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FATE OF THE ANTIFUNGAL DRUG CLOTRIMAZOLE IN AGRICULTURAL SOIL

LYNE SABOURIN,† ABDUL JABBAR AL-RAJAB,† RALPH CHAPMAN,† DAVID R. LAPEN,‡ and EDWARD TOPP*†
†Agriculture and Agri-Food Canada, London, Ontario
‡Agriculture and Agri-Food Canada, Ottawa, Ontario

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Abstract—Clotrimazole is a broad-spectrum antimycotic drug used for the treatment of dermatological and gynecological infections; it is incompletely broken down during sewage treatment and could potentially reach agricultural land through the application of municipal biosolids or wastewater. In the absence of any environmental fate data, we evaluated the persistence and dissipation pathways of ^{3}H-clotrimazole during laboratory incubations of agricultural soils. Clotrimazole was removed from a loam (time to dissipate 50% = 68 d), a sandy loam (time to dissipate 50% = 36 d), and a clay loam (time to dissipate 50% = 55 d), with formation of nonextractable residues being the major sink for ^{3}H. Their parent compound had no significant mineralization, as evidenced by the lack of formation of $^{3}H_2O$. Up to 15% of the applied radioactivity was recovered in the form of [^{3}H]-(2-chlorophenyl)diphenyl methanol. The rate of clotrimazole dissipation in the loam soil did not vary with moisture content, but it was slower at a lower temperature (number of days to dissipate 50% = 275.6 d at 4°C). Addition of municipal biosolids to the loam soil did not vary the clotrimazole dissipation rate. In summary, the present study has established that clotrimazole is dissipated in soil, at rates that varied with soil texture and temperature. Clotrimazole dissipation was accompanied by the formation of nonextractable residues and detectable extractable residues of the transformation product (2-chlorophenyl)diphenyl methanol. Environ. Toxicol. Chem. 2011;30:582–587. © 2010 SETAC

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INTRODUCTION

Pharmaceuticals and personal care products in raw sewage vary in their recalcitrance to biodegradation during wastewater treatment, and emissions from wastewater treatment plants are an important source of environmental exposure to pharmaceuticals and personal care products [1,2]. Chemicals that persist during sewage treatment and that are sorbed preferentially to organic matter can be released to the environment through the recovered sludge (biosolids [3]) rather than by the treated aqueous effluent. After application of biosolids to agricultural land, a common farming practice in many jurisdictions, some pharmaceuticals can be detected in surface runoff or leachate [4–7]. Considerable scientific and regulatory interest has arisen in the potential environmental and human health significance of exposure to pharmaceuticals in effluents from land receiving biosolids [8].

The widely used antimycotic agent clotrimazole (diphenyl-[2-chlorophenyl]-1-imidazolylmethane; Fig. 1) is typically administered in topical formulations for dermatophytic infections, and in lozenge or suppository form for oral or vaginal yeast infections [9]. Clotrimazole and other members of the imidazole class of antifungal medicines such as ketoconazole and miconazole inhibit the cytochrome P450–mediated demethylation of lanosterol by CYP51 in the ergosterol biosynthetic pathway, disrupting cell wall integrity and function [10]. Clotrimazole also inhibits other cytochrome P450s, and concern has been expressed that it has the potential to elicit endocrine-disrupting effects in exposed wildlife or humans. Specifically, imidazole antifungal agents inhibit cytochrome P450 aromatases, catalyzing the conversion of steroidal androgens to estrogens in rainbow trout (*Oncorhynchus mykiss*; [11]), frogs (*Xenopus tropicalis*; [12]), and humans [13–15], promote testosterone accumulation in human volunteers [16], and promote testicular development in female salmon (*Oncorhynchus tshawytscha* [17]).

Clotrimazole was detected in estuary surface water samples in the United Kingdom at a median concentration of 7 ng/L [18]. Negligible absorption occurs through the skin of topically applied clotrimazole, and therefore the primary route of entry into the sewage collection system is bathing and showering [19]. Reported concentrations in raw sewage range from 10 to110 ng/L [20]. Reported concentrations of clotrimazole in biosolids range from 190 + 28 to 1,442 + 107 μg/kg [21]. In a survey of clotrimazole fate in Swedish sewage treatment plants, concentrations in biosolids ranged from 30 to 120 μg/kg [22]. Furthermore, the mass of clotrimazole accounted for in biosolids from Swedish treatment plants was sufficient to account for all estimated clotrimazole product sales [22].

Based on clotrimazole's potency as an aromatase inhibitor and the likelihood that land application could be a major route of environmental exposure through leaching or runoff, we sought to determine its persistence in agricultural soil. In the present study, we undertook laboratory incubations with [^{3}H]-clotrimazole to determine the rate of dissipation, and how persistence and dissipation pathways varied with soil type, moisture, and temperature.

MATERIALS AND METHODS

Chemicals

Clotrimazole was purchased from Sigma-Aldrich Canada, and a standard of 2-(chlorophenyl)diphenyl methanol (Fig. 1) was purchased from U.S. Pharmacopeia. The [^{3}H]-clotrimazole (nominally uniformly labeled; radioactive purity 97.2%; specific activity 51.8 GBq/mmol) was purchased from Moravek Biochemicals. Stock solutions of ^{3}H-labeled (final radioactive concentration 8.72 KBq 100 μl) and unlabeled (200 μg/mL)

* To whom correspondence may be addressed (ed.topp@agr.gc.ca).
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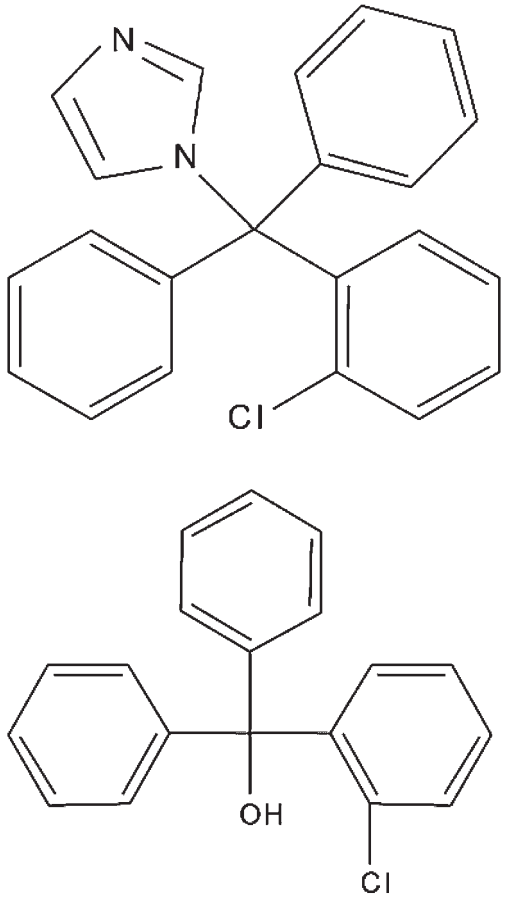

Fig. 1. Structures of clotrimazole and the transformation product (2-chlorophenyl)diphenyl methanol. Commercially purchased [^{3}H]-clotrimazole was nominally uniformly labeled.

clotrimazole were prepared in ethanol and stored at −20°C until used in experiments.

Soil and biosolids

Three agricultural soils (depth, 0–20 cm) were used in the current study: a loam soil obtained from the Agriculture and Agri-Food Canada research farm at London, Ontario (42°59′N, 81°15′W), a sandy loam soil obtained from the Agriculture and Agri-Food Canada research farm at Delhi, Ontario (42°51′N, 80°29′W), and a clay loam soil obtained from the Essex Region Conservation Authority research farm at Holid Beach, Ontario (42°2′N, 83°3′W). Key properties of these soils are described in Table 1. All soils were obtained from areas that were under sod and had never received biosolids. Soils were sieved to a maximum particle size of 2 mm and were stored at 4°C for periods of up to one year before experimentation.

Table 1. Key properties of the soils used in this study

Soil	Clay (%)	Silt (%)	Sand (%)	OM[a] (%)	CEC[b] (mEq 100/g)	pH
Loam	9.2	27.6	63.2	3.7	13.5	7.5
Sandy loam	3.7	7.8	88.5	1.5	5.4	6.1
Clay loam	32.8	40.0	27.2	3.7	16.6	5.2

[a] Organic matter content.
[b] Cation exchange capacity.

The liquid municipal biosolids used in the current study were obtained from the Adelaide pollution control plant in London, Ontario, Canada. The liquid municipal biosolids was return activated sludge from a secondary treatment; it had been aerobically digested, and it had the following characteristics: pH 6.6; dry matter content, 0.5%; organic matter content, 0.4%; carbon to nitrogen ratio, 6:1.

Laboratory dissipation studies

Soils were incubated in microcosms with radiolabeled clotrimazole, either added directly to the soil or applied with spiked liquid municipal biosolids. Microcosms consisted of 150 ml baby-food jars incubated in sealable glass 1-L Mason jars. Unless otherwise indicated, microcosms contained 50 g treated soil. A scintillation vial containing 10 ml water was placed in each jar to maintain a humid atmosphere and prevent desiccation of the soil. Soils were supplemented with ^{3}H-labeled and unlabeled clotrimazole by adding stock solutions (in ethanol) to 1-g portions of pulverized air-dried soil, allowing the solvent to evaporate, and thoroughly incorporating this into 49 g (moist wet) portions of the corresponding soil, to give a total of 50 g. Unless otherwise indicated, soils received 0.1 μg clotrimazole/g, and 166.67 Bq/g wet weight and soil moisture contents were normalized to 15% in all treatments. Triplicate microcosms were prepared for each treatment, and these were incubated in the dark at 30°C unless otherwise specified.

Extraction of soils

To estimate the portion of radioactivity remaining in the extractable fraction during the incubation, 5-g portions of soil were periodically removed from each microcosm with a spatula. Soils were extracted three times with 15 ml methanol. For each extraction, samples were shaken automatically for 10 min on a Burell wrist action shaker. Samples were then centrifuged for 10 min at 1,800 *g* in an HL-4 swinging bucket rotor in a Sorvall GLC-1 centrifuge (Fisher Scientific). The supernatants were transferred to a clean 250-ml glass round bottom bottle and a portion counted by liquid scintillation counting as described later. The extract was dried down to 1 ml using a rotary evaporator (Buchi Rotavapor R110), and 0.5 ml was removed for determination of radioactivity by liquid scintillation counting as previously described. The remaining extract was transferred to a 1.8-ml glass high-performance liquid chromatography (HPLC) vial and stored at 4°C until analysis. The difference in radioactivity counts in soil extracts and after evaporation was taken to be evaporative loss of ^3H_2O produced by the mineralization of clotrimazole. The extraction efficiency of this procedure was established in preliminary experiments by supplementing triplicate 5-g portions of soil with 25 μl of an ethanol solution containing 670 Bq [^{3}H]-clotrimazole. The methanol extraction method described recovered 87.8 ± 1.5, 87.7 ± 3.7, and $91 \pm 7\%$ of the radioactivity of applied clotrimazole in the loam soil, sandy loam soil, and clay soil, respectively. On this basis, the extraction method was considered satisfactory.

Analytical methods

Radioactivity extracted from the soil was quantified by liquid scintillation counting, using a Beckman Coulter Model LA 6500 instrument. Each sample was added to 10 ml ICN UniverSol scintillation cocktail in a plastic scintillation vial and counted 24 h later to allow for chemiluminescence to subside. Data were corrected automatically for quenching.

The disposition of extracted radioactivity in parent compound and potential transformation products in extracts was

determined as follows. Standards of clotrimazole and the potential transformation product 2-(chlorophenyl)diphenyl methanol were resolved and detected by HPLC (Waters) with ultraviolet (UV) detection (HPLC-UV). An Agilent Zorbax Eclipse XDB C-18 column, (4.6 mm × 250 mm, 5-μm pore size) was used, and the UV detector (Varian 9050 Variable Wavelength UV/Vis Detector) was set at 210 nm. The mobile phase consisted of 75:25 acetonitrile:water adjusted to a pH value of 3.5 with phosphoric acid. With solvent delivered at 1 ml/min, the retention time (RT) of clotrimazole was 8 min, and the RT of (2-chlorophenyl)diphenyl methanol was 11 min.

The mass balance of radioactivity in parent compound and in potential transformation products was established by injecting 50 μl concentrated soil extract (containing a known amount of radioactivity determined by liquid scintillation counting) in the HPLC operated as indicated previously, and fractionating the exit flow at 1- or 2-min intervals into scintillation vials. The radioactivity of each fraction was then determined by liquid scintillation counting, and the retention time of the recovered ^{3}H referenced to the retention time of the standards established by HPLC-UV. The fraction of radioactivity in parent and product was expressed as a percentage of the total radioactivity recovered in each sample. In all cases, the total radioactivity collected through fractionation corresponded closely to the known quantity injected. To identify potential transformation products of clotrimazole, selected soil extracts were analyzed by HPLC mass spectrometry (HPLC-MS). The HPLC-MS system consisted of an Alliance 2690 HPLC/autosampler and an LTC orthogonal acceleration time-of-flight mass spectrometer (Waters/Micromass). Samples were analyzed isocratically on a 2 × 150 mm, 5-μg Prodigy ODS3 column (Phenomenex) with 25:75:0.1 water-acetonitrile-formic acid flowing at 0.2 ml/min. The total effluent from the column was introduced into the standard ion source of the mass spectrometer operating in positive ion mode. Flow from the column was diverted from the MS between 1 min and 4.5 min when analyzing soil extracts to reduce contamination of the ion source. Source conditions were as follows: capillary, 3,000 V; desolvation temperature, 250°C; desolvation gas flow, 450 L/h; and sample cone, 20 V. Mass spectra were acquired over the range 100 to 1,500 m/z. Instrument control, data acquisition, and processing were accomplished using MassLynx 4.0 (Waters/Micromass). Under the conditions used, clotrimazole and (2-chlorophenyl)diphenyl methanol had RTs of 2.2s and 8.2 min, respectively. Extracts for HPLC-MS analysis were obtained from soil incubations with unlabeled clotrimazole executed in parallel with incubations of soil amended with both labeled and unlabeled clotrimazole.

Calculations and statistics

Dissipation rates for parent compound were estimated on the basis of removal of [^{3}H]-clotrimazole from the extractable phase established by liquid scintillation counting and by high-performance liquid chromatography-radiation detection (HPLC-RD). Raw data analysis was conducted using Microsoft Excel 2002 (Microsoft Canada). The HPLC-RD data were captured and evaluated using Waters Empower2 software. Dissipation curves were plotted using SigmaPlot (Version 10, Systat Software). Data in figures represent the means and standard deviations of triplicate samples. Significant treatment effects on dissipation rate constants were evaluated using the Student's *t* test [23] at 95% confidence.

RESULTS AND DISCUSSION

The dissipation of clotrimazole was evaluated in a loam, a sandy loam, and a clay loam soil (Fig. 2). At the start of the experiment, approximately 65% of the [^{3}H]-clotrimazole was extractable. Clotrimazole is a weak base with a reported pK_a of 6.12 [19]. In the unprotonated form the log K_{OW} is 4.1 [19], but the K_{OW} decreases with pH as the molecule becomes charged [24]. These characteristics suggest that both non-ionic and pH-dependent ionic interactions can influence the sorption of clotrimazole to soil, with the strength varying with soil pH, organic matter, and clay content. The discordance between the extraction efficiencies obtained in preliminary experiments and the apparent extraction efficiency in the microcosm experi-

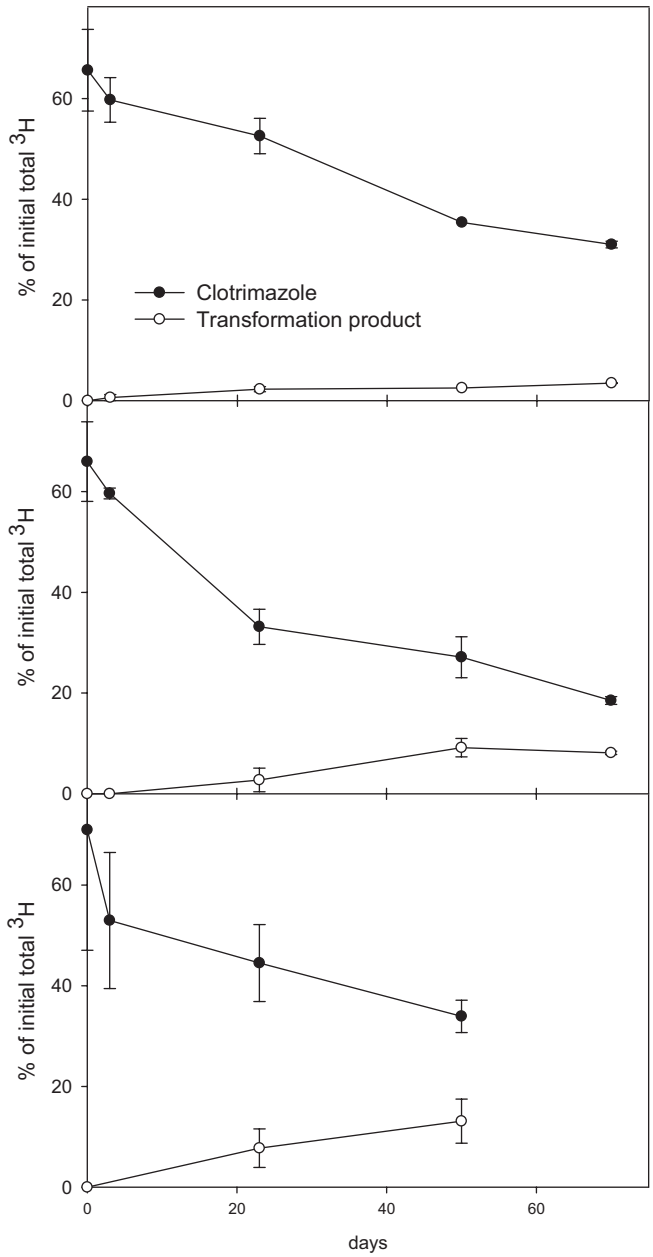

Fig. 2. Dissipation of [^{3}H]-clotrimazole in three agricultural soils incubated in the laboratory. Soil moisture content was adjusted to 15% before incubation at 30°C. High-performance liquid chromatography was used to resolve the extractable radioactivity in each extract into that portion carried by the parent clotrimazole and the transformation product (2-chlorophenyl)diphenyl methanol. Data represent mean ± standard deviation for triplicate microcosms.

Table 2. Summary of kinetics of clotrimazole dissipation in soils of varying texture (data from Fig. 2), and in the loam soil incubated with varying temperature (Fig. 3), moisture (Fig. 4), and in the presence of biosolids (Fig. 5)

Variable	Treatment	DT50 (d)	k (/d)	r^2
Soil type	Loam	68.0 ± 5.8^{a}	0.0102 ± 0.0008	0.9288 ± 0.0957
	Clay loam	54.7 ± 28.2^{ab}	0.0158 ± 0.0095	0.8766 ± 0.0920
	Sandy loam	36.2 ± 1.7^{b}	0.0192 ± 0.0009	0.9424 ± 0.0113
Temperature	30 °C	57.1 ± 10.6^{a}	0.0124 ± 0.0021	0.7793 ± 0.0825
	20 °C	144.4 ± 106.3^{ab}	0.0065 ± 0.0035	0.3769 ± 0.2653
	12 °C	235.3 ± 37.0^{b}	0.0030 ± 0.0005	0.2080 ± 0.0715
	4 °C	275.6 ± 96.4^{b}	0.0028 ± 0.0011	0.2631 ± 0.2509
Moisture	Air-dry	114.1 ± 23.1^{a}	0.0063 ± 0.0014	0.3279 ± 0.0201
	7%	81.7 ± 11.2^{a}	0.0086 ± 0.0012	0.4340 ± 0.1752
	15%	95.9 ± 37.4^{a}	0.0079 ± 0.0026	0.6611 ± 0.0465
	Water holding capacity	130.8 ± 49.5^{a}	0.0060 ± 0.0029	0.3254 ± 0.2636
Biosolids	+ Biosolids	65.5 ± 10.8^{a}	0.0108 ± 0.0018	0.7788 ± 0.1005
	−Biosolids	63.9 ± 17.2^{a}	0.0115 ± 0.0036	0.6568 ± 0.2713

Calculations of the number of days to dissipate 50% (DT50) of the initially applied ^{3}H-clotrimazole and rate constants were based on the assumption that dissipation was first order, and the goodness of fit to the first-order kinetic model is expressed with the coefficient of determination. r^2 = coefficient of determination; k = rate constant in reciprocal days. The superscript letter following the rate constants indicates if that parameter is significantly different from the other treatments using a Student's t test.

ments was most likely attributable to the difficulty in supplementing and sampling the microcosm soils uniformly.

During incubation of soils at 30°C, clotrimazole was removed slowly from the extractable phase (Fig. 2, Table 2). The time to dissipate 50% in the loam soil was 68.1 ± 5.8 d, in the sandy loam soil 36.1 ± 1.7 d, and in the clay loam soil 54.7 ± 28.2 d. The r^2 values ranged from 0.8766 with the clay loam to 0.9424 with the sandy loam, indicating that the fit to a first-order kinetic model was generally good (Table 2). No significant radioactivity was lost on evaporation of any of the extracts, indicating no $^{3}H_2O$ was produced through mineralization of [^{3}H]-clotrimazole during the incubation (data not

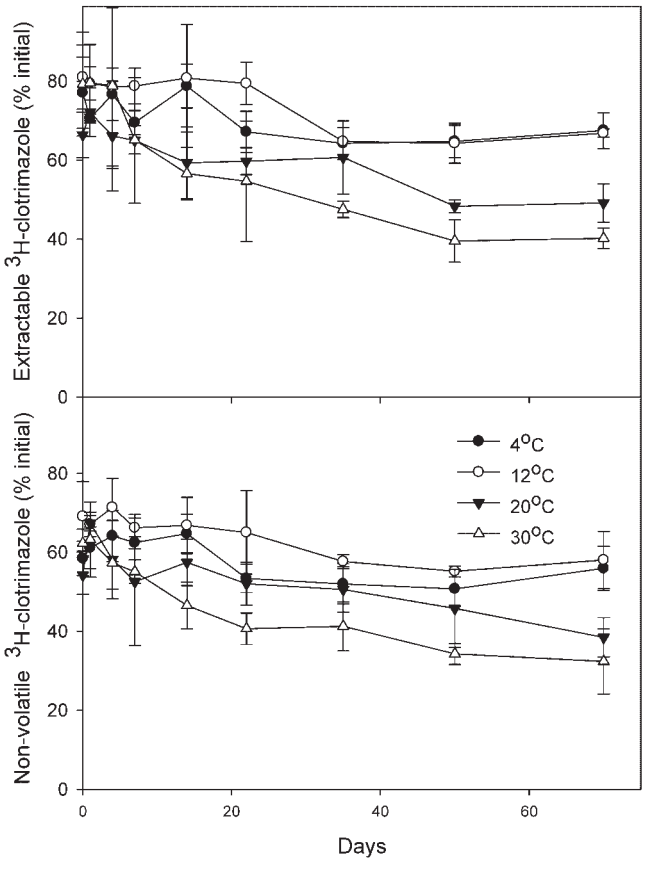

Fig. 3. Dissipation of [^{3}H]-clotrimazole in a loam soil incubated at various temperatures. Soil moisture content was adjusted to 15% before incubation. Total radioactivity in soil extracts before (top panel) and after (bottom panel) evaporation was determined to establish what fraction of the [^{3}H]-clotrimazole was mineralized to $^{3}H_2O$. Data represent mean ± standard deviation for triplicate microcosms.

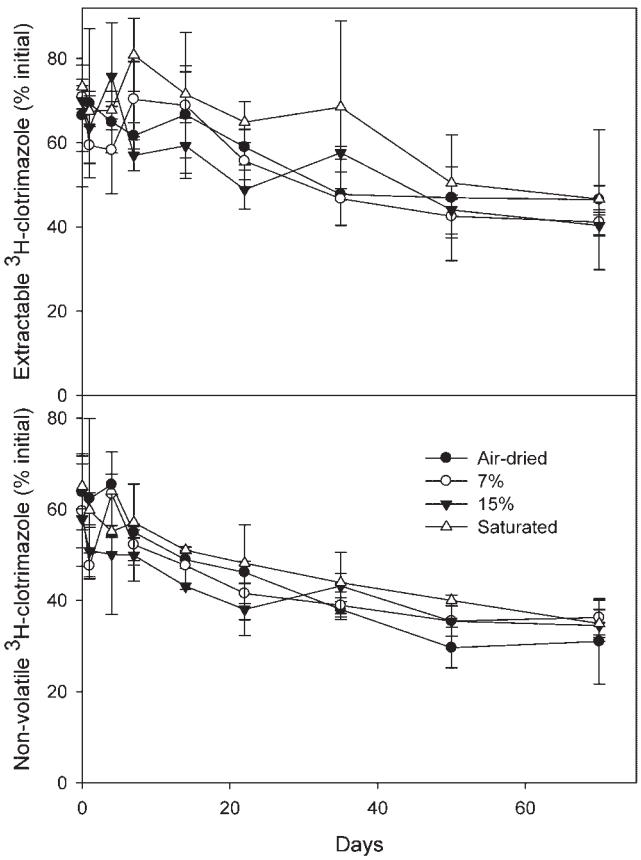

Fig. 4. Dissipation of ^{3}H-clotrimazole in a loam soil incubated at 30°C with varying moisture content. Total radioactivity in soil extracts before (top panel) and after (bottom panel) evaporation was determined to establish what fraction of the [^{3}H]-clotrimazole was mineralized to $^{3}H_2O$. Data represent mean ± standard deviation for triplicate microcosms.

shown). Analysis of extracts by HPLC-RD indicated that as the experiment progressed some portion of the recovered radioactivity did not co-elute with a clotrimazole standard (RT = 8 min), but rather was found in a product with an RT of 11 min. This retention time corresponded to that of a standard of (2-chlorophenyl) diphenyl methanol, a clotrimazole degradation product found in commercial formulations (Fig. 1; [25]). The transformation product had a mass spectrum identical to that of a standard of (2-chlorophenyl)diphenyl methanol on HPLC-MS analysis (data not shown). By the end of the incubation, the accumulated (2-chlorophenyl)diphenyl methanol represented up to approximately 15% of the initially applied radioactivity (Fig. 2). The substituted imidazole structure of the molecule confers antifungal activity; therefore, the methanol transformation product generally will not be inhibitory to cytochrome P_{450}s [26].

The impact of the key variables, soil temperature and moisture on clotrimazole dissipation, was evaluated in the loam soil. As expected, clotrimazole residues were more persistent at lower temperatures, with the time to dissipate 50% increasing from 57.1 ± 10.6 d at 30°C to 275.6 ± 96.4 d at 4°C (Table 2, Fig. 3). At the end of the incubation, the extractable methanol metabolite did not exceed 5% of the initial ^{3}H applied in any of the treatments (data not shown). None of the radioactivity was lost when extracts were dried, indicating no ^3H_2O in the extract, as would be produced by [^{3}H]-clotrimazole mineralization (Fig. 3). Varying soil moisture content had no significant effect on the clotrimazole dissipation rate (Table 2, Fig. 4). None of the radioactivity was lost when extracts were dried, indicating no significant clotrimazole mineralization (Fig. 4).

The addition of municipal biosolids (5% liquid municipal biosolids v/v) to the loam soil had no effect on clotrimazole dissipation and did not promote [^{3}H]-clotrimazole mineralization (Table 2, Fig. 5). All radioactivity in the extracts comigrated with clotrimazole in HPLC-RD (data not shown).

In summary, at 30°C clotrimazole dissipated in three different soils with number of days to dissipate 50% in the range of one to two months. In the loam soil, clotrimazole dissipation rates decreased with incubation temperature. At colder temperatures or under drier soil conditions, the goodness of fit to the first-order model was quite poor. This is consistent with clotrimazole dissipation under these circumstances being rate limited by a factor (temperature or moisture) other than, or in addition to, the bioavailable concentration of the parent compound. The clotrimazole transformation product (2-chlorophenyl)diphenyl methanol was detected in soil extracts, and no detectable mineralization of [^{3}H]-clotrimazole residues was found under any conditions.

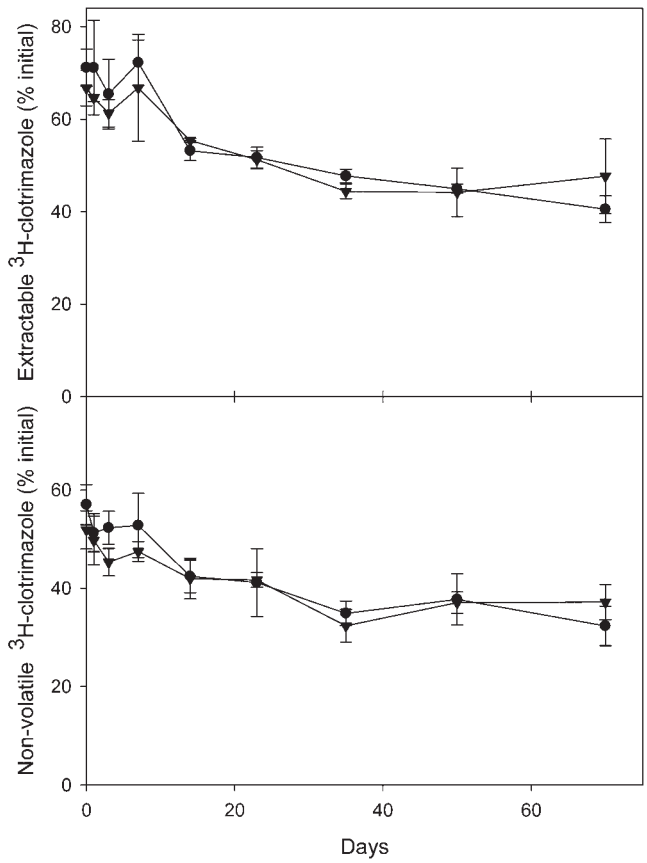

Fig. 5. Impact of supplementation with liquid municipal biosolids on the dissipation of ^{3}H-clotrimazole in a loam soil incubated at 30°C. Liquid municipal biosolids (closed circle) was added at a final concentration (v/v) of 5%, with control soils receiving the same volume of additional water (closed triangle). Total radioactivity in soil extracts before (top panel) and after (bottom panel) evaporation was determined to establish what fraction of the [^{3}H]-clotrimazole was mineralized to ^3H_2O. Data represent mean ± standard deviation for triplicate microcosms.

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