

EFFECT OF FLUBENDIAMIDE ON MORPHOLOGY, AVOIDANCE BEHAVIOUR AND ACETYLCHOLINESTERASE ACTIVITY IN EARTHWORM *EUDRILUS EUGENIAE*

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ABSTRACT

Objective: Flubendiamide is extensively used in agriculture practices as foliar application pesticide. Due to long persistency in the soil, flubendiamide pose serious health concern in non-target organisms. Our main objective was to examine flubendiamide impact on burrowing animal *Eudrilus eugeniae* with special emphasis on avoidance behaviour and neurotoxicity.

Methods: Acute toxicity study of flubendiamide (Diamide pesticide) was conducted on earthworm, *Eudrilus eugeniae* through direct paper contact method and artificial soil method. Median lethal concentration (LC₅₀) of flubendiamide was calculated by following probit analysis. The neurotoxic potential of flubendiamide was studied with marker enzyme Acetyl cholinesterase (AChE) levels in both *In vivo* and *In vitro* experiments.

Results: LC₅₀ in earthworms was found to be 94.4 µg cm⁻² at 48h paper contact test and 332.21 mg kg⁻¹ and 238.31 mg kg⁻¹ respectively at 7 and 14 d artificial soil exposure. Morphological and physiological alterations in earthworms attribute to inhibition of AChE levels. The kinetic study of AChE activity in presence and absence of inhibitor suggests the enzyme reaction is competitive in nature.

Conclusion: Present study establishes concentration-dependent flubendiamide toxicity in earthworm *E. eugeniae*. No clear conclusive remarks were made on earthworm avoidance behaviour as the worms were located both in toxic and control soil after 48h of exposure. Further studies may be needed in this aspect to establish clear understanding on avoidance nature of *E. eugeniae* in different soil types.

Keywords: Earthworm, Acetylcholinesterase, Flubendiamide, Enzyme kinetics, Avoidance behaviour, *Eudrilus eugeniae*

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INTRODUCTION

Phthalic acid diamide is a new class of insecticide used extensively as a substitute for organochlorine and organophosphate pesticides for agriculture applications. This is due to high specificity to Lepidoptera insect larvae and lower persistence in the environment. These insecticides work by regulating ryanodine receptor affecting calcium levels in a cell. Ryanodine receptor activation results in rapid cessation of feeding behaviour resulting in starvation and death of larvae [1]. Use of phthalic acid diamide insecticide in agriculture practices has already crossed its threshold and resulting in environmental contamination. Foliar application of pesticides leaves their trail in residual soil by precipitation or surface runoff, which tend to accumulate with the successive application.

Pesticides in soil pose adverse physiological effects to many non-target organisms [2]. Earthworms are considered farmer's friendly in that they act as bio-fertilizers and composting agents [3]. Earthworms also act as nature's plough, aerator and moisture retainer [4]. Use of vermicompost, a product of vermiculture has been useful to many farmers in reaping organic crop with limited or no fertilizers during crop cultivation [5]. Worm population is mainly depended on nutrient recycling, moisture content, organic matter and presence of pesticide or fertilizers in soil [6]. The presence of pesticides in soil may affect earthworms in the vicinity of pollution. Earthworms may get exposed to these pesticides either by surface contact or through feeding on contaminated organic matter in the soil. Such scenario may pose severe consequences to earthworm population and at the same time to ecological balance. There are several toxicity studies have been conducted on non-target organisms with selected pesticides, but very few of them are on earthworm population [7]. The present study was an attempt of adding to our current knowledge on pesticide impact on earthworms. We hypothesized, based on available literature that, repeated exposure to flubendiamide may have a detrimental effect on physiology and morphology of earthworms. Special emphasis was given on earthworm avoidance behaviour, which was originally

developed by Yearley *et al.* [8], is one of the easy to perform a test to study earthworm avoidance behaviour. The principle of the test is to expose the earthworms simultaneously to soil samples spiked with a chemical of interest and a control soil. After 48 h location of earthworms is determined. The existing study protocols [9] were standardised according to test species chosen, substrate and conditions suitable for the sub-tropical region.

Flubendiamide, "N²-[1,1-Dimethyl-2-(methylsulfonyl)ethyl]-3-iodo-N¹-[2-methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl) ethyl] phenyl]-1,2-benzenedicarboxamide" is commercially used in India for the foliar application of cotton, chickpea and many vegetable crops. Hence, it is evocable to study toxic effects of flubendiamide on earthworm *Eudrilus eugeniae*. This species is most commonly found in Indian subcontinent. The present study examined the acute toxicity of flubendiamide in *E. eugeniae* using paper contact and artificial soil test methods with special emphasis on avoidance behaviour and acetylcholinesterase (AChE) enzyme kinetics.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents used were procured from Sigma-Aldrich, and were of analytical grade and used without further purification. Earthworm *E. eugeniae* were procured from department of vermiculture, University of Agriculture Sciences, Dharwad, India. They were brought to the laboratory within 1 h of procurement in a moist organic soil. Before commencing the experiment, these earthworms were acclimatised for 10 d in a feed box containing red soil at the bottom, a layer organic waste, 16-inch alluvial soil and finally dried leaves on top. Feed boxes were covered with perforated polyethylene covers for aeration.

Determination of median lethal concentration (LC₅₀)

Acute toxicity tests were conducted by two methods, direct filter paper contact test (48h) and artificial soil test (14-Day) according to

OECD guideline [10] and as followed by Venkateswara Rao and Kavitha, [11]. Briefly, for filter paper test, flat bottomed glass vials were lined with Whatman filter paper No 1 (surface area, 63.77 cm²) without overlapping. Test chemical, flubendiamide, was dissolved in water at predetermined values (from maximum tolerance dose test) 3.0 mg/ml, 6.0 mg/ml and 12.0 mg/ml to get 54 µg/cm², 94 µg/cm² and 188 µg/cm² concentration respectively. Controls were also run parallel with water alone. Earthworms from moist soil taken out and randomised for a group of 20 earthworms per treatment group (1.44±0.29 g in weight).

The artificial soil test system was prepared by evenly mixing dry weight mixture of 68% mesh silica, 20% kaolin clay and 10% sphagnum peat as described in OECD [10]. Different concentrations of flubendiamide (100, 150, 250, 300, 350, 450 and 550 mg/kg) was mixed with artificial soil. 35% moisture and pH 6.0±0.5 (by adding calcium carbonate) were maintained throughout the exposure period. Total soil mixture was divided into four portion and 10 earthworms of uniform size were administered into each portion soil at a frequency of 1 earthworm per 100 g soil. Test system was set up in earthly pots covered with a perforated plastic sheet for proper aeration and to prevent animals from escaping. Morphological abnormalities and percentage kill were monitored at 7 and 14 d of exposure. Probit analysis [12] is used to calculate median lethal concentration (LC₅₀) at both exposure tenures.

Avoidance test

The avoidance test was performed according to the method first developed by Yearley *et al.*, [8] and previously described by Garcia *et al.*, [13]. A fresh batch of artificial soil was prepared and different concentrations used in a median lethal test system with control were set up for avoidance test. Plastic vessels (11 X 15.5 cm area, 6 cm height) was separated into to half using a piece of plastic fitted transversely in the vessel. One-half is filled with artificial soil mixed with different concentrations of flubendiamide and another half with uncontaminated soil. Then the plastic separator was removed and 10 earthworms of *E. eugeniae* were put on the separating line. Soil pH and moisture were monitored throughout the experiment. At the 48h, the control and contaminated soil were carefully separated and a number of earthworms in each section were counted. Worms found on the central line of two were counted according to the direction to which they were moving based on the position of anterior portion. Any missing animal is considered dead or escaped.

Test was conducted in triplicate for each concentration and mean was taken as reading.

AChE activity

Earthworms from artificial soil exposure were carefully taken out and preserved for AChE activity. Anterior portion of four earthworms was dissected out and washed in saline buffer and homogenised in 0.1 M phosphate buffer. Homogenate was centrifuged at 10,000 rpm for 15 min and the supernatant was recentrifuged at 10,000 rpm for another 10 min. The resultant supernatant was used as enzyme source of AChE and is stored in 4 °C. Protein portion was estimated by the method of Lowry *et al.*, [14], and AChE assay was performed according to the method of Ellman *et al.*, [15] and the activity was expressed as mol/min/gram tissue.

Simultaneously, unexposed earthworms were used to study *in vitro* evaluation of AChE activity. The maximum velocity of the reaction (*V*_{max}) and the substrate required to attain half of maximum reaction velocity (*K*_m) were estimated by plotting Lineweaver-Burk plots using different substrate concentrations (0.01, 0.025, 0.030, 0.035, 0.040, 0.050, 0.1 and 0.2 mmol), 0.16 mmol DTNB, and known amount of protein in 3 ml assay volume. Mode of AChE inhibition was assessed by allowing different concentration of flubendiamide (3.75, 7.50 and 12.0×10⁻⁵ M) to react with an enzyme in a reaction mixture along with substrate. The inhibitory constant *K*_i was determined graphically by plotting slopes of intercepts of different inhibitors against concentration [16].

Statistical analysis

Data are expressed as mean±SD of three separate experiments. The experiments were repeated three times in triplicate and the data were analysed by analysis of variance. The individual means were compared using Duncan's test for multiple comparisons. A probability of *P* ≤ 0.05 was selected as statistically significant.

RESULTS AND DISCUSSION

Acute toxic effect of flubendiamide was recorded at 48h of paper contact test and 7, 14 d of an artificial soil test. The calculated median lethal concentration (LC₅₀) at 48h paper contact test was 94.4 µg cm⁻² and 332.21 and 238.31 mg kg⁻¹ respectively, for 7 and 14 d of artificial soil test (table 1). These values suggest lower concentrations of flubendiamide are enough to cause 50% mortality in earthworms exposed for 48h, 7 d and 48 d.

Table 1: Median lethal concentration (LC₅₀) of flubendiamide to earthworm *E. eugeniae* in paper contact and artificial soil tests

Median lethal concentration (LC ₅₀)		Artificial soil method	
Paper contact method			
48 h	94.4 µg cm ⁻²	7 d	332.21 mg /kg
	60.1 mg /l	14 d	238.31 mg /kg ⁻¹

There was a progressive morphological degeneration observed among earthworms exposed at varying concentration of pesticide in paper contact test method. Signs of toxicity includes sluggish movements, excessive mucous secretion and swelling of clitellum at concentrations of 54–94 µg cm⁻². Blood lesions were observed

along with excessive bodily mucous secretions at higher concentration (150 µg cm⁻²). Morphological changes such as coiling and swelling of clitellum were prominent at 24h of exposure and degenerative lesions were observed till 48h of exposures (fig. 1B and 1C).

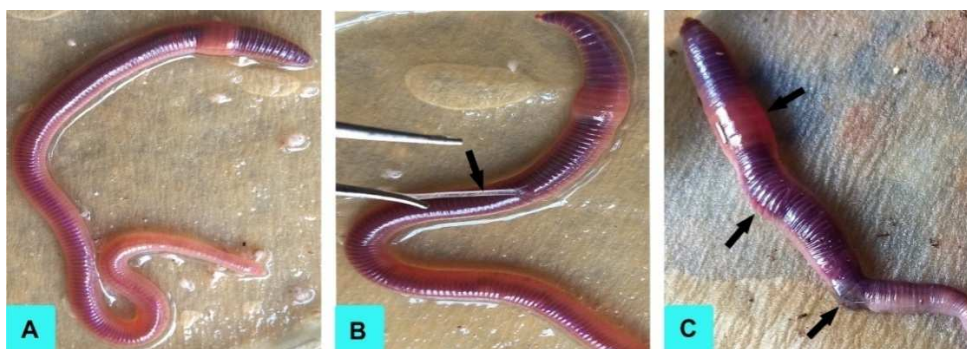


Fig. 1 (A-C): Morphological changes in earthworm *E. eugeniae* after 48h exposure to Flubendiamide using paper contact test

According to metabolic cost hypothesis, major energy reserves are affected when an organism is under toxic stress [17]. Proteins and carbohydrates happen to be a prime energy source in burrowing animals and probability of toxicants targeting these energy reserves is certain [18]. Sluggish movements observed in earthworms exposed to flubendiamide and production of excessive mucous could be attributed to the fact that defensive mechanism of earthworms against pesticide induced stress. Animals tend to recuperate from pesticide stress by utilizing energy reserves such as stored proteins. However, pesticide toxicities beyond defence threshold could possibly lead to autolysis of own body tissues as observed in the

present study. A similar observation was made by Ramaswami and Subbram, [19] in *Polypheretima elongata* exposed to textile dyes. In the case of artificial test system two-thirds of test animals were found dwelling at the bottom portion of test container.

This could be due to clitellum swelling which may have prevented free movement of earthworm. In contrast, earthworms in control soil were found burrowing freely throughout the container. At higher concentration of flubendiamide (>350 mg kg⁻¹) loss of pigmentation was prominent among earthworms whereas control animals exhibited no such notable morphological differences.

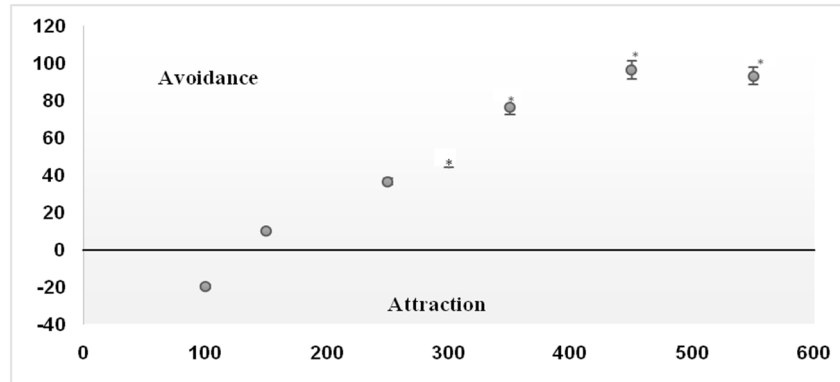


Fig. 2: Percentage avoidance pattern of earthworm *E. eugeniae* to different concentrations of flubendiamide in artificial soil (data shows mean response and percentage error bars). * indicates significant value at $p \leq 0.05$

The effect of pesticide avoidance behaviour is summarized in fig. 2. In artificial soil, earthworm *E. eugeniae* response is significant at 300 mg kg⁻¹ concentration and this trend was seen till 550 mg kg⁻¹ concentration. Whereas, at a lower concentration of 100 mg kg⁻¹ non-significant attraction towards flubendiamide treated soil was observed. According to available literature, avoidance behaviour is a sensitive parameter when detection of a pesticide in soil is a major objective [9]. This implies physical parameters such as temperature, potential H⁺ ion level and type of soil plays a definitive role in concluding avoidance studies. Reinecke et al., [20], studied avoidance pattern of *E. fetida* in artificial soil and documented avoidance behaviour against fungicide Mancozeb, whereas, affinitive behaviour towards lead contaminated soil. This suggests the extent

of avoidance or attraction is dependent on a respective chemical to be tested. The aim of performing avoidance study in natural soil is to obtain toxicity data of the relevant compound. However, no encouraging data on *E. eugeniae* avoidance behaviour in sandy clay loam (which is common to subtropical countries like India) soil is available for the best of our knowledge. The present study is one step in this direction to understand nature of the behavioural response of *E. eugeniae* in flubendiamide contaminated soil. Furthermore, avoidance behaviour of different earthworm species in sandy clay loam soil is unsuccessful so far [13]. Therefore there cannot be any conclusive remarks on *E. eugeniae* response to flub-diamide and further studies in this regard may be required to know the precise behavioural response of *E. eugeniae* in different soil types.

Table 2: Treatment wise AChE inhibition among earthworm exposed to different concentrations of flubendiamide for 7 and 14 d

Treatment concentration	Mean AChE (U/mg protein)	Mean AChE (U/mg protein)
	Day 7	Day 14
C (0.0 mg kg ⁻¹)	0.354±0.011	0.354±0.011
100.0 mg kg ⁻¹	0.305±0.006*(13.84)	0.248±0.006*(29.94)
150.0 mg kg ⁻¹	0.282±0.022*(20.33)	0.222±0.005*(37.28)
250.0 mg kg ⁻¹	0.273±0.010*(22.88)	0.194±0.007*(45.19)
300.0 mg kg ⁻¹	0.221±0.007*(36.72)	0.181±0.006*(48.87)

Data represents mean±SD where $p \leq 0.05$ considered as significant. (Values in parentheses indicate percentage decrease).

Table 3: Kinetic constants of *In vitro* AChE activity in presence of different concentrations of flubendiamide

Pesticide concentration	Intercept	Slope	K_m (1/Intercept)×(Slope)	V_{max} (1/Intercept)
Control	0.82	0.025	0.030	1.219
3.75×10 ⁻⁵	0.78	0.036	0.046	1.282
7.50×10 ⁻⁵	0.74	0.046	0.062	1.351
12.0×10 ⁻⁵	0.71	0.082	0.115	1.408

In vivo AChE activity in earthworms treated with flubendiamide for 7 and 14 d in artificial soil is illustrated in table 2. The enzyme inhibition is evident in both exposure period and in a concentration dependent manner. The percentage inhibition of enzyme is 13.84% in 100 mg kg⁻¹, 20.33% in 150 mg kg⁻¹, 22.88% in 250 mg kg⁻¹ and 36.72% in 300 mg kg⁻¹ on 7th day of treatment.

The percentage inhibition of AChE was further increased on 14th day of exposure by 49% at a highest concentration of flubendiamide. *In vitro* dissociation constant (K_m) known as Michaelis-Menten constant was determined by plotting curves of reciprocals of substrate [1/S] against reciprocals of reaction velocity [1/V] (fig. 3a-3d).

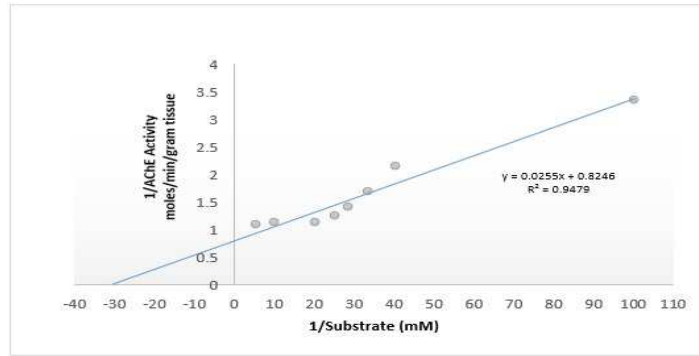


Fig. 3a: Lineweaver-burk plots of AChE activity of earthworms in absence of flubendiamide as a function of substrate concentration

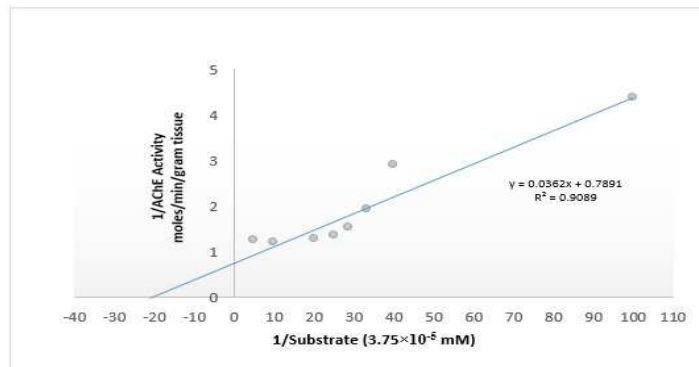


Fig. 3b: Lineweaver-burk plots of AChE activity of earthworms in presence of 3.75×10^{-5} mmol flubendiamide as a function of substrate concentration

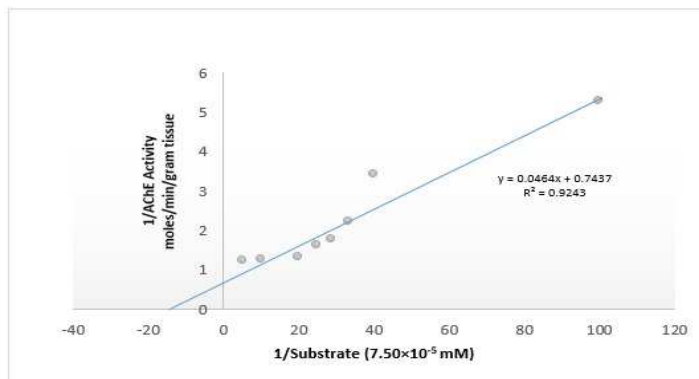


Fig. 3c: Lineweaver-Burk plots of AChE activity of earthworms in presence of 7.50×10^{-5} mmol flubendiamide as a function of substrate concentration

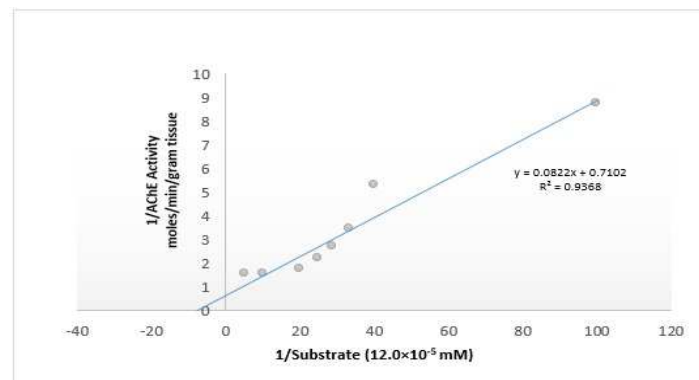


Fig. 3d: Lineweaver-Burk plots of AChE activity of earthworms in presence of 12.0×10^{-5} mmol flubendiamide as a function of substrate concentration

The maximum velocity V_{max} and K_m values of AChE activity in control and various treated concentrations of flubendiamide were determined by regression equations of their respective curves (table 3). The estimated V_{max} and K_m values of AChE in control earthworms were 1.219 and 0.030 respectively. Constant increase in K_m values with increase in inhibitor concentration indicates a

close structural resemblance of inhibitor to the substrate. Thus this kind of inhibition by flubendiamide could be competitive in nature. In addition, inhibition constant (K_i) of entire reaction is derived by plotting the graph of slope (K_m/V_{max}) against different inhibitor concentration [16]. The inhibition constant for flubendiamide is 4.4×10^{-5} moles (fig. 4).

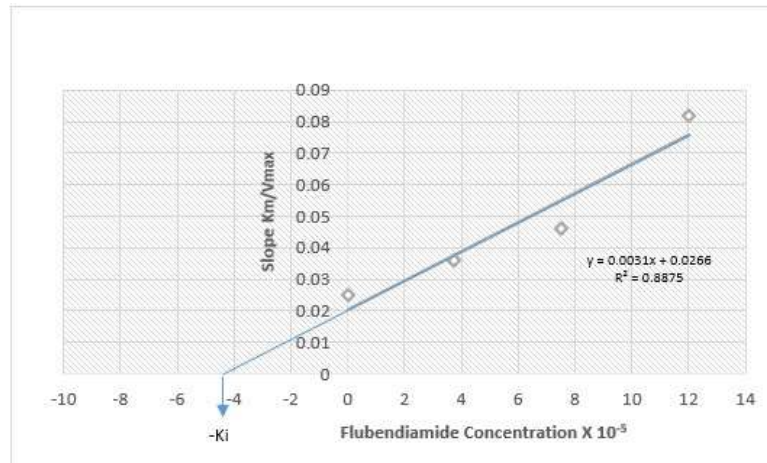


Fig. 4: Slopes of lineweaver-burk plots at different concentrations of flubendiamide

CONCLUSION

Present study demonstrates toxic effects of flubendiamide on *E. euginae* increases with increase in pesticide concentration and exposure period. This indicates accumulation of flubendiamide is a measure of toxicity with inhibition of AChE enzyme. The paper contact test is more suitable than artificial soil test in observing morphological changes in earthworms. An artificial soil test is close to reality but physiological alterations like excessive mucous secretion, extrusion of coelomic fluid and formation of bloody lesions are clearly visible in paper contact test. Earthworms tend to avoid flubendiamide contaminated soil at higher concentration, but at lower concentrations, more attraction towards contaminated soil finds no conclusive remark. Further studies may be required in this aspect of the study. From enzyme kinetic data, AChE inhibition at a higher concentration of flubendiamide suggests the inhibition is competitive in nature. Based on present enzyme kinetics, it is recommended to study the effect of other diamide pesticides on AChE activity in earthworms. The present study and generated data will be helpful in understanding toxic potentials of diamide pesticides by employing paper contact test prior to conducting artificial soil tests. The study may also be helpful in assessing the impact of similar chemical compounds on population and toxicity of earthworms.

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AUTHOR CONTRIBUTION

Each author contributes equally for the conduct of this work.

CONFLICT OF INTERESTS

Authors declare no conflicts of potential interest with this article.

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