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Acute toxicity evaluation of Tricyclozole 75% WP on earthworms *Eisenia fetida*

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Earthworms (*Eisenia foetida*) were exposed to Tricyclozole 75% WP pesticides in an acute earthworm toxicity test as per OECD guideline 207. Four replicates of ten clitellated adult earthworms each were exposed to the concentrations of 31.25, 62.5, 125, 250, 500, and 1000 mg/kg dry soil. The control artificial soil with acetone and quartz sand had four replicates, with ten earthworms in each replicate. Earthworms were assessed for behavioural effects at 0, 7, and 14 days of exposure, mortality after 7 and 14 days of exposure, and the earthworm biomass was assessed on day 0 and on day 14. The results of the study revealed acute toxicity of Tricyclozole 75% WP to the earthworm *Eisenia fetida* in artificial soil

Keywords: Tricyclazole 75% WP, *Eisenia fetida*, mortality, biomass

INTRODUCTION

Earthworms are important organisms that play a vital role in the development of the nutrient content in the soil. For the earthworm, suitable conditions for living in soil is a pH close to 7.0 ± 0.5. Earthworms act as carriers of nutrients between the soil and the crops. Most of Indian economy rests on agriculture, nearly 70% of the total population depends on it. To eradicate insects and increase the productivity of the crops, farmers spray pesticides. When pesticides are used appropriately, they are eco-friendly. Nowadays, however, the use of pesticides is on the rise and pesticides remain as traces in the soil. This affects the natural living organisms in soils and also has an adverse affect on the human health. Pesticide pollution is the main environmental issue for rice-growing areas, because pesticides in rice fields enter the open environment easily and may affect the quality of such resources as groundwater and surface water (Mohammadian et al., 2015).

Tricyclazole fungicide products are marketed both as granules and wettable powders. Most formulations contain 750 grams of active ingredient per kilogram of product and the product is referred to as Tricyclazole 75% WP. Tricyclazole is slightly soluble in water and moderately toxic to aquatic organisms on an acute basis. Tricyclazole is stable at an ambient temperature and it will degrade slowly under environmental conditions. Tricyclazole ((5-methyl-1,2,4-triazolo[3,4-b]benzothiazole) is a common fungicide used to proctect rice crops (Phong Kh T et al., 2009; Habibzadeh F et al., 2012; Young Deuk Lee et al., 1998). This substance has a relatively high retention in soil and water, and contact with it can lead to health problems (Fattahi et al., 2015; Padovani et al., 2006; Qiu et al., 2013). Studies have shown that this toxin and its metabolites cause more damage in aquatic organisms than to mammals (Jeong et al., 2012; WHO, 2004).

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Eisenia fetida is the standard test organism used in terrestrial ecotoxicology, because it can be easily bred on a variety of organic wastes with short generation times (Shahla Yasmin et al., 2010; ISO, 1993; ISO, 1998; OECD, 1984). Its susceptibility to chemicals resembles that of true soil organisms. Sensitivity tests of multiple earthworm species have revealed that *Eisenia fetida* is comparatively less sensitive (OECD, 2004; Ma et al., 1993; Kula, 1995). Although earthworm species vary in their tolerance, reports have shown a decline in earthworm populations in response to large amounts of organic chemical deposition (Fitzgerald et al., 1996; Bayer et al., 1982).

The current research was conducted in order to evaluate the acute toxicity of Tricyclozole 75% WP to earthworms after 7 and 14 days of exposure and to estimate NOEC (no observed effect concentration) and LC_{50} of the test item to *Eisenia fetida*.

MATERIALS AND METHODS

Materials

Tricyclozole 75% WP with the expiry date of two years from the date of manufacturing was purchased from market having.

2-chloroacetamide was purchased from Sigma Aldrich. The LC_{50} of the 2-chloroacetamide is determined twice in a year as a means of assuring that the laboratory test conditions are adequate and have not changed significantly.

Biological system

Earthworms (*Eisenia fetida*) were procured from Vanishri Agrochem Pvt. Ltd, Pune, India. Earthworms (*Eisenia fetida*) of 3–4 months of age with biomass of 300–600 mg/worm remained in the breeding medium (50:50 ratio of cow manure and coco peat) at 20 ± 2 °C, light regime of 16 h light/8 h dark, and light intensity of 400 to 800 lux. Prior to use, the earthworms were observed for obvious physical or distinct behavioural changes, if any. The earthworms were acclimatized for one day in the artificial soil under environmental test conditions.

Preparation of the artificial soil

The artificial soil was prepared using dry constituents, viz., 10% Sphagnum-peat (air-dried and finely ground with no visible plant remains), 20% kaolin clay (kaolinite content >30%), approximately 69% fine quartz sand (>50% of the particles ranged between 50 to 200 microns). The above ingredients were mixed thoroughly using homogenizer for about 20 minutes. The remaining 1% of sand was added during the test substance application. Before initiating the study, the soil was prepared and stored until use in an airtight plastic container.

Maximum Water Holding Capacity (MWHC): Water content of the study was based on Maximum Water Holding Capacity of the artificial soil. A double-ended open glass tube (10.0 × 5.0 cm) with a cotton cloth covering one of its ends was weighed (T) and artificial soil (approximately 100 g) was filled compactly through the open end to a depth of 5–7 cm of the tube. The tube was then gradually immersed in water contained in a wide-mouth bowl, until the water level was just above the top of the soil. This setup was kept undisturbed for 3 h. The soil sample was then allowed to drain the excess water for 2 h by placing the tube in a bed of fine wet sand contained within a covered vessel (to prevent drying). After this period has lapsed, the sample was weighed (S) and dried at 105 °C for 3 h using a hot air oven.

The sample was weighed after drying (D) and the maximum water holding capacity of the artificial soil was calculated as follows:

$$\text{MWHC \% (dry mass)} = \frac{S - T - D}{D} \times 100$$

where S = water saturated soil + mass of the tube + mass of the cotton cloth (g)

T = Tare (mass of tube + cotton cloth) in g

D = Dry mass of the soil (g)

During test item application, the soil was moistened with de-ionised water corresponding to approximately 52.90%, maximum water holding capacity (66.16%) of the artificial soil. The moisture content of the artificial soil was determined at the start (day 0, after test item

application) and at the end (day 14) of the experiment by the following procedure: a known quantity of soil was uniformly spread in a Petri dish and the initial weight of the soil was noted. The soil was dried by placing the Petri dish in **a** hot air oven at 105 °C for 3 hours. Final weight of the soil after drying was recorded and the moisture content was calculated using the following formula (oven drying method):

$$\text{Moisture content (dry weight basic)} = \frac{I - F}{F} \times 100$$

I = Initial weight of the soil before placing in the hot air oven

F = Final weight of the soil after drying

The moisture content was in the range of 33.73% to 34.95% at the test start (day 0), and 35.50% to 35.83% at the end of the test (day 14). The pH of the artificial soil was determined at the start (after test item application) and end of the experiment. It was in the range of 7.03 to 7.94 at the test start (day 0), and 6.48 to 7.63 at the end of the test (day 14). The complete data is presented in Table 1.

Test conditions

The controlled environment room maintained at 19.8–22.0 °C, at continued light intensity of 444–635 lux on the test container. Test conditions (temperature and light intensity) and test conditions for the soil (pH and water content) were recorded with suitable calibrated instruments at start and at the end of the test.

Test conduct

Healthy earthworms with well-developed clitellum were selected, washed with tap water, blotted carefully with filter paper, weighed as a group of ten worms, and then released onto the surface of the treated artificial soil. The soil water content of each container was checked periodically by weighing, and water was added. Therefore the loss of soil moisture was kept within 10% of the initial soil moisture content for a period of 14 days.

Table 1. **The pH and moisture content during the course of the test**

Treatments (mg/kg dry soil)	Range finding experiment[1]				Treatments (mg/kg dry soil)	Main experiment[2]			
	pH		% moisture content			pH		% moisture content	
	Test start (day 0)	Test end (day 14)	Test start (day 0)	Test end (day 14)		Test start (day 0)	Test end (day 14)	Test start (day 0)	Test end (day 14)
Control (acetone + quartz sand)	6.49	7.35	36.09	35.03	Control (acetone + quartz sand)	7.03	6.48	33.73	35.64
10	7.16	7.96	36.70	35.04	31.25	7.54	7.24	34.95	35.56
33	7.15	8.04	38.10	35.58	62.5	7.66	7.26	34.71	35.80
100	6.99	8.07	35.72	35.03	125	7.49	7.25	34.36	35.50
333	7.06	8.01	34.91	34.63	250	7.94	7.63	34.74	35.83
667	7.00	8.19	35.54	34.00	500	7.73	7.51	34.73	35.67
1000	6.94	8.14	35.27	35.26	1000	7.62	7.62	34.95	35.55
Min.	6.49	7.35	34.91	34.00	Min.	7.03	6.48	33.73	35.50
Max.	7.16	8.19	38.10	35.58	Max.	7.94	7.63	34.95	35.83

[1] Non replicated

[2] Representative samples from 4 replicates

Experimental procedure

Before the start of the experiment, approximately 3–4-month-old healthy (active movement and shiny skin), clitellated earthworms were collected from the breeding box and acclimatized for one day in the artificial soil. The earthworms may typically be exposed to agricultural pesticides and their soil degradates present within the soil profile.

For each test, 550 g artificial soil (dry weight) was filled into each glass container. The artificial soil was moistened with approximately half of the final water content one day before the application. On the day of the experiment, the test item was dissolved in acetone (maximum of 10 ml/kg dry soil) and applied to quartz sand (10 g of quartz sand/kg dry soil) in a Petri dish and left uncovered to enable complete evaporation of solvent from sand. Thus prepared, the test item (Tricyclozole 75% WP) and quartz sand mixture was blended thoroughly with the artificial soil, along with the remaining water, using a laboratory mixer. Control received acetone alone, without the test item. The soil for each concentration was treated in two batches and each batch was further divided into two replicates, thus there were four replicates altogether at each concentration level. The wet weight of the soil was documented at the beginning of the test. The test medium was ventilated before use.

Ten earthworms, which have been acclimatized for a day in the artificial soil, were washed, blotted carefully, and released randomly into the test medium. The containers were covered with perforated plastic lids to prevent the test medium from drying. Non-replicated range finding study was conducted with 10 worms/treatment with 10, 33, 100, 333, 667, and 1000 mg/kg dry soil to arrive at accurate concentrations for the definitive test (Table 2).

Based on the mortality and biomass change from the range finding test, the following concentration viz., 31.25, 62.5, 125, 250, 500, and 1000 mg/kg dry soil were selected in order to demonstrate that the LC_{50} of the test item is more than 1000 mg/kg dry soil. The concentrations are nominal amounts. Deviation to the target concentration was less than 10% (Table 3).

Application order: control (acetone + quartz sand) and the test item (from low to high concentrations).

Parameters observed

The artificial soil was emptied from the beaker and searched for earthworms on days 7 and 14 after test item application. The number of live and dead earthworms in each replicate was assessed on each of these days. Earthworms were considered dead if they failed to respond to gentle stimulation. Due to rapid decomposition under test conditions, missing earthworms were also considered dead.

The number of worms that did not burrow into the soil, if any, was recorded as behavioural abnormalities at the test start (day 0). Also, other behavioural abnormalities and toxic symptoms, if any, were observed during each mortality assessment. After the mortality check on day 7, the live earthworms and

Table 2. **Preparation of test item concentrations for the range finding test**

Amount of test item weighed [mg/0.55 kg dry soil]	Resulting concentration [mg/kg dry soil]	Quantity of deionised water added/0.55 kg Dry soil (g)
5.7	10.36	193
18.5	33.64	193
55.3	100.55	193
183.4	333.45	193
367.0	667.27	193
550.1	1000.18	193

Table 3. **Preparation of test item concentrations for the main test**

Amount of test item weighed [mg/1.1 kg dry soil]		Resulting concentration [mg/kg dry soil]	Quantity of deionised water added/1.1 kg dry soil (g)
Batch I	Batch II		
34.9	34.4	31.5	386
69.0	68.8	62.6	386
137.7	138.0	125.3	386
275.1	276.1	250.5	386
551.1	551.0	501.0	386
1100.4	1103.5	1001.8	386

the artificial soil were returned to the respective test containers. Healthy earthworms with well-developed clitellum were selected, washed with tap water, blotted carefully with filter paper, weighed as a group of 10 worms and then released on the surface of the treated artificial soil.

At the start (day 0) and the end (day 14) of the test, the soil water content and pH in each test concentration and control (representative samples from each treatment group) were determined.

Observations

Since the observed mortality was less than 50% (2.5% in 1000 mg/kg dry soil, the highest concentration tested), LC_{50} analysis was not performed. The LC_{50} was estimated to be more than the highest concentration tested. Fisher's exact test was performed to estimate the NOEC (no observed effect concentration) related to mortality, using TOXSTAT version 3.5 software.

The biomass changes of the treated and untreated series were analysed for homogeneity and normality using Bartlett's and Kolmogorov tests, respectively. Since the data was homogenous and normally distributed, Dunnett's test was performed (multiple comparison, one sided) to estimate the NOEC using TOXSTAT version 3.5 software (Table 4).

Table 4. **Results of the test item with the reference item, 2-Chloroacetamide**

Treatment Group	Control (deionised water)	2-Chloroacetamide [mg/kg dry soil wt.]				
		6	12	24	48	96
Mortality after 14 days (%)[1]	0	$0^{n.s}$	$0^{n.s}$	$0^{n.s}$	100*	100*
Biomass change after 14 days (%)[2]	–6.38	$-0.61^{n.s}$	$-3.26^{n.s}$	$-6.02^{n.s}$	–100*	–100*
14 day LC_{50}	36 mg/kg dry soil					
Fiducial limits (95% confidence)	Could not be determined					
LOEC related to mortality and biomass	48 mg/kg dry soil					
NOEC related to mortality and biomass	24 mg/kg dry soil					

n.s. = no statistically significant differences compared to the control

* = statistically significant differences compared to the control

[1] Fisher's exact test, $\alpha = 0.05$; [2] Dunnett's test, $\alpha = 0.05$

RESULTS AND DISCUSSIONS

Range finding test

Mortality

Fourteen days after exposure to the test item, no mortality was observed in any of the treatments on day 7. However, 10% mortality was observed in test item treatment, 667 mg/kg dry soil on day 14. The details are presented in Table 5

Biomass

The biomass changes of the earthworms in the test item treatment ranged between –42.07% (667 mg/kg dry soil) and –7.70% (10 mg/kg dry soil) from the initial weight. The mean biomass change in the control was –8.39% from the initial weight. The details are presented in Table 5.

Table 5. **Results of the range finding study**

No.	Treatments (mg/kg dry soil)	Behavioural abnormalities [%]			Biomass (mg/worm)*			Mortality (%)	
		day 0	day 7	day 14	Test Start (day 0)	Test End (day 14)	Bio mass change (%)	day 7	day 14
1.	Control (acetone + quartz sand)	0	0	0	393.5	360.5	–8.39	0	0
2.	10	0	0	0	367.6	339.3	–7.70	0	0
3.	33	0	0	0	384.7	341.3	–11.28	0	0
4.	100	0	0	0	359.8	296.4	–17.62	0	0
5.	333	0	0	0	331.6	233.4	–29.61	0	0
6.	667	10	10	0	362.0	209.7	–42.07	0	10
7.	1000	0	0	0	381.8	260.3	–31.82	0	0

Non-replicated; * Mean weight of live earthworm

Behavioural abnormalities

Earthworms that were lying on the top layer of the soil were observed as a behavioural abnormality. 10% of the earthworms were noticed on the top layer of the soil on day 0 and day 7 in the concentration 667 mg/kg dry soil. However, no behavioural abnormality was observed in any other treatments on day 14. The details are presented in Table 5.

Main test

Mortality

After 14 days of exposure to the test item, 2.5% mortality was observed in the highest test item treatment, 1000 mg/kg dry soil. The statistical test (Fisher's exact test) revealed that there was no significant difference in mortality in any of the concentrations tested when compared to control. The details are presented in Table 6.

Behavioural abnormalities

No behavioural abnormality was observed in any treatments on day 0 and day 7. However, 2.5% behavioural abnormality (earthworm lying on the top of the soil) was observed in the concentration 62.5 mg/kg dry soil on day 14. The details are presented in Table 5.

Biomass

The biomass changes of the earthworms in the test item treatment ranged between –26.96% (1000 mg/kg dry soil) and –4.37% (31.25 mg/kg dry soil) from the initial weight. The mean biomass change in the control was –0.47% from the initial weight. The statisti-

Table 6. **Behavioural abnormalities and mortality of the worms from the test item (main experiment)**

No.	Treatments (mg/kg dry soil)	Behavioural abnormalities (%)			Mortality (%)	
		Day 0	Day 7	Day 14	Day 7	Day 14
1.	Control (acetone + quartz sand)	0	0	0	0	0
2.	31.25	0	0	0	0	0
3.	62.5	0	0	2.5	0	0
4.	125	0	0	0	0	0
5.	250	0	0	0	0	0
6.	500	0	0	0	0	0
7.	1000	0	0	0	0	2.5

Mean of 4 replications

cal test (Dunnett's test $\alpha = 0.05$) revealed that there was no significant difference on the biomass change in the concentration 31.25 mg/kg dry soil when compared to control. However, a significant biomass change was recorded from the concentration of 62.5 mg/kg dry soil onwards. The details pertaining to the change in biomass of earthworms after 14 days of treatment with the test item are given in Table 7, and the percentage change in biomass of earthworms after 14 days is presented in Table 8.

Table 7. **Earthworm biomass after exposure to the test item (main experiment)**

No.	Treatments (mg/kg dry soil)	Mean biomass (mg/worm)									
		Test start (day 0)				Mean	Test end (day 14)				Mean
		RI	RII	RIII	RIV		RI	RII	RIII	RIV	
1.	Control (acetone + quartz sand)	406.8	387.4	378.1	396.2	392.1 (12.3)	406.1	404.6	367.4	383.0	390.3 (18.6)
2.	31.25	355.4	406.2	370.8	395.4	382.0 (23.1)	352.8	382.5	358.8	365.0	364.8 (12.8)
3.	62.5	384.7	372.9	378.1	381.0	379.2 (5.0)	342.1	355.2	364.6	347.9	352.5 (9.7)
4.	125	366.0	382.1	378.4	380.0	376.6 (7.2)	318.1	350.8	328.0	326.6	330.9 (14.0)
5.	250	363.6	358.7	400.1	395.4	379.5 (21.3)	295.3	295.7	328.4	326.4	311.5 (18.4)
6.	500	376.0	365.9	382.4	387.7	378.0 (9.4)	303.1	285.9	301.4	305.4	299.0 (8.9)
7.	1000	389.2	380.2	378.8	379.7	382.0 (4.9)	284.2	277.7	293.5	260.6	279.0 (13.9)

R – Replication; figures in parentheses are standard deviation (SD)

Table 8. **Changes in earthworm biomass – main experiment**

No.	Treatments (mg/kg dry soil)	Biomass changes (%)				Mean (%)
		RI	RII	RIII	RIV	
1.	Control (Acetone + Quartz sand)	–0.17	+4.44	–2.83	–3.33	–0.47 (3.6)
2.	31.25	–0.73	–5.83	–3.24	–7.69	–4.37^{ns}, (3.0)
3	62.5	–11.07	–4.75	–3.57	–8.69	–7.02* (3.5)
4.	125	–13.09	–8.19	–13.32	–14.05	–12.16* (2.7)
5.	250	–18.78	–17.56	–17.92	–17.45	–17.93* (0.6)
6.	500	–19.39	–21.86	–21.18	–21.23	–20.92* (1.1)
7.	1000	–26.98	–26.96	–22.52	–31.37	–26.96* (3.6)

R – Replication; Figures in the parentheses are Standard Deviation

n.s. – not significant compared to the control

* – significant compared to control (Dunnett's test, $\alpha = 0.05$)

CONCLUSIONS

It is concluded from the results of this study that the 14-day LC_{50} of Tricyclozole 75% WP was found to be >1000 mg/kg dry soil. No observed effect concentration (NOEC) with respect to the biomass and mortality was found at 31.25 and 1000 mg/kg dry soil, respectively.

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75 % TRICIKLAZOLO MILTELIŲ (WP) ŪMAUS TOKSIŠKUMO POVEIKIS *EISENIA FETIDA* SLIEKAMS

Santrauka

Sliekai yra svarbūs organizmai, išnešiojantys maistines medžiagas dirvožemyje. Palankiausios sąlygos sliekams gyventi yra tuomet, kai dirvožemio pH = 7,0 ± 0,5. Triciklazolo fungicidas yra tiek granulių, tiek miltelių (WP) pavidalo. Dažniausiai viename kilograme produkto yra 750 gramų veikliosios medžiagos (75 % triciklazolo milteliai). Triciklazolas lengvai tirpsta vandenyje ir yra vidutiniškai toksiškas vandens organizmams; jis stabilus kambario temperatūroje ir lėtai yra aplinkos sąlygomis. Šio tyrimo metu sliekai (*Eisenia fetida*) buvo veikiami 75 % triciklazolo milteliais. Su dešimčia subrendusių sliekų bandymas kartotas keturis kartus – kiekvienas iš jų buvo veikiamas 31,25, 62,5, 125, 250, 500 ir 1 000 mg/kg fungicidu sausame dirvožemyje; kontrolinis dirbtinis dirvožemis su acetonu ir kvarciniu smėliu taip pat buvo išbandytas keturis kartus su dešimčia sliekų kiekvieno kartojimo metu. Tyrime buvo vertinama: 1) sliekų elgsena po 0, 7 ir 14 poveikio dienų; 2) mirtingumas po 7 ir 14 poveikio dienų; 3) sliekų biomasė 0 ir 14 dieną. Gauti rezultatai patvirtino 75 % triciklazolo ūmaus toksiškumo poveikį *Eisenia foetida* sliekams dirbtiniame dirvožemyje.

Raktažodžiai: 75 % triciklazolo milteliai (WP), *Eisenia fetida*, mirtingumas, biomasė