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Accelerated degradation of *N*, *N*′-dibutylurea (DBU) upon repeated application

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Abstract

In a recent study on the degradation of *N*,*N*′-dibutylurea (DBU), a breakdown product of benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate], the active ingredient in Benlate® fungicides, degradation half-lives of 1.4–46.5 days were observed across several soils incubated at various combinations of soil moisture potential (−0.03 and −0.1 MPa) and temperature (23, 33, and 44 °C) for a single DBU application of 0.08 and 0.8 μg g^{-1} (Lee et al. 2004). However, Benlate® can be applied as often as every 7 days resulting in the repeated application of DBU likely to be present in the Benlate® over a growing season. In this study, the effect of seven repeated DBU applications on mineralization rate was investigated in two soils, which encompass the range in rates previously observed. For the slower degrading soil, repeated DBU application increased mineralization from 0.029 to 0.99 day^{-1} at the 0.08 μg g^{-1} rate, and 0.037 to 0.89 day^{-1} at the 0.8 μg g^{-1} rate. For the faster degrading soil, effects on mineralization of repeated DBU applications were small to negligible. For the latter soil, the effect on mineralization of applied DBU concentrations from 0.0008 to 80 μg g^{-1} was also investigated. Mineralization rates decreased from 0.43 to 0.019 day^{-1} with increasing DBU concentrations. However, the amount of DBU mineralized by day 70 was similar across concentrations and averaged 83% of applied. Microbial respiration was not affected by increasing DBU concentrations. These findings support the supposition that DBU is readily degraded by soil microorganisms, thus unlikely to accumulate in agricultural soils.

Introduction

Persistence of *N*, *N*′-dibutylurea (DBU), a breakdown product of benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate] the active ingredient in Benlate® fungicide, is of particular concern, because DBU has been shown to damage plants under specific conditions invoked in laboratory studies (Calmon & Sayag 1976; Moye et al. 1994; Shilling et al. 1994; Tolson et al. 1999). DBU can be formed by conversion of benomyl to 2-benzimidazole-carbamate and *n*-butylisocyanate (BIC) followed by BIC reacting with water to form butylcarbamic acid, which quickly decarboxylates to give CO_2 and *n*-butylamine (BA), which can react with any remaining BIC to form DBU. It is known that DBU can form in the manufacturing of benomyl, and in commercial benomyl formulations stored under conditions of high humidity and temperature. DBU also has been shown to form on plant leaves from deposition of the precursor BIC vapors from solution (Moye et al. 1994). Sassman et al. (2004) investigated the potential for DBU to be formed in soils at different water potentials and temperatures after either application of commercial benomyl formulations or the immediate precursor BIC. DBU formation was observed in only a few cases with the maximum DBU concentration observed of 0.41 μg g^{-1} (0.65 wt% of applied benomyl at 62.8 μg g^{-1}) after application of Benlate® 50 DF at higher than the recommended drench rate followed by a rapid

dissipation of the DBU formed. This maximum observed concentration is well below those currently reported to cause adverse effects to plants.

Lee et al. (2004) investigated the degradation of DBU in several soils incubated at various combinations of DBU concentration (0.08 or 0.8 μg g^{-1}), soil moisture potential (−0.03 and −0.1 MPa), and temperature (23, 33, and 44 °C). DBU half-lives ranged from 1.4 to 45.6 days over all the conditions investigated, indicating that persistence of DBU in soils is unlikely. However, all half-lives reported by Lee et al. (2004) were determined from a single application of DBU. According to the product label, Benlate® can be applied as often as every 7 days if needed (E.I. du Pont de Nemours and Company 2000); therefore, DBU present in the formulation may be applied to soils repeatedly over a growing season. Repeated applications of pesticides to soils have been shown to increase the mineralization rates resulting in accelerated losses with subsequent applications of the pesticide, or in some cases, structurally related compounds (Racke & Coats 1990; Roeth 1986; Turco & Konopka 1990). The development of adapted microbial community and resultant enhanced degradation has been noted for benomyl and its degradation product, methyl benzimidazol-2-ylcarbamate (MBC), in soils with a previous treatment history of benomyl (Aharonson & Katan 1993; Yarden et al. 1985, 1987, 1990). Additionally, organisms capable of degrading benomyl and methyl benzimidazol-2-ylcarbamate (MBC) in soil have been isolated (Ali & Wainwright 1994; Helweg 1972).

Studies on the effect of pesticide applications to soils have found variable responses of the soil microbial community to the pesticides (Greaves 1979; Hart & Brookes 1997). Studies have shown that there was no discernable effect, an increase or decrease in measures of microbial activity or biomass carbon (Ahtiainen 2003; Anderson 1992; Chen et al. 2001; Duah-Yentumi & Johnson 1986; El-Ghamry et al. 2002; Haney et al. 2002; Hart & Brookes 1996; Martikainen et al. 1998; Prado & Airoldi 2001; Voets et al. 1974). The conclusion of several researchers has been that although an effect might be measurable in the short term, long-term effects of pesticide applications to soils are transient (El-Ghamry 2002; Greaves 1979; Hart & Brooks 1996). Several studies have also found that that total soil respiration measures in particular may not be affected by pesticide treatments (Ahtiainen et al. 2003; Hart & Brooks 1996). Respiration would indicate a holistic effect of a compound upon the soil microbial microbial community as opposed to measures which target a particular segment of the microbial community such as fungi. Additionally, applications of pesticides may both decrease and increase activity or biomass-C (Greaves 1979; Hart & Brooks 1996).

Application rates of Benlate® vary depending on the crop and the disease pressure. Typical field application rates of Benlate® are 0.56–2.2 kg ha^{-1} (0.5–2.0 lbs $acre^{-1}$), although past ornamental uses were labeled at a drench rate of 122 kg ha^{-1} (109 lbs $acre^{-1}$). Complete conversion of all the benomyl in a Benlate® formulation (50% by weight) will yield a calculated theoretical maximum of 14.7% DBU (by weight), although this highly unlikely to occur under any conceivable circumstance. Previous work conducted with a single DBU application indicated that DBU degrades relatively quickly and is unlikely to persist in soils (Lee et al. 2004). In the current study, we investigated the effect on DBU mineralization of 1–7 repeated applications of DBU at rates of 0.08 and 0.8 μg g^{-1} in two soils that represent the range in rates observed in our previous study (Lee et al. 2004). For one soil, which exhibited a slight negative concentration effect upon DBU mineralization in our previous study, we also investigated in more detail the effect of DBU concentrations from 0.0008 to 80 μg g^{-1} on DBU and glucose mineralization, a measure of microbial respiration.

Materials and methods

Soils

Collection and characterization of soils was described in Lee et al. (2004) (Table 1). Prior to beginning each experiment, triplicate soil samples (10 g dry weight equivalent) were weighed into 125 ml jars and brought to moisture content equivalent to a −0.03 MPa moisture potential and incubated for at least 3 days at 23 °C. Loss of water due to evaporation over the course of experimentation was monitored gravimetrically and water was added as needed to maintain a moisture potential of −0.03 MPa.

Table 1. Soil properties[a]

Soil ID	pH[b]	Type	Clay(%)	OC[c] (%)	CEC[d]	Total N[e] (mg kg^{-1})	P (Bray, Olsen)[f] (mg kg^{-1})	Site	DBU mineralization rate (day^{-1})[a]
7CB	6.3	Sandy loam	6.8	9.1	41.0	5200	11, 4	N. Costa Rica	0.231[g] 0.118[h]
9RD	7.9	Sandy loam	16.4	3.7	13.0	1818	115, 158	S. Florida	0.045[g]

[a]Lee et al. (2004).
[b]pH at 1:1 g ml^{-1} in water.
[c]determined by the Walkley–Black permanganate oxidation method (Nelson & Sommers 1982).
[d]Cation exchange capacity (meq 100 g^{-1}) determined by NH_4OAc extraction at pH7.
[e]Kjeldahl (wet oxidation) method.
[f]Available P determined using NH_4F-HCl extraction (Bray) and $NaHCO_3$ (Olsen) (Olsen & Sommers 1982).
[g]at 23 °C, 0.08 μg g^{-1}.
[h]at 23 °C, 0.8 μg g^{-1}.

DBU repeated application

Soil samples were treated with DBU by mixing into the soil sample silica gel, 100–200 mesh (EM Science, Gibbstown NJ) coated with DBU such that DBU final concentrations in the soil samples of either 0.08 or 0.8 μg g^{-1} were achieved. Control samples (no-DBU) were treated by adding the same mass of silica gel not treated with DBU. Silica gel was used in order to eliminate the repeated introduction of a carrier solvent in the application process, as well as to aid in the even distribution of the DBU throughout the sample. Enough samples were prepared so that three treated samples and three controls could be sacrificed at each time interval and assayed for DBU mineralization. One set of samples was sacrificed and treated at Day 0 (0th treatment frequency) so that mineralization rates using the silica carrier application process could be compared to the previous study where DBU was applied using methanol as a carrier. DBU treated silica was prepared by applying a solution of DBU in methanol (ACS Grade, Fisher Scientific, Pittsburg, PA) to silica gel, and adding an excess of methanol to create a slurry. The methanol–silica gel slurry was stirred to evenly distribute the DBU and the methanol was allowed to evaporate. The DBU treated silica was slurried with methanol once more to ensure even coverage of the chemical onto the silica. At the time of the initial application of DBU-silica, and at each subsequent treatment time (every 5–7 days) thereafter, three treated and three control samples were sacrificed to perform a DBU mineralization rate assay. The remaining samples were treated again with the DBU-silica or plain silica and placed in an incubator at 23 °C. In this manner, samples were treated in a staggered scheme such that seven total applications of DBU were applied to the final triplicate set of samples.

Determination of DBU mineralization rates after repeated application

To assess mineralization rates of DBU after each treatment, sacrificed samples were treated with DBU silica at the same DBU application concentration as the corresponding treatment concentration (0.08 or 0.8 μg g^{-1}), but supplemented with 0.03 μCi of [carbonyl-^{14}C] DBU, specific activity 55 mCi $mmol^{-1}$ (NEN Life Science Products, Boston, Massachusetts). The combined ^{12}C- and ^{14}C-DBU silica was prepared as described above for the nonradiolabelled DBU silica with the exception of the added ^{14}C-DBU. Silica gel containing only 0.03 μCi ^{14}C-DBU was applied to sacrificed control samples (approximate concentration 0.009 μg g^{-1}). A small vial containing 1M KOH (10 ml) was placed in the jar with the ^{14}C-DBU treated soil and the jars were sealed. DBU mineralization was monitored for 35 days by sampling vials at 1–5 days intervals. KOH solution (1 ml) containing the trapped $^{14}CO_2$ was placed in a scintillation vial (22 ml) with an aliquot of Econosafe scintillation cocktail (15 ml) (Research Products International Corp., Mt. Prospect, IL). Vials were kept in the dark for 24 h prior to LSC and ^{14}C determined by liquid scintillation

counting (LSC) on a Packard Model 1600TR LSC using external standard quench correction. Samples treated with ^{14}C-DBU were monitored over a period of 35–50 days and data was cumulated to generate mineralization curves.

DBU mineralization at different initial concentrations in soil 7CB

Soil (10 g dry equivalent weight) was weighed into 125 ml jars in triplicate, brought to −0.03 MPa water potential and pre-incubated at 23 °C at least 3 days prior to beginning the experiment. Samples were treated with DBU-silica to achieve six different concentrations, 0.0008, 0.008, 0.08, 0.8, 8, and 80 μg g^{-1} soil and a control set with no DBU pretreatment was treated with methanol washed silica. For the 0.0008 and the 0.008 μg g^{-1} concentration, only ^{14}C-DBU was used to treat the soil, resulting in an application of 0.0027 and 0.026 μCi, respectively. The high treatment concentrations were achieved using 0.026 μCi ^{14}C-DBU supplemented with nonradiolabeled DBU to achieve the appropriate concentrations. The DBU-silica was added to each soil sample and stirred well to mix. A vial containing 1 M KOH (10 ml) was added to each jar and the jars were returned to the 23 °C incubator. The KOH traps were sampled periodically and the KOH traps replaced with fresh solution. Evolved $^{14}CO_2$ was determined by liquid scintillation counting as described previously.

Microbial respiration in soils treated with different concentrations of DBU

Soil samples were prepared, pre-incubated, and treated with DBU-silica as described above for DBU mineralization, again using concentrations 0.0008, 0.008, 0.08, 0.8, 8 and 80 μg DBU g^{-1} soil, and a set of control samples which received no treatment with DBU prior to determination of respiration. Samples were allowed to incubate after DBU treatment for either 24 or 48 h. Each sample was then treated with 600 μg g^{-1} glucose and with 0.08 μCi ^{14}C-uniformly ring labeled (UL)-glucose (187 mCi mmol^{-1}; Sigma-Aldrich, St. Louis, MO). The concentration of glucose to be used was determined in a preliminary experiment where five different concentrations of glucose were applied and the maximum CO_2 evolution rate after a 4 h incubation time was determined (Horwath & Paul 1994). The final soil moisture potential for these two experiments was −0.03 MPa. Microbial respiration was monitored by determining evolved $^{14}CO_2$ from ^{14}C-UL-glucose, as described above. Respiration in the samples was compared by computing the glucose consumed by the microbial community.

Mass balance determination

At the conclusion of each incubation, soils were analyzed for ^{14}C remaining by combustion using a Packard Model 307 Sample Oxidizer (Perkin Elmer, Boston, MA). Soils were frozen and then lyophilized to remove water. Subsamples were weighed in triplicate (0.2–0.5 g dry weight equivalent) into a paper cup, mixed with approximately 200 mg powdered cellulose, and combusted for 3 min. Carbosorb E (10 ml) reagent was used to trap $^{14}CO_2$ and Permafluor E+ scintillation cocktail (10 ml) was added to the sample. Samples were left in the dark for 24 h before ^{14}C determination by LSC. Quenching was compensated by using a quench curve constructed with Carbosorb E and Permafluor E+. Mass balance was determined by summing the quantity of radioisotope recovered after combustions and the total recovered as $^{14}CO_2$ and comparing to the original amount applied to the soils.

Data analysis

Statistical analysis was performed using Microsoft Excel, Redmond WA (ANOVA, *t*-test), Systat 7.0, SPSS, Chicago, IL (ANOVA, GLM pairwise comparisons) or SAS, SAS/STAT User's Guide, Version 8, 1999, SAS Institute Inc., Cary, NC (ANOVA, Boxcox, and significance testing). Data were log transformed where necessary and interactions were all tested at $p < 0.05$ probability level.

Results and discussion

DBU repeated application

Mineralization rates after repeated application of DBU at two concentrations (0.08 and 0.8 μg g^{-1}) were determined in soil 9RD and 7CB 23 °C and a

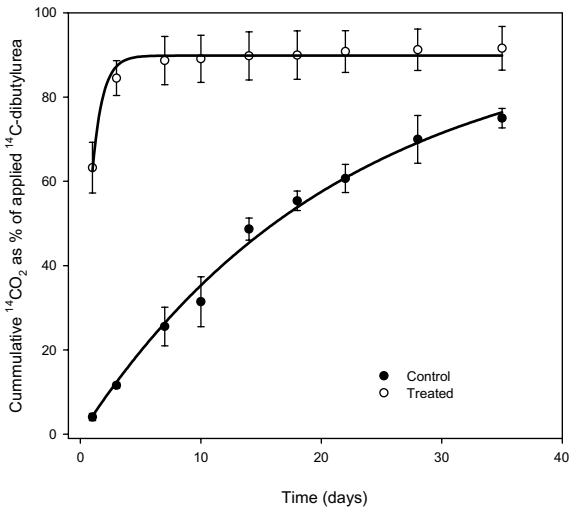

Figure 1. Degradation of dibutylurea (DBU) in soil 9RD after the 7th repeated application of DBU at 0.8 μg g^{-1}.

water potential of −0.03 MPa. The two DBU concentrations used here were the same as in our previous study (Lee et al. 2004). DBU mineralization rates were determined independently for each application by least squares fitting of the cumulative ^{14}C-CO_2 evolution data to the first order rate equation $\ln(C_t/C_0) = -kt$ where C_t and C_0 are the concentrations at time *0* and time t, respectively, and k is the first order rate constant. Average mass balance recoveries across all experiments was >98%. A representative plot of cumulative ^{14}C-CO_2 evolution over time is shown in Figure 1 and mineralization rates are summarized in Figures 2 and 3 for soils 9RD and 7CB, respectively.

Evaluation of treatment methodology

Silica gel was used as an inert carrier of DBU to soils to avoid the use of solvents while ensuring compound distribution throughout the soil sample (Anderson & Domsch 1978; Turco & Konopka 1990). To account for any effect of silica additions on DBU mineralization, a set of control samples, which received repeated additions of silica containing no DBU, were carried with each soil-treatment experiment. After each treatment time, a set of controls were sacrificed, spiked with only ^{14}C-DBU-silica, and mineralization monitored. The impact on DBU mineralization rates were small to negligible with each addition of silica gel across all experiments except for the 0.08 μg g^{-1} DBU level experiment with soil 7CB (Figures 2

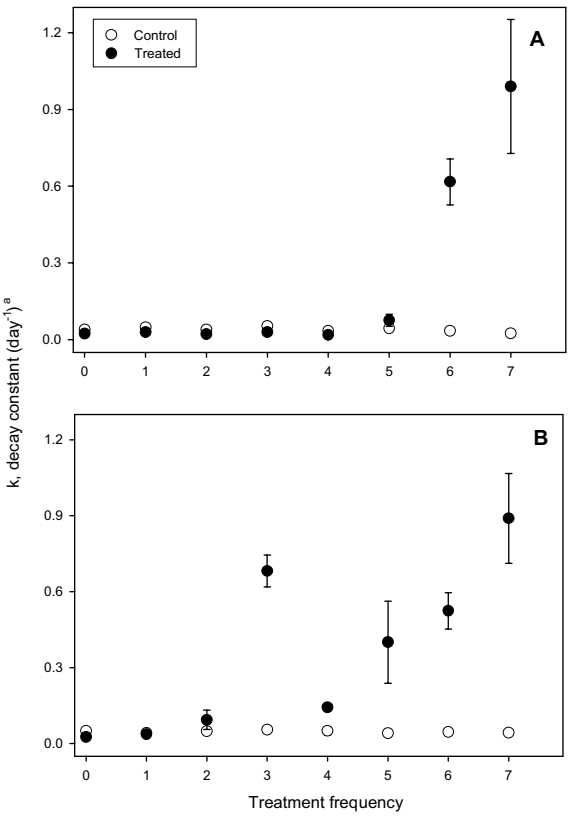

Figure 2. Dibutylurea (DBU) mineralization rate as a function of treatment frequency in soil 9RD at DBU application rates of (A) 0.08 μg g^{-1} and (B) 0.8 μg g^{-1}. Error bars depict standard deviation.

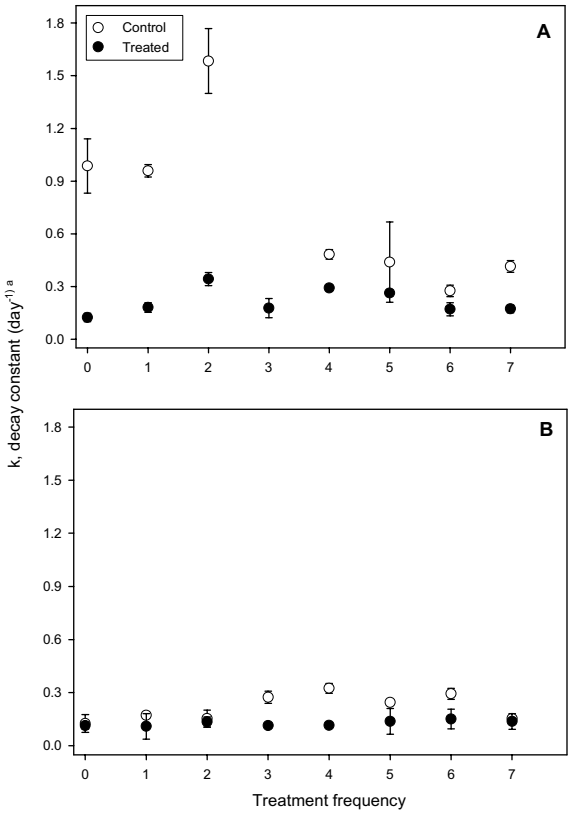

Figure 3. Dibutylurea mineralization rate as a function of treatment frequency in soil 7CB at (A) 0.08 μg g^{-1} and (B) 0.8 μg g^{-1}. Error bars depict standard deviation.

and 3). In the latter, the mineralization rates measured after the first no-DBU silica additions (0th treatment frequency) and the first two repeated additions (1st and 2nd treatment frequencies) are three to five times higher than observed in subsequent no-DBU silica additions (Figure 3) and in our previous study (Lee et al. 2004) (Table 1). A lower mineralization rate with the addition of DBU-silica would have been expected if DBU release from the silica was hindered. On the other hand, methanol as our carrier of DBU in our previous study may have negatively impacted the initial activity of the microbial communities responsible for DBU degradation. However, given that the DBU degradation rates observed for no-DBU silica additions after the 2nd treatment frequency and in all of the treatment frequencies with DBU-silica additions are within a factor of 2 of what was observed in our previous study, a further interpretation of the 0.08 μg g^{-1} DBU level soil 7CB controls (Figure 3A) would be tenuous.

Effect of repeated application on mineralization of DBU

For samples receiving repeated applications of DBU, mineralization rates in soil 9RD increased four fold after the fifth 0.08 μg g^{-1} application of DBU (Figure 2). After the 6th and 7th repeated DBU application, mineralization rates were nearly 20 and 34 times higher, respectively. At the higher concentration (0.8 μg g^{-1}), rate constants were nine times higher after the 5th and 6th treatments and 24 times higher after the 7th repeated application. Significant differences in mineralization constants for soil 7CB treated repeatedly at the 0.08 μg g^{-1} level were also observed, but the numerical differences in the rates were quite small compared to the increases observed in soil 9RD. The previously reported mineralization rate for DBU in soil 9RD (at 23 °C, −0.03 MPa, and 0.08 μg DBU g^{-1} application rate) was 0.045 day^{-1}, the slowest observed under these conditions for this soil. In the current study, the repeated application of DBU to soil 9RD clearly increased the ability of the soil microbial community to degrade DBU. Two explanations for the increase in DBU mineralization rates are plausible: (1) a specific microbial community was selected by repeatedly applying DBU to the soil; or (2) the overall microbial community in the soil was stimulated by the addition of nutrients in the form of DBU. DBU contains 65% C and 8% N and added at a rate of 0.08 μg g^{-1} to soil would yield an application of 0.0064 μg g^{-1} N and 0.05 μg g^{-1}C. These amounts are small relative to the total N and C content of these soils (Table 1), although not all of the totals reported in Table 1 will be readily available nutrients. Enhanced mineralization of pesticides has been well described in soils for many different pesticides (Kaufman et al. 1985; Racke & Coats 1990; Roeth 1986). So, it seems most likely that the specific DBU degrading microorganisms increased after the repeated application, leading to an increase in mineralization rates.

DBU mineralization at different initial concentrations in soil 7CB

Previous experiments had shown that DBU mineralization in soil 7CB was slower for the 0.8 μg g^{-1} application rate compared to the lower rate of 0.08 μg g^{-1} (Table 1). Therefore, minerali-

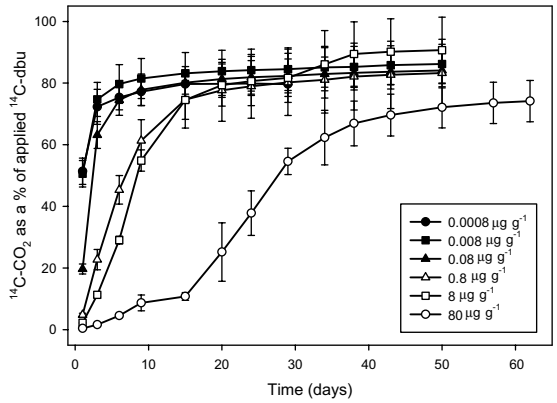

Figure 4. Dibutylurea mineralization at different application rates in soil 7CB at 23 °C and −0.03 MPa moisture potential. Error bars depict standard deviation.

zation rate constants were calculated for an expanded range of DBU concentrations from a single application to soil 7CB (Figure 4 and Table 2). For 0.0008 and 0.008 μg g^{-1} application rates, which would represent application concentrations of DBU from commercial benomyl formulations that may be realistic, no significant differences in mineralization rate were observed ($p < 0.05$). At all higher application rates, mineralization was significantly lower. However, the quantity of DBU degraded after 29 days was not significantly different for the lowest five application concentrations and averaged 81.7% of the total applied across the five lowest concentrations. After 70 days of incubation, when the last experiment was terminated, the quantity of DBU degraded was not significantly different for any of the six concentrations.

Microbial activity in soils treated with different DBU concentrations

The significant decrease in mineralization rates of DBU at the higher application concentrations studied suggests the possibility that application of DBU to these soils at high concentrations might have a detrimental effect on soil microbial community. Therefore, the impact of DBU concentration on overall microbial activity was assessed based on respiration of glucose (Vose 1980). Soils were pre-treated with DBU for either 24 or 48 h prior to measurement of microbial respiration to expose the microbial community to the chemical. There was no significant difference ($p < 0.05$) in respiration rates measured at 24 and 48 h after treatment with DBU for any of the DBU application rates. A preliminary study in which DBU was applied 1 h prior to assessing respiration also showed no differences in respiration at the different application concentrations (data not shown). Microbial activity was not significantly different in the 24 h pre-incubation study vs. samples pre-incubated for 48 h (ANOVA, $p < 0.05$). Average microbial activity in DBU treated soils was 0.0609 μmol glucose consumed g^{-1} h^{-1} (standard error = 0.0029) in samples which were preincubated with DBU for 24 h prior to measuring microbial respiration, and 0.0576 μmol glucose consumed g^{-1} h^{-1} (standard error = 0.0017) in samples treated 48 h prior to analysis. These values fall within an expected range for soils (Vose 1980).

Determining the exact cause of the decrease in mineralization rate in soil 7CB at higher application concentrations would likely be difficult and would have to be conducted at unrealistically high

Table 2. DBU mineralization rates in soil 7CD at six different application concentrations

Application concentration (μg g^{-1})	Mineralization rate (day^{-1}) (SE[a])	DBU degraded after 29 days % of applied (SE[a])	DBU degraded at termination of each experiment % of applied (SE[a])
0.0008	0.429 (0.093)	79.7 (2.3)	79.7 (2.3)
0.008	0.474 (0.092)	84.5 (4.0)	86.2 (3.7)
0.08	0.225 (0.028)	82.3 (3.2)	84.1 (3.3)
0.8	0.0871 (0.014)	80.2 (6.2)	83.3 (6.3)
8	0.0718 (0.0069)	81.7 (4.6)	90.7 (6.2)
80	0.0188 (0.0021)	54.6 (2.5)	74.5 (3.8)

[a]Standard error.

application rates. The end result observed here, that greater than 80% of the DBU was degraded at all application rates of DBU, indicates that although the initial effect of concentration on mineralization rate was significant, microbial mineralization proceeded to a similar endpoint. The probability that DBU would be inadvertently applied to soils at the levels included in this study is quite unlikely, since even the application rates of 0.08 and 0.8 μg g^{-1} used in this and the previous study were high for application of DBU to soils. The rates applied here were only used to accentuate the observable changes in microbial respiration, if in fact they existed, between application rates.

Conclusions

In the current study, we investigated the effect of the repeated application of DBU since Benlate®, which could potentially contain DBU, can be applied repeatedly over a growing season as often as every 7 days if needed (E.I. du Pont de Nemours and Company 2000). In the two soils studied here, we observed that repeated application of DBU to the soil (9RD), which had the slowest DBU mineralization rate in previous studies, increased the mineralization rate several fold. For the soil which represented a soil (7CB) with the faster initial mineralization rate in previous studies, the effect on mineralization was minimal. In the same soil, application of unrealistically high concentrations of DBU (0.08–80 μg g^{-1} soil) resulted in a decrease in mineralization rate, but the overall amount degraded did not differ. Note that a drench rate of 122 kg Benlate® ha^{-1} (109 lbs $acre^{-1}$) if all benomyl converted to DBU is less than 0.04 μg DBU g^{-1} soil. Also soil microbial activity assessed by glucose respiration was not affected by high concentrations. Findings from this study support the hypothesis that persistence of DBU in agricultural soils is not likely to occur.

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