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TOXICITY OF 2,3,7,8-TCDD AND SHORT-TERM BIOACCUMULATION BY EARTHWORMS (OLIGOCHAETA)

ADRIAAN J. REINECKE
Department of Zoology, Potchefstroom University, Potchefstroom, South Africa
and
RALPH G. NASH
Pesticide Degradation Laboratory, U.S. Department of Agriculture, Beltsville, MD 20705, U.S.A.

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Summary—The earthworms *Allolobophora caliginosa* and *Lumbricus rubellus* were used to study the toxicity of 2,3,7,8-TCDD (dioxin) for earthworms. The earthworms were exposed to soil containing concentrations ranging from 0.05 to 5.0 $\mu g\,g^{-1}$. No worms were killed or showed any other observable toxicological effects when exposed to concentrations up to 5 $\mu g\,g^{-1}$ for 85 days in soil. The lethal threshold concentration for TCDD to earthworms falls between 5 and 10 $\mu g\,g^{-1}$ in this study. In soils containing 0.05 $\mu g\,g^{-1}$ earthworms accumulated TCDD up to 5 times the original soil concentration within 7 days. Worms were also exposed to TCDD on filter paper to study the behaviour of earthworms and the uptake of TCDD after surface contact. The earthworms did not avoid TCDD in their environment, indicating an indifference to it. No active penetration of TCDD into the body occurred where earthworms were exposed to surface concentrations. No indication was found of possible biological breakdown of TCDD on passing through the earthworm gut, although the search for metabolites was limited to the mono-, bi- and trichlorinated dioxins. There was a steady decrease (a $T_{\frac{1}{2}}$-value of 80–400 days) in the amount of TCDD recovered from worm-worked soil compared to soil without worms.

INTRODUCTION

TCDD (dioxin) is considered one of the most toxic man-made compounds (Higginbotham *et al.*, 1968; Poland *et al.*, 1971). Concern has been expressed over genetic, carcinogenic and teratogenic effects, and the debate over the danger of chemical pollution by TCDD and its attendant biological hazards continues (Pemberton, 1980; Henig, 1980; James, 1979; Zack and Su, 1980; Report to the New Zealand Minister of Health, 1980, Kjellström, 1980). TCDD has not been found in the environment (excepting areas of severe contamination resulting from factory accidents) or in animals, e.g. eagle carcasses (Woolson *et al.*, 1973). Most countries have used and still are using the herbicide 2,4,5-T on a wide scale. This herbicide normally contains low quantities of TCDD.

Woolson *et al.* (1972) examined 129 phenol-derived pesticides for dioxins. They found TCDD in 23 of the 42 samples of 2,4,5-T and the concentration ranged from <0.01 to $>10\,\mu g\,g^{-1}$. TCDD has also been found in older 2,4,5-T formulations in Sweden (Nörstrom *et al.*, 1979) although most producers of 2,4,5-T claim their products contain less than 0.1 $\mu g\,g^{-1}$ TCDD. In 1973, the commercial formulations of 2,4,5-T in the U.S.A. contained $<0.5\,\mu g\,g^{-1}$ chlorinated dioxins (Helling *et al.*, 1973). The current limits set by authorities for TCDD concentrations in 2,4,5-T are not known (if any) for many countries where this herbicide is currently being used. For most western countries the limits are below 0.5 $\mu g\,g^{-1}$.

A normal high application rate of 5 kg 2,4,5-T ha^{-1} containing 0.1 $\mu g\,g^{-1}$ TCDD represents a concentration of about 0.5 pg TCDD ha^{-1} in the surface 10 cm of soil. The lowest rate used in this study (0.05 $\mu g\,g^{-1}$ represented approximately 1×10^6 times the likely TCDD concentration in soil from one application of 2,4,5-T. The analytical limits of determination influenced the selection of these concentrations after preliminary observations showed that the earthworms were not adversely affected by low concentrations of TCDD. It must, however, be kept in mind that in many African and other countries imported chlorinated phenoxy herbicides have been used since the 1950s. Especially 2,4,5-T has been in continual agricultural use since 1957 in widespread areas to control the weed, *Opuntia aurantiaca*. The contamination levels of the formulations were reportedly higher and taking the persistency and build-up potential of TCDD in soil into account (Kearney *et al.*, 1973), levels in excess of those calculated above for one application of 2,4,5-T could still be expected.

We report the toxicity of TCDD to the earthworms *Allolobophora caliginosa* and *Lumbricus rubellus*, and the presence of TCDD in the bodies of these earthworms living in soil amended with TCDD.

METHODS

Surface exposure of earthworms

Earthworms were exposed to filter paper (9 cm dia) treated with TCDD in amounts ranging from 0.9 pg to 199 μg to determine whether TCDD acted as a contact poison. Hexane was used as a solvent. Paper was treated by pipetting 1 ml of a solution with the appropriate concentration so as to cover the whole filter paper surface. This resulted in surface concentrations ranging from 0.02 pg cm^{-2} to 3.13 $\mu g\,cm^{-2}$.

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After evaporation of the solvent the papers were moistened with 2 ml distilled water.

Three worms were placed in each Petri dish containing TCDD-treated filter paper. Petri dishes were divided in groups of 10 replicates for each dose and kept in an environmental chamber set at 20°C.

Worms were collected regularly starting 24 h after exposure with a maximum exposure time of 8 days. Sampled worms were kept on clean, moist filter paper and checked daily for effects. Some of the sampled worms were weighed, placed in hexane and stored at −5°C for later TCDD analysis. Other sampled worms were placed into moist (20–30%) non-treated soil after various periods of exposure to TCDD on filter paper.

A short multiple-choice experiment was undertaken to determine whether the earthworms could detect TCDD in their environment. A filter paper was cut into four quarters. Three of the pieces were treated with different concentrations of TCDD. All four pieces were placed into a Petri dish after the hexane had evaporated and moistened slightly with 2 ml water. Ten worms were placed in the Petri dish consecutively. Each worm was placed on each of the quarters for at least 5 min and its movements were noted. A second batch of 10 worms was used on a different set of treated paper quarters. TCDD concentrations ranging from 3 pg cm^{-2} to 10 $\mu g\ cm^{-2}$ were used. The direction of movement and the possible selection of areas were noted. The sole purpose of this small exercise was to determine whether earthworms could sense and possibly avoid TCDD in their environment.

Preliminary tests with 50 live specimens of *A. caliginosa* and *L. rubellus* were undertaken to ensure that both species would survive the duration of the exposure on moist filter paper without food. *L. rubellus* died after 3 days without food while some *A. caliginosa* (1–2%) died after 20 days. No indication was found of earthworms consuming filter paper.

Exposure of earthworms to TCDD in soil

Thirty-five juveniles of *A. caliginosa* were exposed in groups of seven or eight worms to each of four different concentrations of TCDD (0.05, 0.5, 1.5 and 5.0 $\mu g\ g^{-1}$) in 50 g of soil in Petri dishes at 20°C, thus giving four replicates at each concentration. One batch of seven worms was exposed to 10 $\mu g\ g^{-1}$. The soil was Galestown sandy loam (Psammantic Hapludults) (pH 6.7; organic matter 5.2%; moisture content tension 15.6% at 33 kPa) was sieved (<2 mm), treated with TCDD (using acetone as solvent) and moistened (20–30%) before the worms were introduced. Soil and worm samples were taken from each of the concentrations periodically for analysis (Table 1).

Some soil and earthworm samples were placed in a freezer until TCDD assay. Worms were kept on moist filter paper for 24 h to allow for evacuation of the gut contents before weighing and preservation and checked regularly for mortality. Soil was kept moist through the entire experiment by adding a few millilitres of distilled water every third day. Soil moisture content varied between 20 and 30% (oven-dry method).

Control soils containing earthworms but no TCDD and control soils containing TCDD but no earthworms were established and sampled simultaneously with TCDD treated soil.

Extraction clean-up and analysis

All soil and tissue samples were extracted in Soxhlets for 12 h as in the analytical method of Nash and Beall (1980), except that the NaCl solution was not added. The stored frozen worms were homogenized in hexane then extracted in the same way as soil samples. Care was taken to remove all fibrous material from the homogenizer blade.

The samples were analyzed by ^{63}Ni-electron capture gas–liquid chromatography (GLC). The columns were 1.8 m × 4 mm i.d. glass packed with 3% OV-17 Gas Chrom Q. Column temperature was 215°C. Carrier gas was CH_4-Ar (5/95%) with a flow rate of 50 ml min^{-1}. Relative retention times against standards were used for qualitative analyses and peak heights for quantitative analyses.

A check on the extraction efficiency showed that it was above 75% for both soils and earthworms and frequently much higher than 80%.

RESULTS

Surface toxicity tests revealed that TCDD was not acutely toxic to earthworms of the species *A. caligi-*

Table 1. Proportional recovery (%) of 2,3,7,8-TCDD recovered (soil dry wt) from soils subjected to earthworm activity for various periods

	TCDD soil treatment rate ($\mu g\ g^{-1}$)				
Day	0.05	0.5	1.5	5.0	Control 5.0 (No earthworms)
1	95	75	86	66	84
7	100	75	69	78	92
12	ND	74	ND	ND	ND
20	100	73	63	59	78
50	95	69	54	59	82
55	86	ND	ND	ND	ND
85	72	8(?)	39	33	86
[a]$C_{1/2}$	210	[c]408	85	83	
[b]r^2	0.75*	0.99**	0.94**	0.30	

[a]$C_{1/2}$ = Time in days required to reduce TCDD concentration by one half.
[b]r^2 = Coefficient of determination, when fitted to means, (*) 95 and (**) 99% confidence level.
[c]First 50 days.

Table 2. Earthworm bioaccumulation of 2,3,7,8-TCDD applied to soil as influenced by soil–worm contact time and TCDD application rate

Day	Soil treatment rate ($\mu g\ g^{-1}$) 0·05	0.5	1.5	5.0
7	4.93	1.06	0.86	0.44
20	9.41	0.68	0.64	0.17
55	4.00	0.89	0.48	0.28
85	4.00	0.63	0.40	0.15
$^{a}C_{1/2}$	ND	ND	75	ND
^{b}T	0.36	0.35	0.94*	0.37

[a,b]See Table 1.

nosa or *L. rubellus*. No deaths occurred that could be related to the presence of TCDD. Superficially, worms that had been exposed for up to 191 h on filter paper treated with 199 μg TCDD did not show any observable abnormalities in behaviour and reproduction for up to 85 days after they were removed from TCDD and placed into moist soil. Cocoons were produced and feeding and burrowing activities seemed unaffected. Dissection and observation under a stereo microscope revealed no gross tumours externally or internally although histological sections were not prepared.

The earthworms which were given a choice of various treated or non-treated TCDD filter paper, were unable to distinguish between areas with and areas without TCDD or between various concentrations of TCDD. Since it was clear that the worms moved at random across the treated and non-treated section of the filter paper the matter was not pursued further. These results indicated that earthworms are unable to sense TCDD in their environment at the concentrations used here.

Although traces of TCDD were probably adhering to the body surface after exposure to filter paper treated with 199 μg TCDD for 191 h, no active penetration of the body wall seems to have occurred. Two worms were "washed" by dipping them momentarily into hexane. The hexane subsequently analysed showed extremely small TCDD peaks on the chromatogram while the whole body homogenates appeared to be without any traces of TCDD.

The lethal threshold concentration for TCDD to earthworms in this experiment falls between 5 and 10 $\mu g\ g^{-1}$. Although the earthworms ingested the TCDD-contaminated soil actively, no mortalities occurred after 85 days exposure even at concentrations as high as 5 $\mu g\ g^{-1}$. However, exposure of earthworms to soil containing 10 $\mu g\ g^{-1}$ TCDD caused some mortality after approximately 20 days and 100% mortality by day 30. Dead worms sampled by day 30 contained 1.8 to 3.0 $\mu g\ g^{-1}$ TCDD (expressed in terms of wet wt or biomass).

The amount of TCDD in the environment is usually low, even where 2,4,5-T has been used (Bovey and Young, 1980). The possibility that this chemical could under normal circumstances affect the earthworm populations adversely seems to be remote. However, if exposure to TCDD over an extended period of time could cause a build-up in the earthworm in excess of 2 $\mu g\ g^{-1}$ the effects could be noticeable. Undoubtedly the TCDD ingested with the soil ends up in the body tissue of the earthworm. Worms exposed for 4 days to various concentrations of TCDD in soil still had considerable amounts in their bodies 30 days after they have been removed from contaminated soil and placed into clean soil. Small amounts of TCDD (0.13 ng) were still recovered 2 months after exposure of the worms to TCDD had been terminated. It can be deduced that the TCDD is incorporated into earthworm body tissues as well and does not merely adhere to the external body surface or internal surface of the intestine.

Earthworms are thought not to be very susceptible to pesticides in general (Beyer and Gish, 1980). Earlier reports have shown that lipophilic organochlorine pesticides are incorporated into the body tissues as the worms pass contaminated soil through their intestines. It has been demonstrated that although some chemicals are not acutely toxic to earthworms the sublethal effects on growth and reproduction could affect earthworm populations severely (Venter, 1983).

Our study indicates that the amounts of TCDD in the soil and those in the worms are not necessarily linearly related (Tables 1 vs 2). Proportionately less TCDD accumulated in tissues of worms from soils containing high amounts of TCDD than from soils with low amounts.

In soils containing 0.05 $\mu g\ g^{-1}$ earthworms accumulated TCDD up to 5 times (calculated in terms of biomass) the original soil concentration within 7 days (Table 2). However at 0.5; 1.5 and 5.0 $\mu g\ g^{-1}$ TCDD soil concentrations there was no accumulation. When the data was fitted to a first order equation, no change in the concentration of TCDD was indicated, except in the 1.5 $\mu g\ g^{-1}$ treatment, in which a decrease in concentration occurred with time. This finding is of great ecological importance because earthworms are a major source of food for many birds, several species of mammals and some reptiles. If TCDD is indeed as dangerous to mammals as the literature suggests (0.06 $\mu g\ kg^{-1}$ acute toxicity for guinea pigs, Helling *et al.*, 1973) a build-up of this kind could, if allowed to occur in the environment, pose a problem far more hazardous than those already known for persistent organochlorine

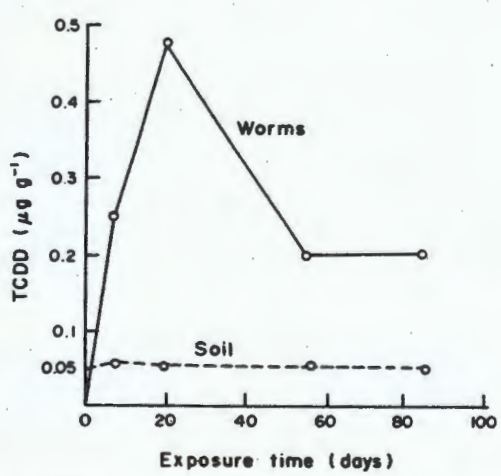

Fig. 1. TCDD concentration in earthworms and soil after a 0.05 $\mu g\ g^{-1}$ soil treatment rate.

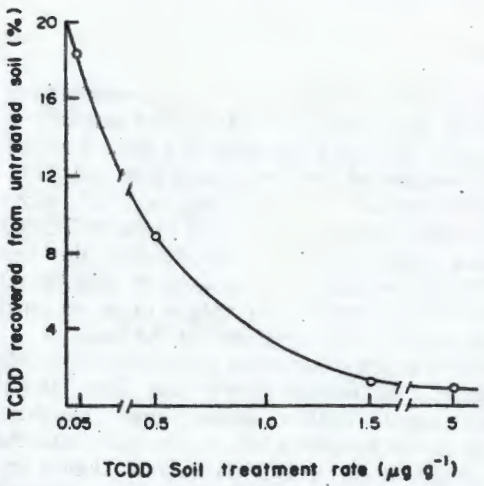

Fig. 2. Soil TCDD removal from earthworms previously exposed for 4 days to soil containing TCDD.

substances. Fanelli *et al.* (1980) concluded from preliminary data from the Seveso, Italy accident that TCDD appears to be accumulating in wildlife.

The absence of a constant relation between TCDD in the soil and that in the worm seems to indicate that the total TCDD content is possibly kept within limits by an excretory mechanism. Figure 1 illustrates the results obtained after analysing worms and soil after various periods of exposure to soil containing $0.05\,\mu g\,g^{-1}$ TCDD. In soils containing $0.5\,\mu g\,g^{-1}$, TCDD accumulated in earthworm tissues to concentrations slightly in excess of that in the soil (Table 2). In soils containing 1.5 or $5\,\mu g\,g^{-1}$ TCDD, concentration in the earthworms did not increase to, or even reach the concentration present in the soil. Hence, the TCDD concentrations in the worms relative to that in the soil at the higher TCDD treatments were much lower than at the lower TCDD soil treatments of 0.05 and $0.5\,\mu g\,g^{-1}$. More striking was that the highest TCDD concentrations in earthworms occurred on day 8 or 20 and not on days 55 or 85. This was true for all treatments. At the lowest TCDD soil concentrations ($0.05\,\mu g\,g^{-1}$) the amount of TCDD accumulated by the earthworms increased to a maximum at day 20. In no instances did live worms contain more than $2\,\mu g\,g^{-1}$ (1.23 ng) TCDD. These results seem to be consistent with an excretion mechanism operating to remove the contaminant.

No chlorinated metabolites of TCDD were identified in either the soil samples or the earthworms. Standards from the United States Food and Drug Administration were used for the mono-, bi- and trichlorinated products. No active metabolic breakdown of the 2,3,7,8-TCDD to any of these compounds was demonstrated. Presumably, if dechlorination takes place, the rate of dechlorination increases with loss of chlorine, thereby no accumulation.

The total amount of TCDD recovered from the soil decreased with time (Table 1). This difference in the amount of TCDD recovered at various periods was in excess of what could be explained by limitations in analytical technique. No similar decrease occurred in controls without earthworms. This is illustrated by the non-significant correlation determined for the control samples when the data was fitted to first-order regression. On the other hand, the treated soils containing earthworms indicated a statistically significant ($\geqslant 95\%$) reduction of TCDD concentration in soil with time. Under these conditions, the time for half of the extractable TCDD to disappear ranged from 85 to 400 days. There is also the possibility that the TCDD was degraded to compounds not recovered by the extraction procedure or of TCDD being "bound" in the soil after passing through the earthworms intestine where soil particles are cemented together.

Earthworm excrements often contain up to 60% more microorganisms than the surrounding soil. The presence of earthworms usually stimulates soil microbial activity, which could in turn be responsible for the lowered recovery of TCDD from the soil.

The earthworms tended to accumulate proportionately more TCDD at the lower levels of exposure (Fig. 2). The resultant contamination of the clean (non-TCDD treated) soil environment by the earthworms was proportionately higher for worms exposed to lower concentrations. Although it is not illustrated in Fig. 2, the equation of the graph shows a log-log relation between the original concentration recovered after one month from "clean soil". This result supports the idea that the total TCDD content of the worm is kept within limits by an excretory mechanism.

If earthworms are also long-term accumulators of TCDD (as is the case for chlorinated hydrocarbon insecticides) from the environment, they may serve as good indicators of environmental contamination in areas where 2,4,5-T has been used extensively. Bioaccumulation in the earthworm body and the resultant biomagnification through the food chain could, if allowed to occur over a prolonged period, provide sufficient grounds for serious concern. More ecological research is required before this possibility can be ruled out entirely.

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