concentration of injected  $^{14}\text{C}$ -DCD into soil rhizosphere microcosms increased ( $p < 0.001$ ) rates of root uptake of the label in both soil types (Table 2). Although a consistently higher mean root uptake rate of DCD was observed from the Eutric Cambisol in comparison to the Sapric Histisol at each

concentration, differences were not significant ( $p > 0.05$ ). After 2 h, wheat roots had acquired  $0.88 \pm 0.12$ ,  $0.96 \pm 0.12$  and  $1.09 \pm 0.21$  % of total applied DCD- $^{14}\text{C}$  (0.01, 0.1 and 1 mM respectively) from the Eutric Cambisol and translocated 5.42–9.13 % to the shoot material (Table 2); the amount acquired by wheat from the Sapric Histisol was ca. half this:  $0.50 \pm 0.08$ ,  $0.53 \pm 0.06$  and  $0.44 \pm 0.06$  % (0.01, 0.1 and 1 mM, respectively), with 7.16–7.80 % translocated to the shoot (Table 2).

Increasing the concentration of injected  $^{14}\text{C}$ -guanidine into the soil-filled microcosms increased rates of root uptake of guanidine- $^{14}\text{C}$  in both soil types ( $p < 0.001$ ; Fig. 5). In the Eutric Cambisol, wheat roots acquired less ( $p < 0.01$ ) of the  $^{14}\text{C}$  label added as DCD in comparison to guanidine at 0.01 mM, yet uptake rates were similar ( $p > 0.05$ ) at 0.1 and 1 mM. In the Sapric Histisol, wheat roots acquired less ( $p < 0.05$ ) of the  $^{14}\text{C}$  label added as DCD in comparison to guanidine at 0.01

**Fig. 5** Uptake of  $^{14}\text{C}$ -dicyandiamide (DCD) or  $^{14}\text{C}$ -guanidine (0.01, 0.1 and 1 mM) by wheat plants grown in either a Eutric Cambisol (Panel A) or Sapric Histisol (Panel B), legend applies to both panels. Values represent means  $\pm$  SEM ( $n=4$ ) and different letters indicate significant differences between means of different compounds, concentrations and between each soil type (Fisher's LSD;  $p < 0.05$ )





and 1 mM, however, uptake rates were similar at 1 mM ( $p>0.05$ ; Table 2). After 2 h, wheat roots had acquired  $2.21\pm 0.38$ ,  $1.54\pm 0.33$  and  $1.00\pm 0.09$  % of total applied guanidine- $^{14}\text{C}$  (0.01, 0.1 and 1 mM respectively) from the Eutric Cambisol and translocated 0.71–2.09 % to the shoot material (Table 2); the total amount acquired by wheat from the Sapric Histosol was  $1.26\pm 0.03$ ,  $1.05\pm 0.14$  and  $0.50\pm 0.08$  % (0.01, 0.1 and 1 mM, respectively), with 0.94–2.24 % translocated to the shoot (Table 2). The percentage of acquired guanidine- $^{14}\text{C}$  which was translocated to the shoot was lower than that of DCD- $^{14}\text{C}$  in both soil types ( $p<0.05$ ; Table 2).

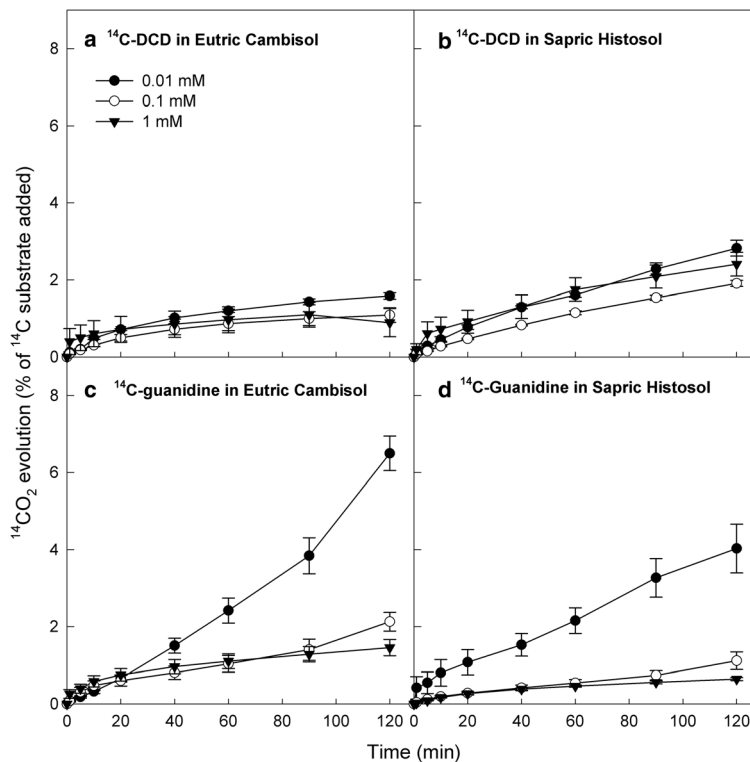
Unlabelled DCD was detected within the wheat shoot extracts by HPLC, confirming intact uptake and translocation of the molecule to shoot material. The concentrations of DCD recovered in shoots following injection of 1 ml of 1 mM DCD and a 2 h incubation period were  $79.7\pm 19.2$  and  $43.7\pm 3.75$  nmol DCD  $\text{g}^{-1}$  shoot DW in the Eutric Cambisol and Sapric Histosol, respectively. DCD concentrations in wheat shoots calculated from the  $^{14}\text{C}$  data showed the same higher shoot recovery in the mineral soil and were of the same order at  $25.8\pm 4.71$  and  $9.48\pm 2.31$  nmol DCD  $\text{g}^{-1}$  shoot DW in the Eutric Cambisol and Sapric Histosol, respectively. Some

disparity in the absolute values may have resulted from the different groups of plants used for the two methods of measurement.

#### Microbial mineralization of DCD and guanidine within soil

Over 2 h, the amount of  $^{14}\text{C}$ -DCD and  $^{14}\text{C}$ -guanidine mineralized in soil was small (Fig. 6; Table 3). After 2 h in the Eutric Cambisol, less ( $p<0.01$ )  $^{14}\text{C}$ -DCD was mineralized in comparison to  $^{14}\text{C}$ -guanidine, at 0.01 and 0.1 mM. Less  $^{14}\text{C}$ -DCD was also mineralized at 1 mM, however, differences were not significant ( $p>0.05$ ). In the Sapric Histosol, more ( $p<0.01$ )  $^{14}\text{C}$ -guanidine was mineralized in comparison to  $^{14}\text{C}$ -DCD at the lowest concentration (0.01 mM); at 0.1 and 1 mM less ( $p<0.05$ )  $^{14}\text{C}$ -guanidine was mineralized in comparison to  $^{14}\text{C}$ -DCD. After 2 h, greater amounts of  $^{14}\text{C}$ -DCD were degraded in the Sapric Histosol in comparison to the Eutric Cambisol at all concentrations ( $p<0.05$ ); conversely, greater amounts of  $^{14}\text{C}$ -guanidine were degraded in the Eutric Cambisol as opposed to the Sapric Histosol at all studied concentrations ( $p<0.05$ ).

**Fig. 6** Mineralization of a)  $^{14}\text{C}$ -dicyandiamide (DCD) in a Eutric Cambisol b)  $^{14}\text{C}$ -DCD in Sapric Histosol c)  $^{14}\text{C}$ -guanidine in Eutric Cambisol and d)  $^{14}\text{C}$ -guanidine in Sapric Histosol at three concentrations (0.01, 0.1 and 1 mM). Legend applies to all panels and symbols represent means $\pm$ SEM ( $n=4$ )



**Table 3** Summary of results for microbial mineralization, microbial uptake and sorption of  $^{14}\text{C}$ -DCD and  $^{14}\text{C}$ -guanidine (0.01, 0.1 and 1 mM) in a Eutric Cambisol and a Sapric Histisol after 2 h. Values represent means $\pm$ SEM ( $n=4$ )

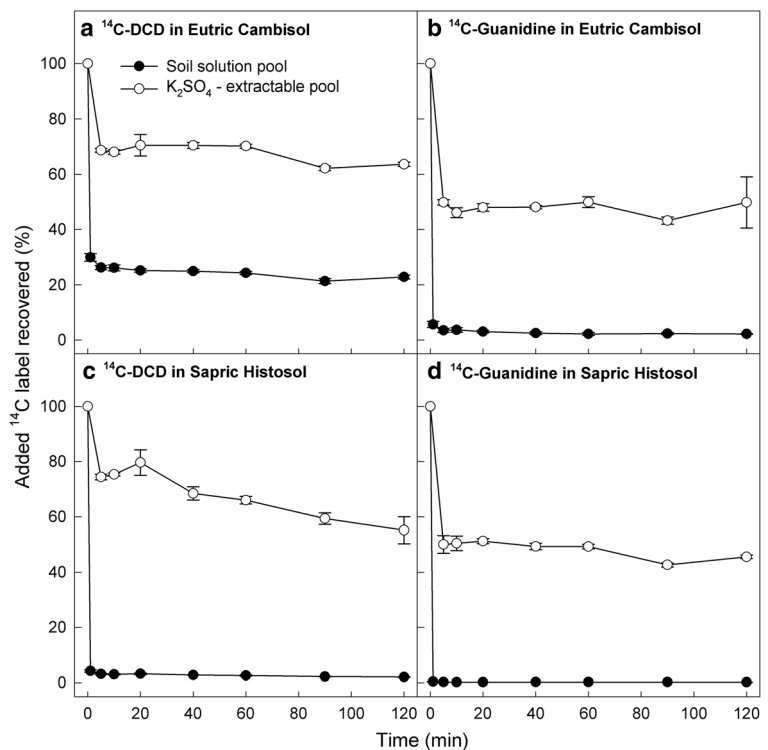
	Soil Type	$^{14}\text{C}$ -DCD			$^{14}\text{C}$ -Guanidine		
		0.01 mM	0.1 mM	1 mM	0.01 mM	0.1 mM	1 mM
Mineralization (% of applied label)	Eutric Cambisol	1.58 $\pm$ 0.08	1.09 $\pm$ 0.08	0.89 $\pm$ 0.37	6.50 $\pm$ 0.45	2.13 $\pm$ 0.24	1.46 $\pm$ 0.21
Mineralization (% of applied label)	Sapric Histisol	2.82 $\pm$ 0.21	1.91 $\pm$ 0.08	2.40 $\pm$ 0.31	4.02 $\pm$ 0.63	1.12 $\pm$ 0.23	0.64 $\pm$ 0.04
Microbial uptake (nmol g $^{-1}$ soil DW)	Eutric Cambisol	0.19 $\pm$ 0.004	1.68 $\pm$ 0.14	15.2 $\pm$ 0.75	0.24 $\pm$ 0.02	1.48 $\pm$ 0.09	10.8 $\pm$ 0.98
Microbial uptake (nmol g $^{-1}$ soil DW)	Sapric Histisol	0.63 $\pm$ 0.01	5.97 $\pm$ 0.18	60.6 $\pm$ 1.64	0.68 $\pm$ 0.01	5.09 $\pm$ 0.14	50.1 $\pm$ 1.36
Sorption (nmol g $^{-1}$ soil DW)	Eutric Cambisol	0.28 $\pm$ 0.002	3.03 $\pm$ 0.14	32.0 $\pm$ 0.57	0.28 $\pm$ 0.02	3.78 $\pm$ 0.07	41.3 $\pm$ 0.04
Sorption (nmol g $^{-1}$ soil DW)	Sapric Histisol	0.60 $\pm$ 0.01	6.31 $\pm$ 0.16	62.2 $\pm$ 1.68	0.56 $\pm$ 0.01	7.33 $\pm$ 0.12	74.1 $\pm$ 1.06

### Microbial uptake and sorption of DCD and guanidine within soil

The amount of  $^{14}\text{C}$ -substrate added as DCD or guanidine present in the soil solution pool and the  $\text{K}_2\text{SO}_4$ -extractable pool is shown in Fig. 7. As the trends in the data were similar across all concentrations, only the 0.01 mM data are presented. Increasing applied DCD or guanidine concentration from 0.01 to 1 mM increased ( $p<0.001$ ) the amount of DCD taken up by microbes in

both soil types, at all concentrations (Table 3). Greater amounts of DCD and guanidine were taken up by microbes in the Sapric Histisol in comparison to the Eutric Cambisol at all studied concentrations ( $p<0.001$ ). In the Eutric Cambisol a greater amount of added guanidine was taken up by microbes in comparison to DCD at 0.01 mM ( $p<0.001$ ), and less guanidine was taken up in comparison to DCD at 0.1 and 1 mM ( $p<0.001$ ). In the Sapric Histisol no difference was observed in the amount of substrate taken up by microbes as DCD or

**Fig. 7** Amount of added  $^{14}\text{C}$  label recovered in the soil solution pool and in the 0.5 M  $\text{K}_2\text{SO}_4$ -extractable pool from **a**)  $^{14}\text{C}$ -dicyandiamide (DCD; 0.01 mM) added to Eutric Cambisol **b**)  $^{14}\text{C}$ -DCD (0.01 mM) added to Sapric Histisol **c**)  $^{14}\text{C}$ -guanidine (0.01 mM) in Eutric Cambisol and **d**)  $^{14}\text{C}$ -guanidine (0.01 mM) in Sapric Histisol. Legend applies to all panels and symbols represent means $\pm$ SEM ( $n=4$ )



guanidine at 0.01 mM ( $p > 0.05$ ) and a lower amount of guanidine was taken up by microbes at 0.1 and 1 mM ( $p < 0.01$ ), in comparison to DCD (Table 3).

Increasing either DCD or guanidine concentration from 0.01 to 1 mM increased the amount of substrate sorbed to both soil types at all studied concentrations ( $p < 0.001$ ; Table 3). A greater amount of either DCD or guanidine sorbed to the Sapric Histosol in comparison to the Eutric Cambisol at all studied concentrations ( $p < 0.001$ ). No difference was found in the amount of DCD or guanidine sorbed in either soil type at 0.01 mM ( $p > 0.05$ ), but greater amounts of guanidine sorbed in comparison to DCD in both soil types at concentrations of 0.1 and 1 mM ( $p < 0.001$ ; Table 3).

## Discussion

### Plant uptake

Our results clearly demonstrate that in the absence of competing physical and biological soil processes (e.g., sorption, microbial uptake and microbial degradation), wheat roots can acquire DCD and guanidine from solution. Interestingly, rates of uptake ( $34.0 \pm 6.29 \mu\text{mol DCD g}^{-1} \text{ root DW h}^{-1}$ ;  $14.3 \pm 4.72 \mu\text{mol guanidine g}^{-1} \text{ root DW h}^{-1}$ ) at 1 mM under similar experimental conditions, were of a similar magnitude to other small N and C containing molecules found in soil (e.g., *ca.*  $25 \mu\text{mol NO}_3^- \text{ g}^{-1} \text{ root DW h}^{-1}$ ; *ca.*  $23 \mu\text{mol alanine g}^{-1} \text{ root DW h}^{-1}$ ; Hill et al. 2013); however, rates of uptake were greater for DCD and guanidine when considering moles of N acquired ( $136 \mu\text{mol DCD-N g}^{-1} \text{ root DW h}^{-1}$  and  $42.9 \mu\text{mol guanidine-N g}^{-1} \text{ root DW h}^{-1}$  compared to  $25 \mu\text{mol NO}_3^- \text{-N g}^{-1} \text{ root DW h}^{-1}$  and  $23 \mu\text{mol alanine-N g}^{-1} \text{ root DW h}^{-1}$ ; Hill et al. 2013).

The mechanisms of DCD uptake and subsequent translocation within plants remain unknown. Being a synthetic compound, we hypothesise that no DCD-specific membrane transporters exist, however, it is possible that uptake could be facilitated by transporter proteins for structurally similar, naturally occurring molecules. A recent study by Eggen and Lillo (2012), found that a pharmaceutical drug used for diabetes II, metformin, had a high bioconcentration factor within seeds of *Brassica napus*. Like DCD, the drug metformin is structurally similar to the naturally occurring molecule,

guanidine. The proposed mechanism of metformin entry to plant cells was via organic cation transporters (OCT), which transport naturally occurring N-containing compounds across the cell membrane. Expression of OCT has been demonstrated within several *Arabidopsis* tissues, including root tissue (Lelandais-Brière et al. 2007; Küfner and Koch 2008; Eggen and Lillo 2012). Substrates for plant OCT have not been well characterised, however, guanidine has been identified as a substrate for mammalian OCT (Cova et al. 2002). Assuming that DCD is an analogue of guanidine and acquired by the same mechanism, we would expect similar rates of uptake for both compounds. However, under sterile conditions we observed lower uptake rates of DCD compared to guanidine at 0.01 mM, similar rates at 0.1 mM and higher rates in comparison to guanidine at 1 mM. This suggests that the transporter affinity differs for the two compounds, and may also suggest there are other alternative transport pathways which need to be further investigated.

### Plant translocation and assimilation

Lower amounts of DCD- $^{14}\text{C}$  were recovered in the shoot in comparison to guanidine- $^{14}\text{C}$ . However, the DCD- $^{14}\text{C}$  appeared to be more evenly distributed within the shoot tissue. The low rates of mineralization of DCD- and guanidine- $^{14}\text{C}$  within the plant over the short incubation time employed here may suggest saturation of metabolic pathways and thus that plant capacity to use the C and N acquired in these molecules is low. Recovery of intact DCD in wheat shoots by HPLC perhaps supports this view. However, preferential accumulation of structurally similar metformin, in seeds of *Brassica napus* and the occurrence of other guanidine derivatives, including arginine, in seeds may suggest storage of DCD and guanidine i.e., a lack of metabolic pathways which are directly connected to respiration (Ngamga et al. 2007; Eggen and Lillo 2012).

### Competition for DCD and guanidine in the rhizosphere

Wheat was able to acquire *ca.* 0.5–1.0 % of the  $^{14}\text{C}$  applied as DCD and *ca.* 0.5–2 % of that applied as guanidine from soil within 2 h when in competition with the rhizosphere microbial community and sorption processes. Although realistic DCD soil solution

concentrations were chosen for this study, direct injection into the rhizosphere may have resulted in a greater amount of root surface area exposed to NI-containing soil solution than may be expected under field conditions. Actual DCD concentrations in the rhizosphere may vary according to NI application method, weather conditions, crop type, soil temperature and moisture and time since application. As only the C within molecules was isotopically labelled and detected within plant tissue, the results of the  $^{14}\text{C}$ -DCD uptake study do not unequivocally demonstrate that DCD was taken up intact from soil without prior lysis by soil microbes, or whether it remained intact once inside the plant without further degradation or transformation of the DCD molecule. Detection of unlabelled DCD within wheat shoots via HPLC, however, shows that intact uptake of DCD by plants took place.

Our results suggest that soil type is a regulator of DCD and guanidine bioavailability, with wheat acquiring consistent numerically (although not statistically) greater amounts of DCD- $^{14}\text{C}$  and guanidine- $^{14}\text{C}$  from the mineral Eutric Cambisol in comparison to the organic Sapric Histosol. A combination of a greater microbial uptake and sorption in the Sapric Histosol similarly suggests a lower availability for plant acquisition when compared to the Eutric Cambisol. The Sapric Histosol has a greater cation exchange capacity and more soil organic matter (which has been identified as important source of DCD binding domains (Jacinthe and Pichtel 1992; Zhang et al. 2004)) in comparison to the Eutric Cambisol, which may have led to greater sorption in this soil. The Sapric Histosol also had a greater microbial biomass compared to the Eutric Cambisol on a soil weight basis, which may have caused greater amounts of DCD and guanidine to be taken up by microbes in this soil.

In comparison to some other simple C substrates (e.g., amino acids, sugars) and the level of sorption to soil particles, the mineralization of DCD and guanidine by soil microbes was very slow (Hill et al. 2008; Wilkinson et al. 2014). This might be expected considering that neither soil has previously been exposed to the synthetic nitrification inhibitor, DCD. The dissimilar pattern of DCD and guanidine mineralization, particularly at low concentrations in the Eutric Cambisol supports the tenet that DCD is not rapidly extracellularly degraded to guanidine. The greater degradation of DCD in the Sapric Histosol in comparison to the Eutric Cambisol may be explained by the greater microbial

biomass on a soil weight for weight basis, although reasons for the lower rates of guanidine mineralization in the organic soil compared to the mineral soil are unclear. The low rates of guanidine uptake and mineralization at higher guanidine application rates ( $\geq 0.1$  mM) may also suggest that although naturally found in the microbial community, the capacity to internally assimilate high amounts of guanidine limits its metabolic conversion to  $\text{CO}_2$ . This supports the conclusions of Rajbanshi et al. (1992) in that the microbial community requires longer time periods than 2 h to adapt to utilising DCD in soil. However, we cannot exclude the possibility that both DCD and guanidine are metabolised largely by pathways which do not feed into respiration.

Our results support the suggestion of Kelliher et al. (2014) that plants may play a role in reducing the half-life of DCD within soils, and this may be more pronounced in mineral soils as opposed to organic soils where plant uptake rates were greater due to lower amounts being sorbed and taken up by microbes. Interestingly, they also suggest that plants are able to derive some additional N from soils where DCD has been applied, and that guanidine (and perhaps other similar N-rich molecules) can be added to the growing list of naturally occurring N forms which plants are able to acquire from soil and metabolise. However, we hesitate to suggest that this has a significant role in plant N nutrition.

#### DCD entry into the food chain

There are three obvious routes of entry for DCD into meat or milk products: by i) consumption of pasture which has intercepted DCD on the foliage during spray application of the NI ii) direct livestock consumption of pasture or forage (which has acquired DCD from the soil as demonstrated here) and iii) ingestion of soil particles containing DCD.

Based on a dairy cow consuming  $15 \text{ kg DM day}^{-1}$  of grass (McDonald et al. 1996), with a standing biomass of  $2000 \text{ kg DM ha}^{-1}$  (O'Donovan and Dillon 1999), following a spray application of DCD ( $10 \text{ kg ha}^{-1}$ ), with 5 % of the total applied DCD intercepted via the canopy (Kim et al. 2012) we estimate that  $3.5 \text{ g of DCD cow}^{-1} \text{ day}^{-1}$  could be consumed if allowed to graze immediately following application. Assuming that wheat is representative of other grasses and extrapolation of uptake and respiration rates to 24 h (with 50 %

translocation to shoots over this period), we estimate that 0.43 mg DCD cow<sup>-1</sup> day<sup>-1</sup> and 0.15 mg DCD cow<sup>-1</sup> day<sup>-1</sup> could be ingested following consumption of grass grown on the Eutric Cambisol and the Sapric Histosol, respectively. To estimate the amount of DCD consumed via soil ingestion we assume an even distribution of DCD in the top 10 cm of soil (soil bulk density of 1.10 and 0.31 g cm<sup>-3</sup> for the Eutric Cambisol and Sapric Histosol, respectively) with removal due to plant uptake as above, and microbial mineralization at 1.30 and 5.93 % (Eutric Cambisol and Sapric Histosol, respectively) of applied DCD over 24 h (measured over this period by Scowen, M; unpublished). Based on 2 % of the cow's DM intake being soil (Thornton and Abrahams 1983), 2.68 and 9.1 mg DCD cow<sup>-1</sup> day<sup>-1</sup> could be ingested with soil in the Eutric Cambisol and Sapric Histosol, respectively. Our calculations are based on a number of assumptions and upscaling laboratory data to field conditions, therefore, care should be taken when considering these estimations. Further research is required to elucidate how application methods (e.g., liquid vs. granular formulations) and environmental conditions (e.g., movement of NI into the root zone due to irrigation or rainfall) may influence pasture plant acquisition of this NI under field conditions.

Based on our estimates the magnitude of risk for DCD entry into the food chain via cattle follows the trend: pasture interception of DCD > soil ingestion of DCD > plant acquisition of DCD. However, the greatest risk pathway (canopy interception) would be transient (e.g., under high rainfall) and easily controlled by preventing grazing immediately following DCD application to pasture. Controlling the amount of DCD ingested beyond this point represents more of a challenge for out-grazing livestock and plant uptake may be a more significant pathway over longer periods. The potential for DCD to enter raw foods destined for direct human consumption (e.g., salad vegetables) also requires further investigation as the concentrations of DCD may be higher than in dairy products. Further, if like some other guanidine derivatives DCD accumulates in seeds, application of DCD to arable grain, oilseed or pulse crops could result in further direct DCD entry into the food chain due to the capacity of plants to acquire DCD from soil through roots (Ngamga et al. 2007; Eggen and Lillo 2012).

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