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Editors
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Oral Presentations

O-14

An Effective Approach to Maintain Homogeneity of Test Chemical in Soil for Earthworm (*Eisenia fetida)* Toxicity Test

Jigarkumar R. Rana*, Dipak K. Patel, Minal K. Kamle, Kathirvelu Baskar and Nadeem A. Khan

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Abstract: The method of exposure plays an important role in evaluating the toxicity of chemicals. The present study was performed as per the test guideline OECD 222. The guideline suggests analytical measurement of soil, while testing chemicals which are volatile, unstable, and readily degradable, but information on analytical verification of test chemical, which are soluble and insoluble in solvent or distilled water and, stable test chemicals, is not provided. Therefore, this study was carried out to identify an effective method for homogeneous mixing of test chemical in artificial soil without analysis using Carbendazim (CBD). CBD is a systemic fungicide, a metabolite of benomyl, and it is recommended by OECD 222 as reference substance. *Eisenia fetida* has been extensively used as a standard test organism for the risk assessment of pesticides due to its high sensitivity to chemical. Due to lesser solubility of CBD in distilled water, acetone was used as vehicle. CBD was dissolved in 40 mL of acetone and mixed with 100 g of industrial sand and mixed with 500 g of artificial soil thoroughly. The end points derived from this recording were growth, mortality, and reproductive output of *E. fetida*. The body weight of earthworms, in CBD exposure, was inversely proportional to the concentration of the CBD, when compared with the vehicle control group. The 28 day LC_{50} and the 56 day EC_{50} of reproduction were calculated as 2.81 and 0.68 mg/kg of the artificial soil, respectively. The no observed effect concentration (NOEC) for body weight changes, mortality, and reproductive output was observed at concentrations of 0.2, 1.4 and 0.4 mg/kg of the artificial soil, respectively. Based on the result it can be concluded that the individual replicate exposure method is more reliable, appropriate and cost effective in the absence of analytical verification.

Key Words: Body weight; EC_{50} and LC_{50}; Juveniles; NOEC

Introduction

In the fast growing world population, there is need to provide adequate food to human-beings and livestock. For the improvement of the agricultural productivity, many agrochemicals are used. Among the chemicals, carbendazim (CBD) is used to control plant diseases on arable crops, fruits, and vegetables. The toxicity of CBD has been widely studied. It is a common active ingredient in benzimidazole fungicides and is a metabolite of benomyl (Boudina *et al.*, 2003). The pesticides, carbendazim and dimethoate, treatment exhibited negative impact on the growth and reproduction of earthworms (Yasmin and D'Souza, 2007).Field studies have reported CBD to cause significant reduction in earthworm population and chronic effects on earthworm reproduction (Holmstrup, 2000). Due to the toxic effect of CBD, current ecotoxicological guidelines also recommend the use of CBD as a reference chemical.

Earthworms are one of the most suitable organism for assessing the ecological risks of pesticide residues (Reinecke and Reinecke, 2007; Landrum *et al.*, 2006). Earthworms were used for assessment of the toxicity of many chemicals *i.e.*, lindane, dimethoate, copper sulphate, carbendazim and benomyl (Loureiro *et al.*, 2005). The toxic effect of pesticides were studied by

2000 laboratories against invertebrate; among the pesticides, 96% were toxic to arthropod and oligochaetes. The toxicity differ from organism to organism (Framptom *et al.*, 2006). Among the different earthworm species, *Eisenia fetida* has been extensively used as standard test organism for the risk assessment of pesticides and is widely used to assess its sensitivity to chemical pollution (OECD, 1984; Edwards and Bohlen, 1992; ISO, 1998; OECD 2016). Baskar *et al.* (2016) studied the sensitivity of *E. fetida* towards chloracetamide. They reported that *E. fetida* is sensitive organism and its growth and survival were affected by chloracetamide.

The different way of exposure is given in test guideline OECD 222 (2016) based on the solubility of test chemicals. If the test chemical is soluble in water, use water as vehicle, if soluble in organic solvent, use solvent as vehicle and if insoluble in both water and solvent, use industrial quartz sand as vehicle. Guideline suggests to analyse artificial soil at the start, during and at the end of the experiment for volatile, unstable, and readily degradable chemicals. It lacks the information for the analysis of artificial soil, when test chemical is stable. Homogeneity of active ingredient can be confirmed by the analysis of artificial soil but in the absence of suitable method and analysis, there is a question for the homogeneity of test chemical. In this case, there must be suitable and accurate method, which gives homogenous mixing of test chemical. Due to the different mode of exposure, toxicity to the organism also differs. Most of the researchers are using the conventional method for the mixing of the test chemical in the artificial soil in bulk. Hence, the present research concentrated about individual replicate exposure of CBD and its effect on *E. fetida*.

Materials and Methods

Test Chemical

Carbendazim, (Sigma-Aldrich, CAS Number -10605-21-7, purity of 99.3%) was used as test chemical.

Test Organism

In-house bred 8 months old *E. fetida* with well-developed clitellum between the body weight of 300-600 mg were used for the present research.

Artificial Soil

Ingredient Used	Quantity (kg)	
	Acclimatisatoin, water holding capacity and control	**Treatment and vehicle control**
Sphagnum peat (Finely ground with no visible plant remains)	0.8	2.76
Kaoline clay (99.39% Kaolin content)	1.6	5.52
Industrial sand (predominantly 50% of the particles < 170 µm)	5.6	14.72*
Total	8	23

Note:

1. All the ingredients of artificial soil were dried before use.
2. Ratio of sphagnum peat, kaoline clay and industrial sand was 1:2:7.
3. Amount of artificial soil used for acclimatization was 2000 g.
4. Amount of artificial soil used for control group was 700 g. 100 g of artificial soil was used from three randomly selected replicates of control group and used for determination of moisture content (100 g from rest of five replicates were discarded).
5. Amount of artificial soil used for treatment group was 600 g (500 g artificial soil and 100 g industrial sand).

*6. For treatment and vehicle control, actual quantity of sand needed was 19.32 kg. The remaining sand was added at the time of treatment (100 g industrial sand was used for treatment for each replicate).

Acclimatization: One day before, the healthy worms were collected from culture andkept for acclimatisation.

Water Holding Capacity: Thewater holding capacity of artificial soil was determined as 40%.

Stock Solution: To prepare the stock solution of 0.225 mg/mL (stock A), an amount of 112.56 mg carbendazim was dissolved in acetone in 500 mL of volumetric flask and volume was made up to the mark. Volumes of 22.14, 12.27, 6.94, 3.74, 2.14, 1.07, 0.54, 0.27 mL were taken from the stock A and made up to 40 mL to achieve the concentrations of 0.1245 (stock B), 0.069 (stock C), 0.039 (stock D), 0.021 (stock E), 0.012 (stock F), 0.006 (stock G), 0.003 (stock H) and 0.0015 (stock I) mg/mL.

Test Concentrations and Exposure: The test concentrations selected for present research were 0.1, 0.2, 0.4, 0.8, 1.4, 2.6, 4.6, 8.3 and 15.0 mg carbendazim/kg artificial soil along with control and vehicle control groups. There were four replicates for treatment group and eight replicates for

control and vehicle control groups with 10 earthworms in each replicate. From each stock solution, 40 mL solution were mixed with pre-weighed 100 g of industrial quartz sand and allowed to evaporate acetone. After complete evaporation, treated 100 g industrial quartz sand was further mixed with 500 g of artificial soil to achieve the test concentrations of 15.0, 8.3, 4.6, 2.6, 1.4, 0.8, 0.4, 0.2 and 0.1 mg/kg artificial soil in individual replicates. For vehicle control group, same procedure were used but without test chemical. For control group, only artificial soil was used. To achieve final moisture content of 24% (40 to 60% of the maximum water holding capacity), 144 mL water was added in each replicate of treatment and vehicle control group. The quantity of 168 mL water was added in each replicate of control group. For treatment and vehicle control groups, 600 g artificial soil was used per replicate and for control group; 700 g artificial soil was used per replicate. The quantity of 100 g of artificial soil was used from three randomly selected replicates of control group and used for determination of moisture content (100 g from rest of five replicates were discarded).

On the day of treatment, the worms were separated, washed with water and kept on tissue paper to remove soil particle and excess water. Earthworms were weighed individually and a group of 10 earthworms were released in respective groups of glass beakers after the addition of thee treated soil (filled with 600 g artificial soil which is approximate 1/4 of the beaker) and covered with perforated and transparent polythene film. The mean body weight of earthworms was between 337.8 and 574.5 mg.

Feeding:

Cow dung was used as feed for earthworms. An amount of 5 g of freshly prepared dry cow dung moistened with 5 mL of water. It was provided first four weeks (weekly base) and on day 28 after removal of adults.

Observation

Symptoms and Mortality

On day 28, artificial soil containing earthworms and cocoons were transferred to a clean tray. Earthworms were counted, observed for changes in behaviour and for morphology. Any earthworm not found at this time of observation was recorded a dead, since it was assumed that such earthworms died and decomposed prior to the assessment.

Body Weight

Body weight of each earthworm was recorded individually on day 0 (prior to release) and 28 (at 4thweek).

Juveniles

On day 28, separated cocoons were counted and released into the test soil for four additional weeks under the same test conditions except that feeding only took place on one occasion at the start of this phase. On day 56, numbers of juveniles were counted by sieve method (OECD, 2016).

Statistical Analysis

The median lethal concentration (LC_{50}) and effect concentration (EC_{50}) of carbendazim was calculated by using the Probit analysis method (Finney, 1971). Data for body weight, body weight change and juveniles were subjected to Bartlett's test to meet the homogeneity of variance before conducting analysis of variance (ANOVA) and Dunnett's "t" test, where the data did not meet the homogeneity of variance Student's "t" test was performed to evaluate the data.

Results and Discussions

The method of exposure plays an important role to evaluate the toxicity of chemicals. The present study was performed in accordance with the test guideline OECD 222. The guideline suggests analytical measurement of soil for volatile, unstable, and readily degradable substances at the start of test, during and at the end of the test. No details have given for analytical verification of test item in soil for soluble, insoluble and stable substance. In the current research, we have made an attempt to identify an effective method for homogeneous mixing of test item in the absence of analytical method and analysis.The water holding capacity of the artificial soil was 40%. The initial moisture content of the artificial soil was 23.6% andat the end of the experiment it was 23.9%. The pH of soil was 6.25 and 6.21 at the start and the end of the test, respectively. The light intensity was maintained between 540-593 lux and the temperature of artificial soil was between 18.5-19.6°C.

Table 1 show smean body weight of earthworms on days 0 and 28. The minimum mean body weight of earthworms was 397.06 mg and maximum body weight of 423.99 mg was recorded at the concentrations of 0.2 and 2.6 mg/kg artificial soil on 0 day, respectively. No statistical difference in mean body weight of earthworms was observed in any of the tested concentrations when compared with the vehicle control group on day 0. On day 28 and between days 0-28, statistically significant decrease in mean body weight and in percent mean body weight change were observed at the test concentrations of 0.4, 0.8, 1.4 and 2.6 mg carbendazim/kg artificial soil, when compared with the

vehicle control group. No statistical analysis was performeddue to 100% mortality at the concentrations of 4.6, 8.3 and 15.0 mg/kg artificial concentrations.

Table 1 shows percent body weight changes, at maximum body weight of 6.23% was increased at the concentration of 0.4 mg/kg artificial soil followed by control group (5.22%). There was no statistical difference was found, upto 0.2 mg/kg artificial concentration when compared with the vehicle control group. Maximum reduction in body weight was found at 2.6 mg/kg of artificial soil. Based on the body weight changes, no observed effect concentration (NOEC) and lowest effect level concentration (LOEC) were 0.2 and0.4 mg/kg of artificial soil, respectively. In the present study, carbendazim reduced the growth of *E. fetida* during the exposure period. Similarly, Yasmin and D'Souza (2007) stated that carbendazim significant reduced the growth of *E. fetida*.

Table 2 shows toxic effect of carbendazim on earthworm. On day 28, 100% mortality was found at the concentrations of 8.3 and 15.0 mg/kg of artificial soil; also at the concentration of 4.6 mg/kg artificial soil exhibited more than 95% mortality of adult earthworm. There was no mortality found upto the concentration of 0.8 mg/kg artificial soil.Based on the adult mortality, the lethal concentration (LC_{50}) was 2.81 mg/kg artificial soil with regression equation of $y = -20.99 + 7.53\ x$ (Table 2). For mortality, 1.4 mg/kg artificial soil concentration of carbendazim considered as NOEC and 2.6 mg/kg of artificial soil concentration was the LOEC (Table 2).Similarly, Huan *et al.* (2016) reported that carbendazim was toxic to the earthworms with LC_{50} value of 8.6 mg/kg dry soil for 14 days mortality. Carbendazim exhibited acute toxicity to earthworm and it also altered the enzyme activity including histopathological changes of the treated *E. fetida*.

Sign of toxicity, 12.82 and 3.33% sluggish (slow movement) was recorded at the concentrations of 1.4 and 2.6 mg/kg artificial soil. The toxic sign of 15.15 and 100% shrink (reduction in length) was observed at the concentrations 2.6 and 4.6 mg/kg artificial soil (Table 2).The present study was coincide with earlier findings of Bharathi and Subbarao (1984), who stated that the pesticides affect the behaviour of the earthworms.

Table 3 exhibits reproductive output of earthworm after 28 days exposure of carbendazim at differentconcentrations. Control, vehicle control, 0.1 and 0.2 mg/kg artificial soil concentration of carbendazim produced more than 80 number of juveniles, while 0.4 mg/kg artificial soil produced 65 number of juveniles. It was statistically similar to control and vehicle control. Juvenile reduction was maximum (91.83%) at 2.6 mg/kg artificial soil followed by 1.4 mg/kg of artificial soil (90.95%). More than 70% reduction in juvenile was recorded at the concentration of 0.8 mg/kg artificial soil.Our finding is in agreement with finding of van Gestel (1992), who stated that cocoon

production of *E. andrei* was reduced drastically when concentration of Benomyl was increased.Lower number of juveniles of *E. fetida* were produced with the treatment of Bisphenol A, when compared to control (Verdu *et al.,* 2018).

The effective concentration 0.68 mg/kg artificial soil with regression equation of y = -4.04 + 3.19x for 50% reproduction inhibition of earthworm. Table 3 clearly indicates that carbendazim was highly toxic to earthworm. Based on the statistical analysis, the NOEC and the LOEC was 0.4 and 0.8 mg/kg artificial soil, respectively. Based on the percent reduction over control NOEC was 0.2 mg/kg artificial soil and the LOEC was 0.4 mg/kg artificial soil.

Different methods were used for mixing of test chemical during the study. In the present study, required quantity of carbendazim was dissolved in 40 mL of acetone, mixed with 100 g of industrial quartz sand and finally mixed with 500 g of artificial soil. The ratio of the industrial sand was maintained 70%. Our LC_{50}value was 2.81 mg/kg artificial soil for mortality and EC_{50} value was 0.68 mg/kg artificial soil for reproductive output. Our individual replicate exposure method is more sensitive than the method of Shanmugasundaram *et al.* (2014). They exposed carbendazim in 5 g of quartz sand for each concentration and found LC_{50} value of 6.33 mg/kg artificial soil for 28 days with NOEC and LOEC value of 3.70 and 6.67 mg a.i./kg dry soil, respectively.Also they found the EC_{50} value of 2.45 mg a.i./kg artificial soil for reproduction. Ellis *et al.* (2007) whose results also supported our results. They obtained LC_{50} value between 6.04 -16.00 mg/kg soil with exposure of carbendazim on earthworm. Our in-house, validation study (unpublished data - Jigar, 2014) also supported the present method is toxic than conventional method. The carbendazim exhibited LC_{50} (mortality) and EC_{50} (reproduction) valuesof 8.26 and 1.32 mg/kg dry weight, respectively.

Conclusion

The individual replicate exposure of test chemical shows higher toxicity to earthworm for growth, mortality and reproductive output. It has also shown the adverse behavioural symptoms to *E. fetida*. Our findings of LC_{50} and EC_{50} were 2.25 and 3.60 times higher toxic than the result of Shanmugasundaram *et al.*, (2014), respectively. The narrow range of end points indicates that the test chemical has mixed homogenously and it has come out as effective and sensitive method. This method will be helpful even when there is absence of analytical method and absence of analysis of treated artificial soil. The present research has proved that the cost effective and time saving method (to minimize analysis).

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Table1: Effect of Carbendazim on Body Weight (mg) of *Eisenia fetida*

Treatment (mg/kg artificial soil)	Body Weight (mg) on Day		Body Weight Change (%) between days
	0	28	0-28
Control#	419.79 ± 19.25	441.55 ± 17.09	5.22 ± 1.91
Vehicle control#	408.20 ± 11.67	423.55 ± 11.17	3.77 ± 1.14
0.1	401.62 ± 13.24	417.93 ± 6.37	4.12 ± 2.38
0.2	397.06 ± 7.73	421.79 ± 7.52	6.23 ± 0.70
0.4	408.92 ± 13.08	386.81 ± 19.28↓↓	-5.45 ± 1.73↓↓
0.8	408.74 ± 8.45	361.82 ± 3.08↓↓	-11.46 ± 1.37↓↓
1.4	411.00 ± 4.13	328.06 ± 3.53↓↓	-20.17 ± 1.60↓↓
2.6	423.99 ± 12.67	320.44 ± 12.88↓↓	-24.38 ± 3.60↓↓
4.6	408.24 ± 9.48	-	-
8.3	411.49 ± 9.06	-	-
15.0	404.80 ± 14.59	-	-

Mean ± SD of 8 replications (N = 80); Mean ± SD of 4 replicates (N = 40); ↓↓ = significantly lower than the control at 1% level ($p \leq 0.01$)

Table 2: Toxic Effect of Carbendazim on Earthworm

Treatment (mg/kg artificial soil)	Mortality (%)	LC_{50} (mg/kg artificial soil)	95 % Fiducial Limits		Regression Equation (y = a + bx)	Sign of Toxicity	
Control #	0.0 ± 0.0	2.81	*2.05*	3.86	y = -20.99 + 7.53x	-	-
Vehicle control #	0.0 ± 0.0					-	-
0.1	0.0 ± 0.0					-	-
0.2	0.0 ± 0.0					-	-
0.4	0.0 ± 0.0					-	-
0.8	0.0 ± 0.0					-	-
1.4	2.5 ± 5.0					-	-
2.6	17.5 ± 15.0					-	12.82% Sluggish
4.6	97.5 ± 5.0					15.15% Shrink	3.33% Sluggish
8.3	100.0 ± 0.00					100% Shrink	*
15.0	100.0 ± 0.00					*	*
						*	*

Mean ± SD of 8 replications (N = 80); Mean ± SD of 4 replicates (N = 40); - = 100% Normal; * = 100% Mortality

Table 3: Effect of Carbendazim on Reproductive Output on Earthworms, *Eisenia fetida*

Treatment (mg/kg artificial soil)	Juveniles (Number)	% Reduction over Control	EC_{50} (mg/kg artificial soil)	95 % Fiducial Limits		Regression Equation (y = a + bx)
				Lower Limit	Upper Limit	
Control #	85.75 ± 8.81	-	0.68	0.52	0.88	y = -4.04 + 3.19x
Vehicle control #	85.63 ± 13.97	-				
0.1	87.00 ± 4.69	-1.60				
0.2	83.50 ± 2.52	2.49				
0.4	65.75 ± 13.40	23.22				
0.8	22.25 ± 9.74 ↓↓	74.02				
1.4	7.75 ± 3.40 ↓↓	90.95				
2.6	7.00 ± 3.00 ↓↓	91.83				
4.6	-	-				
8.3	-	-				
15.0	-	-				

Mean ± SD of 8 replications (N = 80); Mean ± SD of 4 replicates (N = 40)
↓↓ = Significantly lower than the vehicle control at 1% level ($p \leq 0.01$).

Note: Coefficient variation of reproductive output for control and vehicle control was 10.27 and 16.31%, respectively.