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Response of enzyme biomarker and life-history parameters of epigeic earthworm *Perionyx excavatus* exposed to organophosphate pesticide

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Abstract
Indigenous epigeic earthworm *Perionyx excavatus* was collected from uncultivated grasslands of Midnapore district and was subjected to 28 days exposure to different sublethal doses of the organophosphate Dimethoate, i.e. 25% of LC_{50} value and 50% of LC_{50} value. The objective was to detect the effect of the organophosphate insecticide on the biomass, cocoon production and the acetylcholinesterase activity of the earthworm. At the end of the experimental period, it was observed that there was significant reduction in the aforesaid parameters with increasing concentration of the insecticide compared to the control value. T3 dose showed 57.21 ±3.05% reduction of biomass of the specimens. Cocoon production in T3 dose was dropped to 0.35 ±0.02 compared to the control value, 0.92±0.04. The AchE activity was inhibited by 69.2% in case of T3 dose. Thus, we can use the life-history parameters as potential tools and the enzyme activity as biomarker to detect pesticide pollution in agro-ecosystems.

Keywords: Dimethoate, *Perionyx excavatus*, acetylcholinesterase, cocoon-production, garden soil

1. Introduction
Earthworms play a vital role in the maintenance of soil structure, functions, and fertility [1]. Their activities modify soil aeration, drainage, and availability of nutrients for plants and generally integrate soil organic and mineral elements to form aggregates and improve soil structure [2]. It is of utmost importance to understand as how to achieve sustainable agriculture by knowing the impact of various contaminants in the soil as well as of different agricultural practices on soil ecosystems as such [3]. Insecticides entered into our food chain and were detected in milk products, vegetables [4], fish [5], food grains [6], meat, groundwater and even in human blood and breast milk [7-9].The frequent applications of pesticides have been found to exert adverse effects on the soil development and its functioning in an ecosystem [10]. Now-a-days, the chemical compounds such as pesticides and fertilizers are frequently used and its excess use leads to soil, surface and ground water pollution that affect target organisms along with non-target organisms like earthworms. Earthworms are frequently available in a broad range of soil and may deposit 60% - 80% of the total soil biomass [11-12]. Earthworms act as bioindicator species for the ecotoxicological analysis of pesticide soil pollution [13-15]. The indispensable use of agrochemicals has caused serious threats for earthworms' abundance and population; therefore, several earthworm protocols have gained acceptance for use in tests to assess the effects of soil chemicals on soil organisms [11, 16-17] suggested that acute toxicity is insufficient to predict environmentally acceptable concentrations of chemicals as they do not reveal sub-lethal effects of low concentrations on growth, behavior, and reproduction.
Organophosphate (OP) compounds are extensively used as pesticides and industrial chemicals. Organophosphate (OP) pesticide self-poisoning is an important clinical problem in rural regions of the developing world that kills an estimated 200,000 people every year [18]. Organophosphorus compounds, the anticholinesterases, produce significant morbidity and mortality in India [19]. Organophosphate insecticides replaced the organochlorines largely during seventies because they were less persistent in the environment [20]. Although these xenobiotics degrade under natural conditions, their residues have been detected in soils, sediments, and water due to their non-regulated usage practice. Contaminations of organophosphate residues have also been detected in certain agricultural products like tea, sugar, vegetables, and fruits throughout India.

Presence of their residues in blood, milk, honey and tissues of humans and animals revealed their excessive use and bio-accumulating capabilities [21].

According to a report, pesticides have detrimental effects on earthworm at various levels of organisation which involves change in the behaviour, defile metabolism and enzymatic functioning, enhance mortality, diminish fertility, hamper growth and reproduction [22]. In the present study, experiments are carried out to evaluate the toxic effects of the sub-lethal doses of an organophosphate, Dimethoate, on the biomass, cocoon production and acetylcholinesterase inhibition activity of the epigeic earthworm *Perionyx excavatus*.

2. Materials and Methods

2.1 Selection of study site and time

Field experiments were conducted inside the campus of Raja N. L. Khan Women's College, Paschim Medinipur, West Bengal, India (22° 25'N 87° 19' E). The study site is located in the laterite belt.

The experiment was carried out in the month of September, 2016 i.e. post monsoon season.

2.2 Collection and Culture of Test Specimens

The collection of the test specimens, *Perionyx excavatus*, was done from the natural grasslands around Midnapore district, which has not been cultivated or used for any other agricultural purposes and is totally free from pesticide contamination. In the laboratory, large earthen pots/cement vats were used to culture the specimens collected from the field. Finely grinded soil (collected from the same grasslands) and farmyard manure mixed in the ratio of 1:1 was used as the culture medium (Ismail, 1997) [23]. The culture pots were covered with fine meshed iron nets and kept inside Environmental Chamber at 28 ± 0.5 ºC. An approximate level of 60%-70% moisture was maintained by adding distilled water into the medium. Farmyard manure was added as feed every week during the entire period of culture [24-25].

Studies were performed with age synchronized specimens (250–300 mg). Experiments were conducted in small inert polythene boxes (16 X 12 X 1 cm; total area, 192 cm2) containing soil, collected from grasslands, as the test medium. Soil samples were dried, grinded and sieved to get a particle size of 0.25 mm before filling in the experimental boxes. The moisture content of the soil was measured by Infrared Torsion balance moisture meter [Adair Dutt, Kolkata] [26]. Finally the experimental boxes were kept in an Environmental Chamber at a constant temperature of 28 ± 0.5°C and 60-65% relative humidity. The physiochemical parameters of both the soil media, viz, pH and Organic carbon Content were measured and the temperature and moisture content were kept constant (Table 2).

Table 1: Recommended Agricultural Dose of the selected pesticide

Sl. No	Pesticide Group	Chemical Name	Commercial Name	Source of Procurement
1.	Organophosphorus	Dimethoate (30% EC) RAD-0.001mg/kg soil	ROGORIN	Plant Remedies Pvt. Ltd, Hazipur.

Table 2: Physical parameters of the test media

Physiochemical Parameters	Natural Soil
1. Moisture Content	61.2%
2. pH	7.17
3. Organic Carbon	0.86%

2.3 Experimental Procedures

2.3.1 Chronic Toxicity Test

Bioassays were made with age synchronized specimens in the same small inert polythene boxes as described above. Dry (500 g) finely ground soil (0.25 mm particle size) was laid in the experimental boxes. Soil moisture of the test soil was maintained at 50–60% level for *P. excavatus*. Two sub-lethal doses (T2, T3) of each pesticide were applied based on control (T1), 25% (T2) and 50% (T3) of the LC_{50} value of the respective pesticide for *P. excavatus*.

Ten age-synchronized adult specimens were introduced in each experimental box. Before introduction, the worms were rinsed with water, blotted dry on a filter paper and biomass per ten worms was determined. Three replicates for each dose were maintained and control boxes for each dose were maintained simultaneously. Finally the experimental boxes were kept in an Environmental Chamber at a constant temperature of 28 ± 0.5 ºC. Finely ground cow manure (5 g dry weight) moistened to 50% (w/w) was added each week to provide food for the growing worms. Additional food was given when all the food added was consumed. Moisture loss from the test soil was checked by weighing the test containers at weekly intervals and replenished if needed [25].

2.4 Parameters studied

2.4.1 Biomass change

The worms were weighted on the 28th day to determine the change in biomass and were removed from the test boxes following the protocols recommended by [25].

2.4.2 Reproduction

Reproductive success of the test specimens was determined from the rate of cocoon production of the worm. For this purpose the test soil was carefully examined under a magnifying glass and the number of cocoons was counted every week. The cocoons were left undisturbed in the test boxes after removal of the adults till the 56th day and were housed inside Environmental chamber. The temperature and moisture were maintained at 28 ± 0.5 ºC and at 50 – 60% for *P. excavatus*. Numbers of cocoons were counted each week [25].

2.4.3 Biochemical parameters

Adult earthworm specimens were removed from the test boxes on the 28th day of the experiment to determine acetylcholinesterase [27] of the earthworms.

2.5 Statistical methods

The data on biomass change and reproduction were subjected to ANOVA. The data for the pesticide was analyzed for single factor ANOVA followed by Least Significance Difference (LSD) test to test significant variation between treatments at 5% level of probability. The data on enzyme activity were also analyzed for single factor ANOVA followed by Least Significance Difference (LSD) test between the treatments at 5% level of probability. The statistical analysis was done by using SPSS Version 16.0 software.

3. Results

3.1 Biomass Change

Control worms recorded 31.03 ±2.85% increase in biomass whereas a reduction of 54.4 ±2.25% of biomass was observed

when the earthworms were exposed to T2 dose representing 25% of LC_{50} and earthworms exposed to T3 dose of Dimethoate, 50% of LC_{50}, recorded 57.21 ±3.05% reduction in biomass from their initial value. The values of T2 and T3 doses are significantly different (p<0.05) from control value.

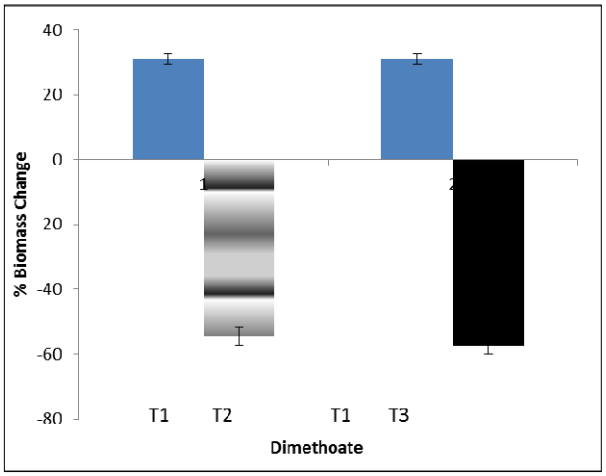

Fig 1: Change in biomass of *P. excavatus* after 28 day exposure to control (T1) and sub-lethal doses (T2 & T3) of Dimethoate.

3.2. Cocoon Production

Cocoon production was 0.92±0.04 in control and was reduced to 0.55 ±0.03 in T2 dose, i.e. 25% of LC_{50} and 0.35 ±0.02 in T3 dose, i.e. 50% of LC_{50}.

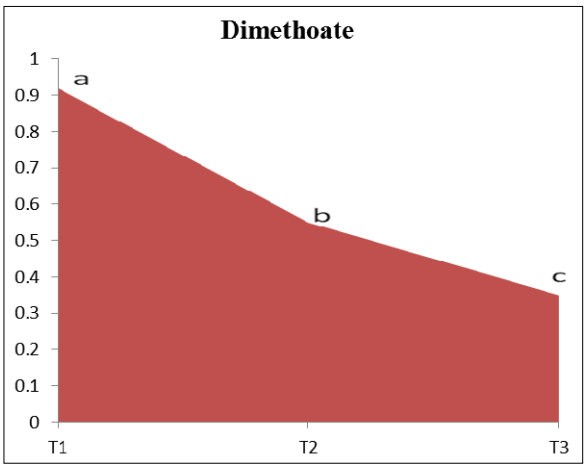

Fig 2: No. of cocoons/worm/week of *P. excavatus* exposed to sublethal doses (T2 & T3) of Dimethoate and the control (T1) with no pesticide. The values of T2 and T3 doses are significantly different (p<0.05) from control value.

3.3. Enzyme activity assay

3.3.1. Acetylcholinesterase

The acetylcholinesterase (AchE) level in control worms was 130.0 ± 2.75 nmoles thiocholine/min/mg of protein whereas the activity was suppressed to 85.0 ±2.55 nmoles thiocholine /min/mg of protein when exposed to T2 dose representing 25% of LC_{50} and earthworms exposed to T3 dose of Dimethoate, 50% of LC_{50}, showed a further suppressed enzyme activity of 40.1 ±1.45 nmoles thiocholine/min/mg of protein (Fig 3). The percentage inhibition of AchE was 34.6% and 69.2% in case of T2 and T3 doses respectively than the control value. The values of T2 and T3 doses are significantly different (p<0.05) from control value.

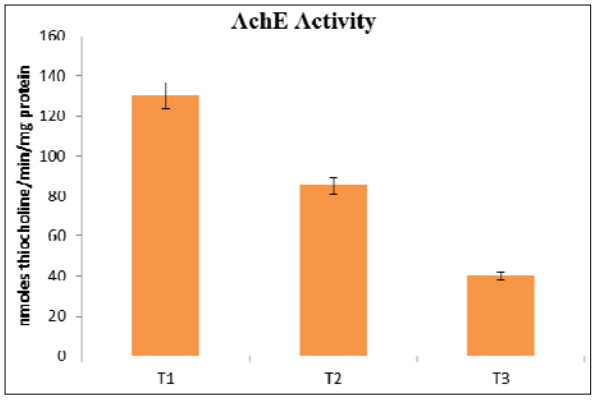

Fig 3: Acetylcholinesterase (AchE) levels of *P. excavatus* exposed to sublethal doses (T2 & T3) of Dimethoate and the control (T1) with no pesticide.

4. Discussion

4.1. Biomass Change

In the present study, *P. excavatus* exposed to sub-lethal doses of the pesticide showed significant alteration in biomass compared to their control values. The epigeic earthworms generally feed on the organic matter in the soil. Since, the soil in the experimental boxes were contaminated with the organophosphate, the released earthworms may have stopped feeding on the soil over time sensing the contamination, which led to their significant reduction in the biomass.

Growth has been reported to be a sensitive parameter to evaluate the toxicity of insecticides on earthworms [28]. The weight loss may indicate feeding inhibition, with the earthworms regulating the intake of insecticides by reducing consumption rate and thus affecting growth rate [29-30]. According to [31] the biomass reduction of the earthworms occurred at lower concentrations of Dimethoate than reduction in survival when parameters like survival and biomass change of the earthworm *Aporrectodeaca liginosa tuberculata* were measured. Reduction in growth of earthworm by sub-lethal doses of insecticides has also been observed in *Eisenia fetida* [32] exposed to dimethoate. [33] observed similar severe effects of chlorpyrifos on growth of earthworm after eight weeks of exposure to 5 mg/kg of the insecticide.

4.2. Cocoon Production

Results of the present study indicated that for *Perionyx excavatus* cocoon production was significantly reduced even at the lowest dose (T2) tested, while increased dose of pesticide led to the severity of reduction of cocoon production. It can be assumed that, since biomass of the earthworms was reduced as a result of the feeding avoidance of the earthworms, it may have affected the cocoon production of the specimens, since considerable amount of energy is spent of reproductive processes with comes from food source.

Application of the organophosphate, malathion declined the reproduction of an earthworm *Drawida willsi*, which is dominant in field crops, following the application of two recommended doses of the pesticide (2.2 mg kg^{-1} – single dose; 4.4 mg kg^{-1} – double dose) as found by [34-35] obtained similar results by exploring the effect of exposure to commercial parathion on the reproductive parameters such as sperm and cocoon production and genotoxicity on male germ cells of *Eisenia fetida* and reported that alterations in reproductive parameters were conspicuous in regard to the number of sperm, cocoons, and worms born. Histological

disorders of spermatheca, alteration of cell proliferation in seminal vesicle and DNA fragmentation in spermatogonia of earthworms as a result of sub-lethal toxicity of organophosphate insecticides resulted in anomalous reproduction in these organisms [36-37, 33] assessed and found chlorpyrifos had adverse effect on fecundity in earthworm exposed to 5mg/kg chlorpyrifos after eight weeks. [38] assessed the effect on fecundity of two organophosphates, chlorpyrifos and diazinon, in the earthworm *Aporrectodea caliginosa* and reported that cocoon production was significantly affected by pesticide exposure ($P<0.05$) and time ($P<0.005$), but there was no evidence for interaction between these factors ($P>0.05$). Earthworms exposed to the high concentration of chlorpyrifos produced fewer cocoons per adult than controls ($P<0.05$) at 4 and 8 weeks during the recovery period, but no other treatments differed from the control ($P>0.05$ for remaining comparisons). There was also some evidence of a dose-related suppression of cocoon production where fewer cocoons were produced at the high concentration of chlorpyrifos compared with the low concentration ($P<0.05$). Furthermore, cocoon production in the resulting adults was reduced by 40% compared with controls, and this effect lasted well into the recovery phase, with full recovery of cocoon production occurring by week 12. It is possible that the significant reduction in cocoon production in the chlorpyrifos-treated earthworms may have been a direct result of the chlorpyrifos-induced lag in maturation. [1] Found the effect of the organophosphate Dichlorovos on the cocoon production of earthworm *Eisenia foetida*. Analysis of cocoon production rate as (number of cocoons/worm/week) in treated worms (*Eisenia foetida)* indicated dose dependent changes. At 19 and 38 mg /kg doses, there were no significant differences compared with the obtained control values. At the 76 mg/kg dose, a decrease in the rate of cocoon production was observed after 7 days of exposure. [35] Observed similar effects on cocoon production, cocoon viability, in *E. foetida* on exposure to commercial parathion at doses of 444, 739, and 1478 mg/kg soil.

4.3. Acetylcholinesterase

The acetylcholinesterase activity of *P. exacavtus* was severely inhibited in both the sub lethal doses, i.e. T2 and T3, of the selected pesticide. The inhibition increased with the increase in concentration of the insecticide. Acetylcholine is a neurotransmitter which is secreted from the neurosecretory cells and it helps in the propagation of nerve impulse through the synaptic cleft of the neuron. When the earthworms are exposed to Dimethoate, it affected the nervous system by inhibiting the breakdown of acetylcholine (ACh). The pesticide binds to the active site of the cholinesterase (ChE) enzyme, which prevents breakdown of ACh in the synaptic cleft. This results in accumulation of ACh in the synaptic cleft causes overstimulation of the neuronal cells, which leads to neurotoxicity of the specimens.

[39] reported that AchE activity was inhibited by 40% in 12h which increased to 79%, 85% and 91% in 24, 36, 72 h when exposed to LC_{50} doses of the organophosphate insecticide Chlorpyrifos in case of *E. fetida*. [40] Studied the toxicity of Azodrine, an organophosphate, on the earthworm *Eisenia foetida.* The neurotoxic potentiality of azodrin was assessed by using a marker enzyme, acetylcholinesterase (AChE; EC 3.1.1.7) in both *in vitro* and *in vivo* experiments. The progressive signs of morphological destruction are correlated with percentage inhibition of AChE in the *in vivo* experiments. The kinetics of AChE activity in the presence and absence of azodrin indicated that the toxicant is competitive in nature. This study demonstrated that azodrin causes concentration-dependent changes in the morphology and AChE activity of the earthworm *E. foetida*. [41] Reported that the amount and activity of acetylcholinesterase was increased when the cotton leaf worm, *Spodoptera littoralis*, larval instars were exposed to chlorpyrifos.

5. Conclusion

It can be concluded from the above experiment that the chronic toxicity studies show that life history parameter like biomass and cocoon production and biochemical parameter like the enzyme, AchE, of the earthworm show significant changes in response to the sublethal doses of the pesticide. Thus, these parameters can be used as potential biomarkers to detect pesticide pollution in agro-ecosystems.

6. Acknowledgement

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