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Assessment of Dimethoate Toxicity on Compost Worm (*Eisenia andrei*) Using Earthworm Avoidance Test

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ABSTRACT. *Terrestrial avoidance behavior, which is an alternative for earthworm acute toxicity test and reproduction test, is considered as a rapid screening tool to assess soil contamination and habitat functions. Short duration and low costs associated with avoidance test makes it ideal for tropical risk assessment of pesticides. In the present study the avoidance test was performed using dimethoate, a frequently used organophosphate insecticide in Sri Lanka as the test chemical and common compost worm, Eisenia andrei as the test species. OECD soil (Organization for Economic Co-Operation and Development) and natural soil (NS) were used as the substrates. Earthworms (n = 10, 4 replicates) were exposed to concentrations ranging from 1 mg / kg dry soil to 300 mg / kg dry soil in two-chamber system kept under tropical conditions (26 ± 2 ^{0}C). No significant difference (p > 0.05) was found in the dual control tests suggesting that soil type did not affect the homogeneous distribution of the worms. The EC_{50} values were very low in both OECD soil and NS (24.06 and 9.73 mg / kg dry soil respectively) indicating very high toxicity. Significant avoidance behavior was observed even in low concentrations in both soil types. The worm distribution in dimethoate treated sides and untreated sides were significantly different at $p < 0.05$ and even at $p < 0.001$ in higher concentrations. Reduced habitat functions were indicated at concentrations > 30 mg / kg dry soil. The difference in toxicity was observed in both soil types and highest toxicity was found in natural soil. The present findings of very high toxicity of dimethoate to earthworms warrant further studies in the field and could be useful in rapid risk assessment.*

INTRODUCTION

Soil ecosystem is a unique and dynamic system in nature, which supports the existence of wide range of flora and fauna. The composition of the system is rather complex and presence of clay and organic matter enhances the retention of soil pollutants such as pesticides. The increasing use of pesticides often disturbs the habitat functions of the soil, hence affecting the soil equilibrium. The habitat functions of soils are often investigated by acute toxicity test (OECD, 1984 and ISO, 1993) and reproduction test (ISO, 1998 and OECD, 2003) using earthworms as representatives of soil biocenois. These tests are very popular and reliable in the temperate regions where large sets of data exist on effects of soil pollutants and now being used even in tropics to asses the effects of pollutants. Constraints such as inability to asses the population effects by acute toxicity test and also the long duration (56 days) and the labor intensive nature of the reproduction test called for rapid assessment methods with short duration and high sensitivity. Therefore, International Standard Organization (ISO) drafted guidelines to determine the habitat functions of the soil through earthworm avoidance test (ISO / DIS 17512-1.2, 2007).

The avoidance test is a complimentary screening test in soil risk assessment (Slimak, 1997; Stephenson *et al.,* 1998; Hund - Rinke *et al.,* 2002) and it has been shown that the sensitivity is as good as reproduction test (Hund - Rinke *et al.,* 2003). It has a very simple test design and relatively short test period which makes it an ideal rapid assessment of pollutants in the soil (Yeardley *et al.,* 1996). The avoidance tests use the presence of chemoreceptors on the anterior segments and sensory tubercles on earthworm's body surface which can detect wide range of contaminants (Reinecke *et al.,* 2002). The habitat function of the soil is considered to be limited if an average of > 80% of the worms is found in control soil when compared to contaminated soil.

Dimethoate (O, O-dimethyl S-[2-(methylamino)-2-oxoethyl] dithiophosphate) is an organophosphate insecticide widely used to kill a broad range of insects. It is an anticholinesterase which disables cholinesterase, an enzyme essential for the functioning of the central nervous system. The formulation is commercially available as Agromet EC 40%, Baur's Dimethoate, Boxer 40, Perfect and some other formulations in Sri Lanka. It has been suggested that dimethoate has low persistence (4 - 16 days) in the soil (Howard, 1991) however a study by Wauchope *et al.* (1992) recorded 120 days of persistence. Although it has been widely used in tropical countries like Sri Lanka, no studies have been performed to investigate the persistence of this chemical in the tropical soils. Although it has been classified as Class II insecticide, the effects on soil organisms such as earthworms have not been studied in the region. Hence this study focuses on the effects of different dimethoate concentrations on the avoidance behavior of the earthworms (*Eisenia andrei*) in two different soil types under tropical laboratory conditions.

MATERIALS AND METHODS

The compost worm (*Eisenia andrei*) was selected as the test species. Adult worms weighing (420 ± 20 mg, wet weight) with well developed clitellum were obtained from synchronized cultures at the Department of Zoology, University of Ruhuna, Matara, Sri Lanka. The selected worms were transferred to the two test substrates, 24 hours before the experiment to get them acclimatized with the substrate. Artificial soil (OECD soil) and natural soil were used as the test soil substrates. OECD soil is composed of 70% fine sand, 20% kaolin, 10% sphagnum peat with small amount of $CaCO_3$ for the adjustment of the pH. This soil acts as the reference substrate for toxicity studies and has a pH of 6.5 - 7.2 and 80% of water holding capacity (WHC). The natural soil was collected from a site near Dickwella, Matara where uppermost soil layer (5 cm) was excavated and sieved (2 mm) in the laboratory to obtain homogeneous soil mixture. The pH of this soil was 6.9 - 7.2 with 45% of WHC.

Dimethoate (98% pure) was obtained from Sigma Aldrich (Amsterdam, The Netherlands). Each of the pesticide concentrations (1, 3, 10, 30, 100, 300, 900 *a.i* mg / kg dry soil) were prepared by dissolving in 50 ml of Acetone. These prepared solutions were added separately to 50 g sample of soil each and kept overnight ensuring the evaporation of the solvent from the soil samples. Control soils were similar to the test soils used, where all characters were similar other than the presence of pesticide.

Two section chambers which were prepared by using plastic trays (30 × 22 × 6 cm), were used as test containers. One side of each container was filled with control soil (500 g dry soil) and the other side with pesticide treated soil prepared by mixing 50 g of pesticide spiked soil with 450 g of dry soil. The two sides were separated by a thin plastic sheet to prevent mixing of the two soils. Final weight of the soil in both sides was 1 kg of soil (dry weight), which was adjusted to 50% of WHC. The random distribution of the worms was ensured by performing dual control tests, which consisted of pesticide free soil in both sides.

After the preparation of the containers, the separator (thin plastic sheet) was removed and 10 adult earthworms were introduced into the separation line. This procedure was followed in all tested concentrations with four replicates and dual control tests. Initially, the containers were kept in the light to ensure their movement into the soil. Then the trays were covered with perforated transparent lids to facilitate the gaseous exchange between the medium and the atmosphere and to facilitate the access of light. Finally, the experimental trays were kept in darkness to avoid the lateral effects of light. The initial moisture content was maintained throughout the study. Then the containers were incubated for 48 hours at 26 ± 2 ^{0}C and the presence of worms in both sides was determined by hand sorting. The worms remained in the separating line were counted by locating the direction of the head. The simplified experimental set up is given in Figure 1.

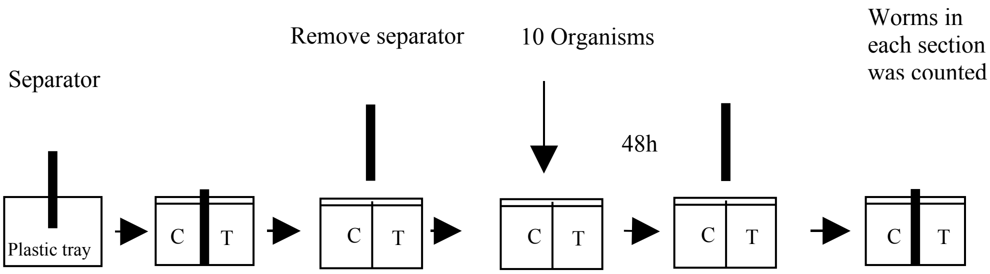

Figure 1. Experimental procedure followed in the avoidance test for earthworms (slightly modified from Hund - Rinke & Wiechering, (2001). C = untreated soil and T = treated soil)

For each replicate the avoidance response was calculated in accordance with the equation given below.

$$A = [\ (C - T) / N] \times 100$$

A = Avoidance response (%) C = Number of worms in untreated soil
T = Number of worms in treated soil N = Total number of worms exposed

Avoidance is indicated by positive response and attraction or non response by negative response. Validity of the avoidance test was checked by the random distribution of the worms in dual control tests where avoidance behavior was absent when the proportion of the earthworms in both sides was not significantly different (student t - test, $p > 0.05$). The tested soils were considered to have limited or reduced habitat function when 80% of the worms stay in control soil compared to the pesticide treated soil. Avoidance response of each of the exposed concentration together with four replicates was also used to determine the EC 50 by Trimmed Spearman- Karber (TSK) method (Hamilton *et al.*, 1977). Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) were calculated using ANOVA and Dunnett's test.

RESULTS AND DISCUSSION

The validity of the test design was determined by counting the number of dead or missing worms in the test containers. The design is considered to be invalid if the number of dead or missing worms is > 10% per treatment (ISO, 2007). No dead or missing worms were found in the test design when OECD soil was used as the substrate and therefore the design used here can be validated. When natural soil was used as the substrate, 100% mortality was recorded in the highest concentration of dimethoate (300 mg / kg dry soil) and this treatment was excluded from the statistical analysis since it influences the validity of the design. Although it has been excluded, it is obvious that mortality could be due to the presence of pesticide in untreated sides by leaching or evaporation. Such concentrations can be extremely toxic to the earthworms in the field. Effective test design also needs to ensure the homogeneous distribution of the earthworms in both sides of the test containers. The dual control tests, which used dimethoate free soil in both sides, did not show any significant preference or aggregation to one side ($p > 0.05$). This indicates homogeneous distribution of the worms in the test containers in spite of different physiochemical characters of the test soils and suitability of this technique in short term risk assessment.

Dimethoate is a volatile chemical that can enter through respiration and ingestion through pore water and soil particles. Previous study by Martikainen (1996) has shown that it can change the behavior of earthworms by acting as an acetylcholinesterase inhibitor. The EC_{50} values for the OECD soil and natural soil were 24.06 mg / kg dry soil (15.52 – 37.31, 95% CI) and 13.76 mg / kg dry soil (9.73 – 19.45, 95% CI) respectively. The No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) calculated using ANOVA and Dunnett's test also reported a significant difference ($p < 0.05$). The experimental set up which used natural soil as the substrate, reported 1 mg / kg dry soil as the NOEC and 3 mg / kg dry soil as LOEC. In OECD soil the NOEC and LOEC values were increased to 10 and 30 mg / kg dry soil respectively.

The EC_{50}, LOEC and NOEC values suggest that dimethoate can be considered as highly toxic to earthworm *Eisenia andrei*. The toxicity of dimethoate depends on the substrate used and the highest toxicity was reported in the natural soil. Loureiro and Soares (2005) used two natural soils found in Portugal to determine the effects of dimethoate on avoidance behavior of *Eisenia andrei*. The reported EC_{50} value in that (50.07, 37.98 – 66.53, 95% CI) is approximately half of the value presented here for the OECD soil. The former experiment was conducted at temperatures $20 \pm 2\ ^0C$ while this study was carried out at a higher temperature ($26 \pm 2\ ^0C$) identical to tropical conditions. Since all the other factors were same in both experiments, it can be suggested that the high temperatures in tropics can induce the avoidance behavior and it may lead to the higher toxicity reported. It also implies the importance of using toxicity data obtained in tropics in the risk assessment of pesticides rather than depends on the toxicity data obtained in the temperate region.

Different concentrations ranging from 1 - 300 mg / kg dry soil were used in the experiment that used OECD soil and natural soil as the substrates. The set up that used OECD soil as the substrate showed avoidance behavior in all tested concentrations except the lowest concentration. The detailed results are presented in Table 1 and Figure 2.

Table 1. Effect of dimethoate on the avoidance behavior of *Eisenia andrei* in OECD soil.

Dimethoate concentration (*ai*.mg/kg dry soil)	Distribution of Earthworms (%)		Net response (%)	Toxicity evaluation
	Control	Treated		
01	52.5	47.5	15[NS]	NRHF
03	65.0	35.0	30**	NRHF
10	62.5	37.5	30*	NRHF
30	67.5	32.5	35***	NRHF
100	92.5	7.5	85***	RHF
300	100.0	0.0	100[ND]	RHF

(* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, NS: not significant at $p > 0.05$, NRHF: no reduced habitat function is considered when > 20% of worms in treated soil and toxic = ≤ 20% of worms in treated soil and RHF: reduced habitat function, ND: not determined due to 100% avoidance or mortality).

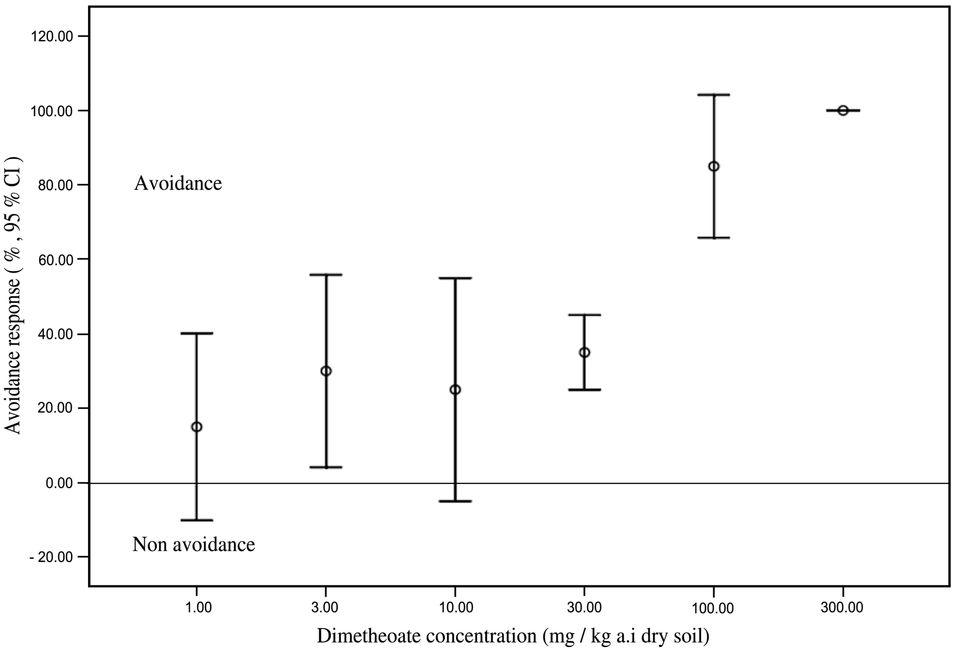

Figure 2. Avoidance response (% ± SE) of *Eisenia andrei* in OECD soil exposed to different concentrations of dimethoate.

There was no significant difference in the worm distribution in the lowest concentration (1 mg / kg dry soil, p > 0.05). In the second lowest concentration (3 mg / kg dry soil), 30% of the avoidance was recorded and worm distribution in the treated and untreated sides differ significantly (p < 0.01). Same response was reported in the concentration of 10 mg / kg dry soil as a result of a negative value (- 20) that was recorded in one replicate out of four replicates. This negative value may be due to the attraction of the worms to the chemical. In spite of that negative value, overall distribution of the worms in two sides was significantly different ($p < 0.05$). The worm distribution in the containers was significantly different in all the concentrations higher than 10 mg / kg dry soil. The tested worms indicated a very strong preference to the untreated side ($p < 0.001$). Two highest concentrations used in the test reported > 80% of avoidance including 100% avoidance in 300 mg / kg dry soil. As the highest concentration reported such value, the t – test to determine the distribution of worms in both sides was not performed. According to the guidelines (ISO, 2007) the concentrations that reported > 80% avoidance can be concluded as the pesticide concentrations which cause reduced habitat functions in the soil. But this criteria is based on the tests carried out in temperate regions (Jones and Hart, 1998; Hund-Rinke and Wiechering, 2001; Hund-Rinke *et al.,* 2003). The implementation of such criteria is rather doubtful in tropics where temperatures play a major role in determination of pesticide toxicity. Therefore, reduced habitat functions that reported significant differences in worm distribution other than the highest concentrations could not be ruled out.

The same concentrations (1- 300 mg / kg dry soil) were used in the natural soil. But 100% mortality in the highest concentration (300 mg / kg dry soil) tested resulted in an exclusion of that concentration from the analysis. The mortality recorded was mainly due to the nature of the high toxicity of dimethoate and assessing mortality is not the objectives of the avoidance test (ISO, 2007). The lowest concentration (1 mg / kg dry soil) reported a similar result with OECD soil where avoidance response was 10% and worm distribution in both sides was not significantly different ($p > 0.05$). The common observation was that toxicity of dimethoate increased in natural soil. High avoidance, which can cause reduced habitat function, was noticed in concentrations above 30 mg / kg dry soil (Figure 3) and 100% avoidance was recorded in the second highest concentration (100 mg / kg dry soil) tested. Also, the worm distribution of treated and untreated sides differs significantly in all concentrations except the lowest concentration ($p < 0.001$, Table 2). The criteria for the reduced habitat function is valid only for the concentrations > 30 mg / kg dry soil but in concentrations where significant worm aggregation was noticed in untreated sides of the experimental set up cannot be ruled out due to the increasing temperatures in the tropics.

Table 2. Effect of dimethoate on the avoidance behavior of *Eisenia andrei* in natural soil.

Dimethoate concentration (*a.i.* mg / Kg dry soil)	Distribution of Earthworms (%)		Net response (%)	Toxicity evaluation
	Control	Treated		
01	52.63	47.36	10^{NS}	NRHF
03	58.97	41.02	17.5^{***}	NRHF
10	65	35	35^{***}	NRHF
30	85	15	70^{***}	RHF
100	100	0	100^{ND}	RHF
300	ND	ND	ND	RHF

(* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, NS: not significant $p > 0.05$, NRHF: no reduced habitat function is considered when > 20% of worms in treated soil and toxic = ≤ 20% of worms in treated soil and RHF: reduced habitat function, ND: not determined due to 100% avoidance or mortality).

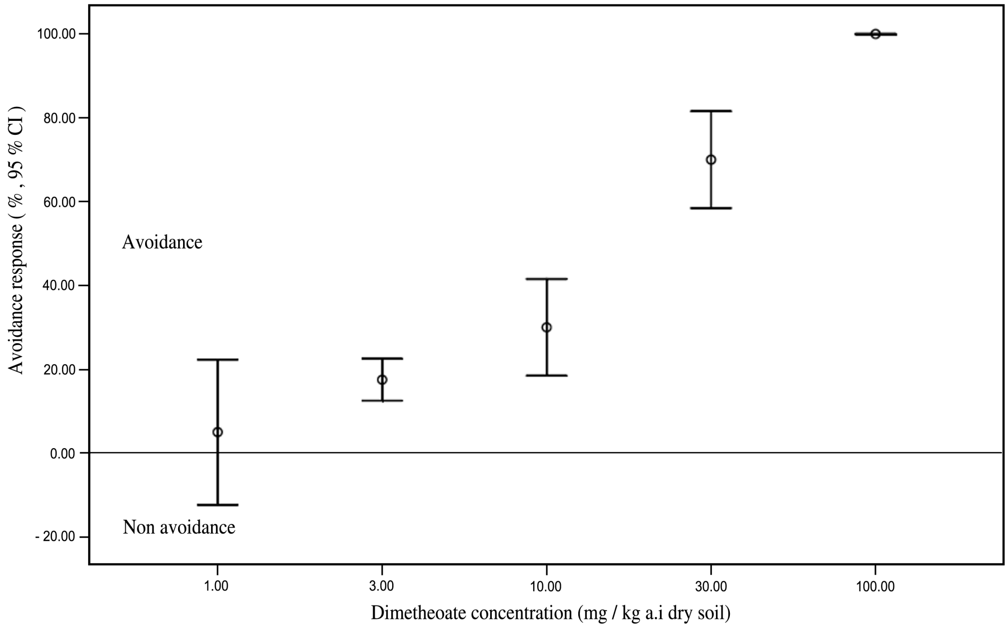

Figure 3. Avoidance response (%, ± SE) of *Eisenia andrei* in natural soil exposed to different concentrations of dimethoate.

The overall results suggest that the toxicity of dimethoate is higher in natural soil than in the OECD soil. This may be due to high percentage of organic matter present in the OECD soil. Organic matter can increase the absorption of pesticides into the soil resulting in limited bioavailability to the soil organisms. Amorim *et al.* (2002) reported that high organic matter content in OECD soil could lead to the underestimation of toxicity of chemicals when compared to natural soils. But it is also necessary to perform such toxicity tests in OECD soil, as it is the standard substrate for toxicity tests and due to the availability of large database of pesticide toxicity in temperate regions.

CONCLUSIONS

The results obtained in this study indicate that dimethoate is highly toxic to earthworms. Therefore, continuous application of such pesticides for higher crop production should be considered seriously. This study also suggests that the risks associated with pesticides to the soil organisms are very complex as the toxicity differs according to the substrate. Nevertheless, avoidance behavior test can be considered as a valuable screening tool to assess toxicity of soil contaminants in the tropics. As this study reported high toxicity of dimethoate to the earthworms, it is obvious that further studies such as reproduction tests should be performed and efforts should be taken to determine the toxicity of dimethoate under field conditions.

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