PATNA WOMEN'S COLLEGE 1940

IRIS
Journal for Young Scientists
ISSN 2278 – 618X **(Print)**
ISSN 2278 – 6384 **(Online)**

http://www.patnawomenscollege.in/journal

Comparative sensitivity of earthworms in avoidance tests

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Received : November 2013
Accepted : March 2014
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Abstract : *We investigated the effects of two different pesticides (Profenofos 50% and Thiophanate Methyl 70%) on the avoidance behavior of Eisenia fetida, Eudrilus eugeniae and Pheretima posthuma under laboratory conditions. The tests were performed according to ISO guidelines 17512.The results indicated that the ecologically relevant and naturally occurring species Pheretima posthuma was most sensitive followed by Eudrilus eugeniae, while Eisenia fetida was the least sensitive. It is suggested that avoidance tests could be used as an initial screening tool for risk assessment of pesticides.*

Key Words : *: Earthworms; Fungicide; Insecticide; Risk assessment; Natural soil; toxicity*

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Introduction :

A greater proportion (>80%) of biomass of terrestrial invertebrates is represented by earthworms which play an important role in structuring of soil, soil fertility and soil permeability. In agricultural areas worldwide, there is an increasing concern about soil contamination due to the widespread use of insecticides (Zhou et al 2006; Reinecke and Reinecke 2007; De Silva and Van Gestel 2009; De Silva et al 2010; Garcia et al 2011; Santos et al 2011).The potential risk of chemicals for the habitat function of soils is often investigated applying acute (OECD 1984; ISO 1993) and reproduction tests (ISO 1998; OECD 2004) using earthworms as representatives of the soil biocenosis. Constraints such as the inability to assess population effects with acute toxicity tests and also the long duration and the labor intensive nature of the reproduction test called for rapid assessment methods with shorter duration but high sensitivity and ecological relevance.

Behaviour therefore is suggested as a sensitive and relevant alternative endpoint for toxicity tests with the soil organisms, like earthworms (Hund-Rinke and Wiechering 2001; Heupel 2002; Natal Da Luz et al 2004; Schaefer

2004; Loureiro et al 2005; Lukkari and Haimi 2005; Aldaya et al 2006; Bareteiro Diogo et al 2007).The avoidance behaviour test is a complementary screening test in soil risk assessment (Slimak 1997; Stephenson et al 1998; Hund-Rinke et al 2003). The Earthworm Avoidance Test, originally developed in USA (Yeardley et al 1996), is proposed as an easy and quick alternative to the other two tests. *Eisenia* spp. is a compost or manure worm, rather difficult to find in natural ecosystem and therefore may not be ecologically relevant, and so is the case of *Eudrilus eugeniae*. Hence, we chose the epigeic *Pheretima posthuma* also as it is a naturally occurring species found in the natural ecosystem. It may contribute to risk assessment of pesticides in the tropics. In the present study, we evaluated the avoidance response of *Eisenia fetida*, *Eudrilus eugeniae* and *Pheretima posthuma* towards the insecticides Profenofos 50% and fungicide Thiophanate Methyl 70% WP using natural soil as test substrate under laboratory conditions.

Materials and Methods :

Adult *Eisenia fetida* (weighing~0.21±0.14g) and *Eudrilus eugeniae* (weighing~0.71±0.10g) with well developed clitellum were obtained from the Vermicomposting Unit of Patna Women's College. Adult epigeic *Pheretima posthuma* (weighing~ 0.43±0.04g) were collected from the upper layer of the soil from Patna Women's College Campus. These worms were acclimatized for 24 hours in the natural soil before the experiment. The natural soil was collected from Patna Women's College Campus. The collected soil was ground and sifted through a fine mesh size seive in order to standardize the grain size and obtain a homogenous soil mixture. The soil pH and moisture were determined according to ISO guidelines.

The pesticides used in the experiment were Profenofos 50% and Thiophanate Methyl 70% WP.

The avoidance behavior of earthworms was tested in response to two concentrations of pesticides: X1 corresponding to single application and X4 corresponding to multiple application of the same pesticide over a short period of time.

The concentrations of pesticides tested for the avoidance behavior in earthworms were calculated as follows:

The application rate of Profenofos 50% is 200ml/acre of land. Assuming that the chemical would disperse into top 5 cm of soil when soil density is 1.5g/cm^2, X1 (single dose) for the experiment was calculated to be 0.34 ml/kg of soil, with X4 (tetra dose) being 1.36 ml/kg of soil.

Similarly, X1 for Thiophanate Methyl 70% WP was calculated to be 0.93 mg/kg of soil (application rate = 500 g/hectare). Four times (X4) for this chemical was 3.7 mg/kg of soil.

The experimental procedure of the avoidance tests was based on ISO (2006) and the dual test developed by Hund-Rinke and Wiechering (2001) was followed. The test containers were plastic trays (30x22x6 cm depth) each with two sections. One side of each container was filled with control soil (500 g dry soil) and the other side with pesticide-treated soil. The two sides were separated by a thin plastic sheet to prevent mixing of the two soils. The random distribution of the worm was confirmed by performing dual control tests, which consisted of pesticide-free soil on 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 for all tested concentrations, each with triplicates. The containers were incubated for 48 hours after which the control and the contaminated soil sections were carefully separated and the number of earthworms was determined in each section of the containers

by hand sorting. Individuals found between the two sections (i.e. on the separating line) were counted according to the direction they were moving, i.e. considered in the section where the anterior part of body was. Dead earthworms were classified as escaped animals.

In these tests, for each replicate the net avoidance response (NR) (expressed as percentage) was calculated as:

$$NR = [(C–T)]/N] \times 100$$

Where, NR = net avoidance response (%), C= number of worms in control soil, T=number of worms in pesticide-amended soil, N=total number of worms exposed. A positive (+) net response indicated avoidance and a negative net response (-) indicated attraction to the chemical tested in a given concentration. The Student's t-test ($p<0.05$ level) was used for comparing the means of proportions of individuals in the two sections (control and treated) of each test tray and to compare the response of earthworms towards lower and higher concentrations of pesticides.

Results and Discussion :

The temperature of the experimental sets ranged from 24.03 to 27.4°C, moisture content ranged from 37.0 to 39.0 and pH ranged from 6.95 to 7.75 (Table 1 and 2).

There was no significant change in the body weight of earthworms when they were exposed to pesticide concentration equivalent to the single (X1) field rate. However, there was significant decrease in the body weight of *Eisenia fetida* and *Pheretima posthuma* when exposed to pesticide Profenofos concentration of four times (X4) the field application rate (Table 3). Further, the weight of *Eisenia fetida* was significantly reduced when exposed to X4 dose of Thiophanate (Table 4).

Table 1.Physico-chemical characteristics of experimental soil with Profenofos 50%

	Lower Dose (X1) Profenos 50%				Higher Dose (X4) Profenos 50%			
	Temperature (°C)				Temperature (°C)			
	Initial	Final	Moisture Content (%)	pH	Initial	Final	Moisture Content (%)	pH
Eudrilus eugeniae	27.3±0.3	27.3±0.3	37.0±1.0	7.71±0.08	25.33±1.33	25.17±1.42	37.0±1.0	7.61±0.19
Eisenia fetida	27.3±0.3	27.4±0.3	39.0±0.5	7.75±0.05	24.5±0.9	24.03±1.01	39.0±0.57	7.32±0.07
Pheretima posthuma	24.3±0.3	24.17±0.4	37.3±0.6	7.71±0.02	27.17±0.44	26.73±0.37	37.3±0.6	7.14±0.06

Table 2.Physico-chemical characteristics of experimental soil with Thiophanate Methyl 70% WP

	Lower Dose (X1) Thiophanate Methyl 70% WP				Higher Dose (X4) Thiophanate Methyl 70% WP			
	Temperature (°C)				Temperature (°C)			
	Initial	Final	Moisture Content (%)	pH	Initial	Final	Moisture Content (%)	pH
Eudrilus eugeniae	27.4±0.4	27.2±0.5	37.0±1.0	7.58±0.12	27.4±0.7	27.1±0.6	37.0±1.0	7.1±0.1
Eisenia fetida	26.1±0.4	25.7±0.5	39.0±0.5	6.95±0.1	26.7±0.49	26.5±0.4	39.0±0.5	7.3±0.1
Pheretima posthuma	27.2±0.5	27.1±0.4	37.3±0.6	7.1±0.12	27.3±0.6	27.3±0.6	37.3±0.6	7.3±0.1

Table 3. Change in body weight of earthworms after 48 hours with Profenofos 50%

Lower Dose (X1)	Higher Dose Profenofos 50%		(X4) Profenofos 50%	
	Initial Weight	Final Weight	Initial Weight	Final Weight
Eudrilus eugeniae	7.26±0.11	6.86±0.18	6.99±0.11	6.05±0.32
Eisenia fetida	2.33±0.14	2.22±0.13	2.63±0.1	2.06±0.02
Pheretima posthuma	3.22±0.05	3.06±0.05	3.12±0.04	2.77±0.05

* Significant at P<0.05

Table 4. Change in body weight of earthworms after 48 hours with Thiophanate Methyl 70% WP

Lower Dose (X1)	Higher Dose (X4) Thiophanate Methyl 70% WP		Thiophanate Methyl 70% WP	
	Initial Weight	Final Weight	Initial Weight	Final Weight
Eudrilus eugeniae	7.09±0.16	6.76±0.27	7.15±0.22	7.05±0.2
Eisenia fetida	2.16±0.08	1.91±0.07	2.18±0.01	2.13±0.01
Pheretima posthuma	3.23±0.05	3.09±0.01	3.32±0.04	3.23±0.03

* Significant at P<0.05

No dead or missing worms were found in case of each of the kinds of species in the test at the lower concentration of the test chemical Thiophanate whereas in higher concentration, few worms of the species *Eisenia fetida* were found to have escaped. Death of *E. fetida* occurred in case of the other test chemical Profenofos both in lower as well as higher concentrations.

For the chemical Thiophanate, *Eisenia fetida* was significantly attracted at lower test concentration (Fig.1) and significant avoidance behaviour was shown by *Eudrilus eugeniae* (Fig. 2) and *Pheretima posthuma* (Fig. 3) both at lower as well as higher concentrations.

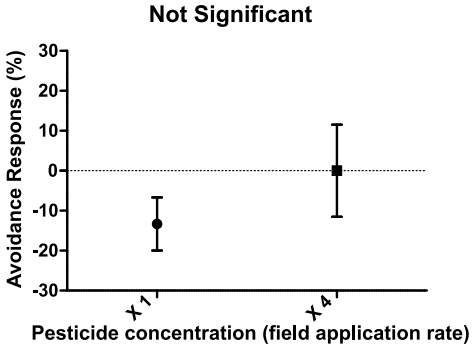

Fig.1. Avoidance or attraction response of tropical *Eisenia fetida* to Thiophanate concentrations in natural soil (mean net response and standard error bars), (statistically non-significant, Student's t-test, p >0.05).

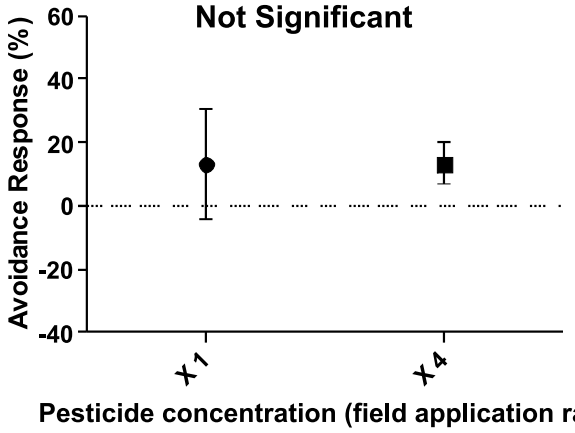

Fig. 2. Avoidance response of tropical *Eudrilus eugeniae* to Thiophanate concentrations in natural soil (mean net response and standard error bars), (statistically non-significant, Student's t-test, p >0.05).

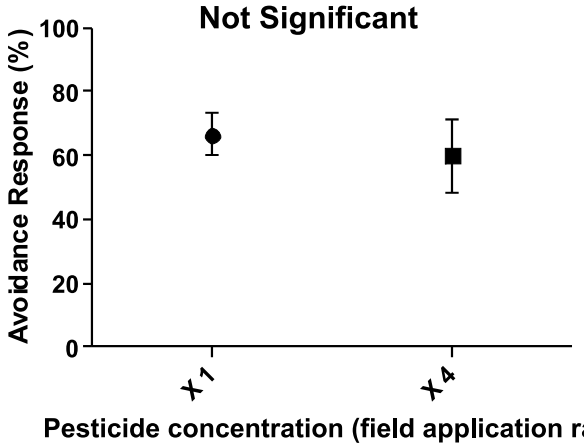

Fig. 3. Avoidance response of tropical *Pheretima posthuma* to Thiophanate concentrations in natural soil (mean net response and standard error bars), (statistically non-significant, Student's t-test, p >0.05).

For the other test chemical Profenofos, all the three species showed avoidance response or attraction response at the lower concentrations while at higher concentrations, they showed significant avoidance response (Fig 5 and Fig 6) except *Eisenia fetida* (Fig 4).The design was considered to be invalid when the number of dead or missing worms was > 10% per treatment (ISO, 2006).This condition was met in the highest concentrations of Profenofos 50% used in case of *Eisenia fetida* and therefore the latter were excluded from the analysis.

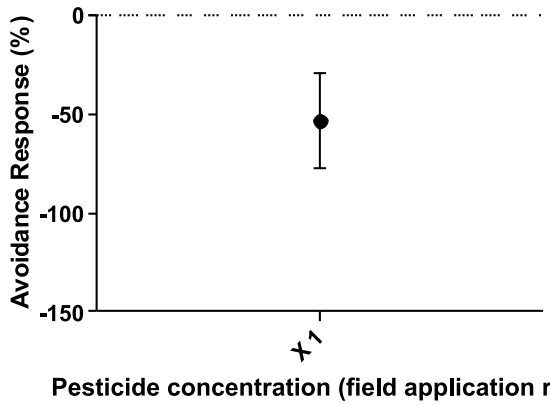

Fig. 4. Attraction response of tropical *Eisenia fetida* to lower concentration (X 1) of Profenofos in natural soil (mean net response and standard error bars). At higher concentration (X 4) 100% mortality occurred.

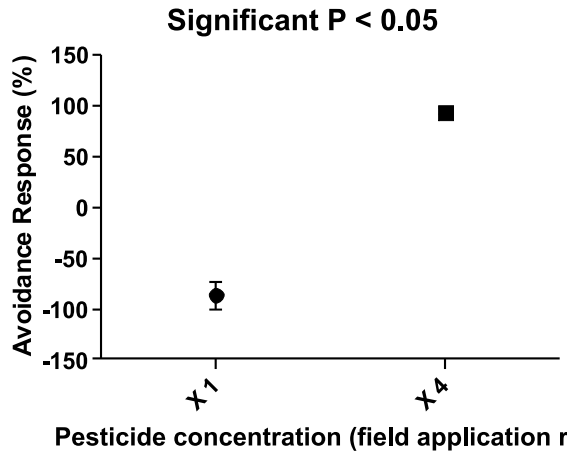

Fig. 5. Avoidance or attraction response of tropical *Eudrilus eugeniae* to Profenofos concentrations in natural soil (mean net response and standard error bars), (statistically significant, Student's t-test, p <0.05).

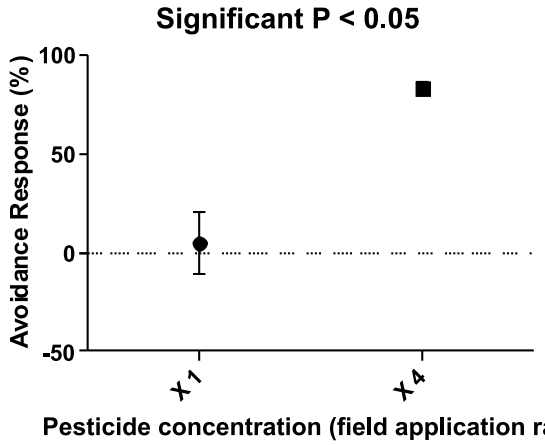

Fig. 6. Avoidance or attraction response of tropical *Pheretima posthuma* to Profenofos concentrations in natural soil (mean net response and standard error bars), (statistically significant, Student's t-test, p <0.05).

Table 5. Comparative Sensitivity of earthworms towards the tested pesticides Profenofos and Thiophanate.

Species	Profenofos 50%		Thiophanate Methyl 70%	
	X1	X4	X1	X4
Eisenia fetida	Attraction	Mortality	Attraction	Neutral
Eudrilus eugeniae	Attraction	Avoidance	Avoidance	Avoidance
Pheretima posthuma	Avoidance	Avoidance	Avoidance	Avoidance

In this study, it was found that out of the three species of earthworms, *Pheretima posthuma* was most sensitive followed by *Eudrilus eugeniae,* while *Eisenia fetida* was the least sensitive (Table 5).

According to the available literature, the avoidance behaviour of *Eisenia fetida* is a very sensitive parameter for the detection of low concentrations of pesticides and other chemicals (ISO, 2006) whereas in our study we found that *Eisenia fetida* showed attraction towards lower dose (X1) of Profenofos and Thiophanate while it showed mortality and neutral behaviour in the X4 concentration of Profenofos and Thiophanate respectively. Ma and Bodt (1993), Kula (1995) and Fitzgerald et al. (1996) also conducted comparative studies on multiple earthworm species and found *Eisenia fetida* to be comparatively less sensitive. Clearly, the extent of the avoidance behavior depends also on the respective chemical to be tested. *Pheretima posthuma* (naturally occurring and ecologically relevant species) showed maximum avoidance response towards both the chemical applications.

Attraction behavior was shown by *Eisenia fetida* and *Eudrilus eugeniae* towards lower concentration of Profenofos. Organophosphates inhibit acetylcholinesterase (AchE) activity (Carmo et al 2010). Profenofos being an organophosphate might have led to the reversibility of the inhibitory pathway thereby, making the sensory receptors of the earthworms not respond.

Other changes seen on exposure to the chemicals were coiling response, shrinkage in size and swollen clitellum. These types of responses exhibited by the earthworms can serve as very useful indicators of different forms of stress in the terrestrial ecosystem since they can be easily detected in the field. The use of behavioural responses by organisms for identifying contaminated sites have also been recognized by other researchers (Otitoloju and Adeoye 2002; Petrauskiene 2003; Untersteiner et al 2003 and Maila and Cloete 2005) as an important tool which can be used singly or in combination with other forms of monitors for detection of stressors in the environment.

Conclusion :

The avoidance tests could be used as an initial screening of pesticide toxicity to earthworms. It is suggested that research should be extended to ecologically relevant species of earthworms and also to other soil fauna to get a comprehensive knowledge on the malfunction in the soil biological processes due to pesticide pollution.

Acknowledgements :

We express our sincere thanks to our respected principal Dr. Sister Doris D' Souza for giving us this wonderful opportunity to undertake the project. We are also thankful to the volunteers of the college nursery for their support throughout our study. Above all, we are grateful to the Research Committee for providing the facilities and financial support.

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