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Evaluation of changes in reproductive and biochemical parameters in indigenous earthworm *Perionyx excavatus* (Perrier, 1872) against impact of selected pesticides

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Abstract
Globally the crisis of food is a major cause of concern. To increase their yield without interference, indiscriminate and unmanaged application of chemical fertilizers coupled with highly toxic pesticides has resulted in irreversible destruction of soil ecology greatly affecting non-target organisms, especially earthworms and deteriorating soil fertility. Ecotoxicological research has helped us in detecting and understanding the mode of action of a varied spectrum of xenobiotics in agro-ecosystem. In the present study acute toxicity of four pesticides *viz*. Pendimethalin, Pretilachlor, Dimethoate and Cypermethrin from different groups was compared on indigenous epigeic earthworm species *Perionyx excavatus* (Perrier, 1872) [23] and the changes in cocoon production and specific activity of acetylcholinesterase enzyme due to long term exposure of sub-lethal doses of pesticides i.e. T1 (25% of LC_{50}) and T2 (50% of LC_{50}) values under controlled laboratory conditions. From the experimental results we could observe that all of the pesticides showed a significant amount of reduction in cocoon production in earthworms. Cypermethrin (LC_{50}- 0.012 mg/kg) was most toxic to earthworms with no cocoon production in T2 dose, but Pendimethalin showed the maximum suppression in the specific activity of acetylcholinesterase with an enzyme inhibition percentage of 63.2% and 72.4% in T1 and T2 doses respectively than control (N) value.

Keywords: Acute toxicity, organophosphate, acetylcholinesterase, *Perionyx excavatus*, biomarker

Introduction
Globally food production involves heavy use of a varied range of pesticides (Fojtov´a *et al*., 2019) [13] which has increased over the last decade (Ozkara *et al*., 2016) [21]. To get the crop yield to a maximum level use of pesticide has considerably been increased over the past few decades *leading* to the incorporation of an array of toxic compounds in ecosystems increasing adverse effects on wildlife and other ecosystem services (Costa *et al*., 2024) [7]. Pesticides are used in agriculture sector to achieve high production, however the pesticide residues increases soil contamination causing stressful environment for soil organisms (Choung *et al*., 2013) [6]. Earthworms play the role of important biological organisms contributing ecosystems functioning and services of terrestrial habitats (Al-Maliki *et al*., 2021) and are the most conspicuous non-target soil organism and worst victims of insecticide application in the agro-ecosystems. Earthworms constitute 92% of the invertebrate soil biomass and are easily susceptible to pesticides due to application in agricultural fields. But presence of earthworms is valuable to agro-ecosystems because they function in complex processes like litter decomposition, nutrient cycling and soil formation (Eriksen-Hamel and Whalen, 2007) [9]. Earthworms should be specifically conserved done for both economic and ecological reasons (Wang *et al*., 2020) [39].

Pesticides affect earthworms at each level like individual, population and community (Uwizeyimana *et al*., 2017) [35]. In recent years, many ecotoxicological studies have been carried out focusing on effect of pesticide (Miglani *et al*., 2019) [18]. Environmental risk assessment (ERA) of soil contamination is done by using earthworms as bioindicators and are recommended as model specimens in ecotoxicological tests because of it is easy of culture, widely available and high vulnerability to chemicals (Calisi *et al*., 2019) [3]. Earthworm toxicity testing procedure recommended by Organization for Economic Cooperation and Development (2004) and USEPA (1996) are globally used in toxicological researches on earthworms. Earthworms living in soil are excellent bio-indicator of soil health specially for evaluating lethal and sub-lethal effects of toxic chemicals present in soil (Uwizeyimana *et al*., 2017) [35].

Four locally available commercial pesticide formulations like Pendimethalin and Pretilachlor (herbicides), Dimethoate (organophosphate) and Cypermethrin (synthetic pyrethroid) having different properties and mode of actions were selected for this study to understand the impacts of these pesticide formulations. This study has investigated acute effects and chronic effects *viz*. mortality, life history parameter like cocoon production and the specific activity of the enzyme acetylcholinesterase (AChE) in indigenous species of epigeic earthworms *Perionyx excavatus*. AChE activity was selected due to its very significant role in the breaking down of acetylcholine, which is a neurotransmitter, from cholinergic synaptic clefts and is widely used as a neurotoxicity biomarker indicating significant chronic effects (Walker *et al*., 2012) [37].

Materials and Methodology

1. Study site and model specimen

Grasslands of Midnapore Town and adjacent areas in Paschim Medinipur district, West Bengal (Fig 1) which was free from crop cultivation, i.e. uncontaminated from direct pesticide application, were chosen as the area of collection of the abundant indigenous species of epigeic earthworm earthworms, *Perionyx excavatus* (Perrier, 1872) [23] used as the model specimens for the experiment (Fig 2).

Fig 1: Paschim Medinipur District, West Bengal, India

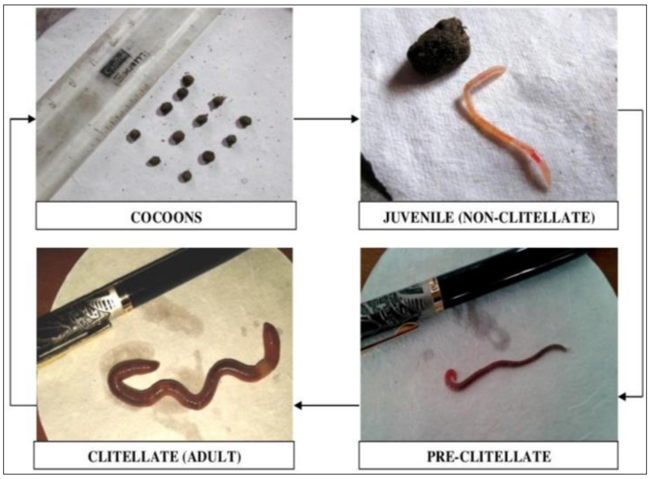

Fig 2: Life cycle stages of *Perionyx excavatus*

2. Culture and maintenance of test specimen

Soil digging was done up to a depth of at least 12-16 cm using a hand spade and the collection of earthworms was done in polythene bags along with a little moist soil and transported to the laboratory for identification and culture (Sanyal and Chakravorty, 2020) [27]. After collection, age synchronized specimens were hand sorted and cultured in large cement vats (Fig 3). Finely grinded and sieved grassland soil and dry cowdung was mixed in equal ratio to make the culture bed (Ismail, 1997) [16]. Shady and cool place was chosen to keep the culture vats and the culture medium was kept moistened at an approximate level of 60-65% by adding water to it under regular supervision keeping the temperature at around 28 ± 0.5ºC. Every week food was added in the form of sun-dried farmyard manure during the culture period. Hand sorting was done to isolate the cocoons, which were kept in separate culture pots and were utilized afterwards as model specimens following guidelines of OECD, (2004).

Fig 3: Culture Vat

3. Test Media

The test medium soil was collected from the grasslands which were selected as the collection site of test specimens, *Perionyx excavatus*.

4. Pesticides Used

Four pesticides from three different groups, used by farmers, which are commercially and locally available, were used in the experiment. The name of pesticides and their respective groups are given in Table 1.

Table 1: The pesticides used in the experiment

Sl. No	Group of Pesticides	Technical Name	Trade Name	Supplying agencies
1	Herbicides	Pendimethalin	Dhanutop	Dhanuka Agritech Ltd, Gurgaon
		Pretilachlor	Racer	Krishi Rasayan, Balasore
2	Organophsphate	Dimethoate	Rogorin	Plant Remedies Pvt. Ltd, Hazipur.
3	Synthetic Pyrethroid	Cypermethrin	Ustaad	United Phosphorus Ltd., Gujarat

5. Physicochemical properties of the test medium

Natural grassland soil was used as the experimental medium. The physicochemical properties of the test medium were evaluated and given in Table 2.

Table 2: Physicochemical properties of the test soil medium

Sl. No	Soil Properties	Value	Methodology
1	Organic carbon content	0.9 %	Walkley and Black, 1934
2	pH	7.20	Piper, 1942
3	Moisture content	61 %	Chakravorty, 1990

6. Acute toxicity test

The median lethal concentrations for all the selected pesticides on *Perionyx excavatus* were evaluated for 96-hr following the OECD guidelines (OECD, 2004).

7. Chronic toxicity study

Ten preclitellar (rate of reproduction) and clitellar (specific activity of AchE) earthworms were released in polyethylene boxes (192 cm^2) (Fig 4) each containing 500 gm of finely sieved garden soil with a 60-70% moisture content. The soil was contaminated uniformly with the sublethal doses of the selected pesticides with the help of a sprayer after half an hour. Three replicates were made for each of control (N), T1 (25% of LC_{50}) and T2 (50% of LC_{50}) doses for each pesticide. All the boxes were kept inside an environmental chamber (Fig 5). The temperature (28±0.5°C) and relative humidity (67%) were maintained at a constant level for 28 days until the evaluation of chronic toxicity bioassays as per the updated standard guidelines of OECD, 2004.

The rate of cocoon production was assessed for 4 weeks by hand sorting and transferred in another box each week following the updated standard guidelines of OECD 2004. The specific activity of acetylcholinesterase enzyme was assessed following the spectrophotometric method of Ellman *et al*., (1961) [8] using first 7 segmental tissue of earthworm, acetylthiocholine as substrate and 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB. After centrifugation the supernatant was taken to carry out the spectrophotometric method. The OD absorbance was measured at 412 nm.

Fig 4: Test Box

Fig 5: Environmental Chamber

8. Statistical Methods

Probit analysis was done to calculate the median lethal concentrations (LC_{50}) of the pesticides obtained after 96 hours of exposure by EPA probit analysis program, version 1.5 (US EPA, 2006).

The data of the entire experimental study for each insecticide was analyzed by two-way ANOVA to evaluate significant variation between control and treatments at 5 % level of confidence using GraphPad Prism 8.0.1 statistical software.

Results

The 96 hours LC_{50} values of the herbicides Pendimethalin and Pretilachlor were found 0.016 and 0.052 mg/kg soil respectively, for the organophosphate insecticide dimethoate was found 0.017 mg/kg soil and for cypermethrin was 0.012 mg/kg soil. Cypermethrin was found as the most toxic amongst the pesticides.

Cocoon production was reduced significantly in all four pesticides at the lowest dose (T1) tested, while increased sublethal dose (T2) of pesticide resulted in the severe reduction in number of cocoons produced (Table 3). Cypermethrin showed the highest toxicity among the pesticides with no cocoon produced in T2 dose (Fig 6).

The specific activity of acetylcholinesterase (AchE) in *P. excavatus* exposed to sublethal doses (T1 and T2) was significantly reduced in case of all the selected pesticides compared to the control (N) value. Pendimethalin showed the maximum inhibition of specific enzyme activity of AchE in both the sublethal dose exposed earthworms, i.e T1 and T2 (Fig 7). The percentage inhibition of AchE as compared to control was 63.2% and 72.4% for T1 and T2 respectively.

Table 3: Rate of Cocoon production (cocoons/worm/week) in *P. excavatus* exposed to sublethal doses of pesticides

Treatments	Pesticides			
	Pendimethalin	Pretilachlor	Dimethoate	Cypermethrin
Control	0.92±0.04^a	0.92±0.04^a	0.92±0.04^a	0.92±0.04^a
T1	0.35±0.03^b	0.75±0.04^b	0.55±0.03^b	0.15±0.02^b
T2	0.25±0.01^c	0.45±0.02^c	0.35±0.02^c	0.00

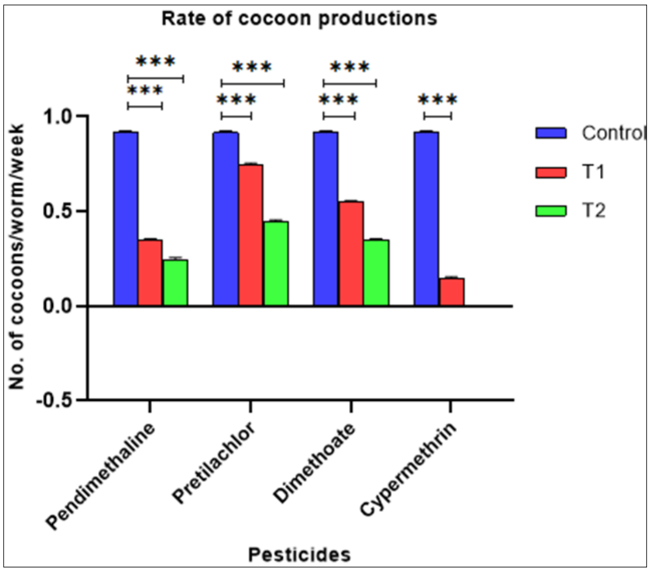

Fig 6: No. of cocoons/worm/week of *P. excavatus* treated with sublethal doses (T1 & T2) of selected pesticides and the control (N). ($p<0.05$)

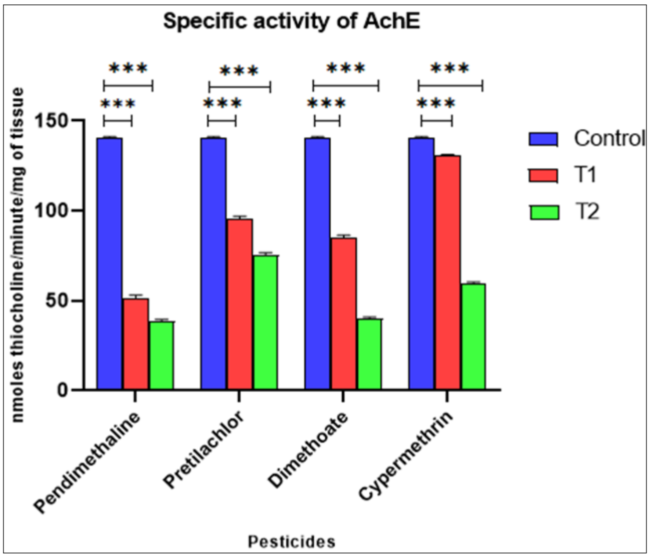

Fig 7: Acetylcholineesterase (AchE) levels of *P. excavatus* treated with sub-lethal doses (T1 & T2) of selected pesticides and the control (N). ($p<0.05$)

Discussion

Pendimethalin and pretilachlor was reported to be toxic by Sanyal *et al*., (2015) [25] to *Perionyx excavatus* under controlled conditions in garden soil. Adelasoye, (2017) [1] reported that the lethal toxicity test for pendimethalin for *Eisenia fetida* and *Libyodrilus violaceus* was 1.77 l/ha and 1.83 l/ha for soil test respectively. When Singh and Singh (2015) [30] applied different concentrations of 2, 4-Dichlorophenoxyacetic acid, a systemic herbicide in loamy soil, clay soil and sandy soil and earthworm *Eutyphoeus waltoni* was exposed, time dependent decreased toxicity on the earthworms was observed. Similarly, Singh and Singh, (2016) [31] reported toxicity of herbicide butachlor on the earthworm *Eutyphoeus waltoni* for loam, clay and sandy soil respectively. Based on LC_{50} value of the organophosphate pesticide dimethoate and comparing the LC_{50} value of the chemical with its RAD, it was found that dimethoate is apparently ecologically safe for the earthworms, because the LC_{50} value is higher than RAD. Considerable toxicity has been observed by Yadav *et al*., (2020) [40] exposing *Eisenia fetida* to the organophosphate chlorpyrifos in a dose dependent manner. Fahreem *et al*., (2014) [10] reported cypermethrin to be most toxic to *Pheretima posthuma* when compared with other pesticides. Cang *et al*., (2017) [4] performed 14 day soil toxicity test on the earthworm *Eisenia fetida* and reported that a comparatively less toxicity was found for *lambda*-cyhalothrin. Sekulić *et al*., (2023) [28] has reported high lethal concentration values of synthetic pyrethroid bifenthrin against *Eisenia fetida* and concluded that the response time of the pesticide is not quick enough but can persist for a longer period in soil harming the earthworms.

We observed that cocoon production was reduced significantly the most in the test specimens, when exposed to Pendimethalin and Cypermethrin even in the lowest concentration of sub lethal doses i.e. T1. In the T2 dose of Cypermethrin, no cocoon was observed in the entire experimental period. For earthworms the mechanism of physiological resistance against toxicants may be expensive as far as energy and other resources are concerned involving reduced ability of investment of energy in reproduction resulting in the inhibited production of cocoons. Muthukaruppan and Gunasekaran, (2009) [19] reported that in *Eisenia fetida* treated with different doses of herbicide butachlor, cocoon production exhibited decreasing trend when the concentrations of butachlor were increased. Farrukh and Ali, (2011) [11] reported dose dependent effect of the organophosphate dichlorovos on the production of cocoons of earthworm *Eisenia fetida*. Pal and Patidar, (2013) [22] have reported decrease in cocoon production and viability in *Eisenia fetida* when exposed to dimethoate. Rosa *et al*., (2016) performed chronic test on the aquatic oligochaete *Lumbriculus variegatus* for 28 days and showed a significant reduction in reproduction when exposed to the neurotoxic pyrethroides fenvalerate. Sanyal *et al*., (2017) [26] reported a significant supression in cocoon production rate in *Perionyx excavatus* when exposed to dimethoate in a dose dependent manner. Earthworms *Eudrilus eugeniae* and *Perionyx barotensis* exposed to varied doses of organophosphate pesticide Monocrotophos for 45 days exhibited abnormal cocoons in earthworms with elevated doses of the pesticide (Gowri and Thangaraj, 2019) [15]. Zhu *et al*., (2022) [41] reported inhibition of acrosomal enzyme activity of sperm of earthworm leading to hindered fertilization and supressed production of cocoons of earthworms when exposed to cypermethrin. Sekulić *et al*., (2023) [28] performed chronic toxicity experiment for 4 and 8 weeks in different concentrations and found significant dose and time dependent suppression in cocoon production in *Eisenia fetida*.

Specific activity of acetylcholinesterase was significantly inhibited in case of all the pesticides in both the sub lethal doses and inhibition was observed in a dose dependent manner, higher the concentration of the pesticide, more significant was the reduction of the activity of the enzyme. The neurotransmitter acetylcholine is secreted from the neurosecretory cells facilitating nerve impulse transmission through the synaptic cleft. When earthworms were treated with pesticides, the nervous system was affected resulting in the inhibition of splitting of acetylcholine (ACh). The pesticides attaches to the active site of the cholinesterase (ChE) enzyme preventing the splitting of ACh in the synaptic cleft leading to ACh accumulation resulting in overstimulation of neuronal cells, which leads to neurotoxicity and subsequent mortality of the specimens.

Fetoh and Asiry, (2013) [12] observed that the level and activity of acetylcholinesterase was elevated when the larval instars of cotton leaf worm, *Spodoptera littoralis*, were treated with chlorpyrifos. Significant inhibition of AChE activities was assessed in earthworms *Eisenia Andrei* and *Lumbricus rubellus* when treated with different concentrations of deltamethrin (0.01, 0.1, and 0.5 mg kg^{-1}) by Velki *et al*., (2014) [26]. Significant supression in the specific activity of AChEwas observed in pre-clitellar, clitellar and post-clitellar regions of earthworm *Eudrilus eugeniae* exposed to cypermethrin were reported by Tiwari *et al*., (2019) [29]. Singh *et al*., (2019) [18] observed the effect of sublethal doses of deltamethrin on the specific activity of AChE in different body segments of earthworm indicating deltamethrin treatment at 5% and 10% of 48 hour LC_{50} showed a supression in AChE activity by 21.79% and 32.61% respectively in pre-clitellar region, 17.86% and 28.13% respectively in the clitellar region, and 23.47% and 42.86% respectively in post-clitellar region ($p < 0.01$) compared to control. Kaur and Hundal (2022) [17] reported inhibition of AChE activity in earthworm species *Eudrilus* *eugeniae* and *Metaphire posthuma* in all the concentrations of imidacloprid, showing evidence of neurotoxicity. Increased chlorpyrifos concentration showed significant decrease in AChE activities as reported by Teng *et al*., (2022) [32].

Conclusion

Thus from this study, we can conclude that, all four pesticides are significantly harmful to the non-target species of earthworms affecting their reproductive physiology as well as inducing neurotoxicity resulting in the death of the test specimens, indicating their potential involvement in soil ecosystem damage. The life cycle and biochemical parameters studied in the experiment can be used as important biomarkers in detection and mitigation of pesticide pollution in agro-ecosystems.

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Conflict of Interest

The authors declare no conflict of interest.

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