



ORIGINAL ARTICLE | ISSN (O): 2582 – 631X DOI: 10.47857/irjms.2020.v01i04.017

Response of Life History and Enzyme Biomarkers in Oligochaete Earthworm Due to Synthetic Pyrethroid Contamination: An Ecotoxicological Study

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ABSTRACT

Environmental conservation has been a burning topic for the past few decades and conservation and health monitoring of soil and soil biota is a domain that needs to be dealt with which is directly related to agriculture, food and human health. Due to indiscriminate use of chemical fertilizers the soil health and the ecology of soil organisms has been drastically deteriorated. Beneficial non-target organisms are harmed as a result of pesticide application, earthworms being the most common and abundant amongst them. Cypermethrin is one of the commonly used synthetic pyrethroid pesticides in agricultural field. *Perionyx excavatus* is an indigenous species of epigeic earthworm found in abundance in the uncultivated grasslands, around Midnapore district of West Bengal, that are free from direct pesticide contamination and are collected from there. In this study, acute toxicity of selected earthworms was evaluated following the 96-hr LC₅₀ test under laboratory conditions. Similarly, chronic toxicity of preclitellate and adult earthworms *Perionyx excavatus* was evaluated by exposing them to sublethal concentrations i.e. 25% of LC₅₀ (T1) and 50% of LC₅₀ (T2) of cypermethrin (Cypermethrin- T1- 3.0 µg/kg soil, T2- 6.0 µg/kg soil) for 28 days to detect their developmental changes and changes in cellular enzyme activities respectively, under laboratory conditions. Our results showed that cypermethrin could lead to significant reduction of biomass, reduced cocoon production and changes in acid and alkaline phosphatase activity. We found that in case of T2 sublethal dose of cypermethrin cocoon production was reduced to nil. In summary, we found that cypermethrin induced both developmental and enzymatic changes in *Perionyx excavatus*, contributing to a more comprehensive evaluation of the safety of the synthetic pyrethroid. These parameters can also be used as effective tools in detecting pesticide pollution in agro-ecosystems.

Keywords: Cypermethrin, synthetic pyrethroid, alkaline phosphatase, natural soil, acute toxicity.

1. INTRODUCTION

Soil pollution has risen to an alarming level for the last few decades due to excessive agricultural use of pesticides and fertilizers. As a result, soil aeration and its fertility has faced a change further leading to an imbalance between flora and fauna residing in the soil (1, 2). Along

with its flora and fauna, soil is a complex mixture of minerals and organic matter. Therefore, being the main consumers and decomposers of soil ecosystem, the soil quality management depends much on its fauna (3). Earthworms act as excellent bio-indicator helping in the evaluation of the health status of soil ecosystem. It has been indicated by earlier

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(Received 25 August 2020; revised 15 September 2020; accepted 21 September 2020)

reports that pesticide application pose threat to their lives as they are exposed to soil contaminated by pesticides (4). They constitute up to 92% of the invertebrate biomass in the soil and become easily susceptible to pesticides applied in agricultural fields (5). Susceptibility and sensitivity to the soil pollutants in earthworms is much more as compared to other soil inhabitants (6). Therefore, for studying the pesticidal impacts on their stress related biochemical parameters, they are considered most suitable organisms.

Cypermethrin is a broad-spectrum insecticide used to control agricultural pests and have been listed as pesticides of potential concern by the US National Oceanic and Atmospheric Administration (7,8). Synthetic pyrethroids are used to control insect pests in agriculture, homes, communities, hospitals, schools and to treat topical head lice and scabies (9). In urban areas pyrethroids are applied primarily for structural pest control, while in agricultural areas these are applied on row crops and orchards, and these insecticides are also used for various household and veterinary purposes (10,11). Goulart and Callisto, 2003 mentioned that non-target species and other factors are contaminated by the pesticides used in agricultural fields (12). Miller, 2004 reported that the quantum of the sprayed pesticides that

reach to these non-target destinations is as high as 98% (13).

In the present study, synthetic pyrethroid cypermethrin (10% EC) [[Cyano-(3-phenoxyphenyl) methyl]3-(2,2dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate; PubChem CID: 2912]] was used for acute and sub-lethal toxicity in indigenous species of earthworm, *Perionyx excavatus*. The life history and bio chemical markers in this experiment have been studied to evaluate the impact of the pyrethroid on the earthworm. 96hr acute toxicity method [Organization for Economic Cooperation and Development (OECD) guidelines, 207] (14) and 28-day chronic toxicity method (15) were implemented to assess the impact of the pesticide on the earthworm.

2.0 MATERIALS AND METHODS

2.1 Test organisms

In India, there are about little more than 500 species of earthworms (16). Seventeen species belonging to 2 orders, 6 families, 13 genera are found in Midnapore district of West Bengal (17). The epigeic earthworm *Perionyx excavatus* (Figure 1), which was selected as the test organism for this study, is a common species in India and of the Midnapore district.

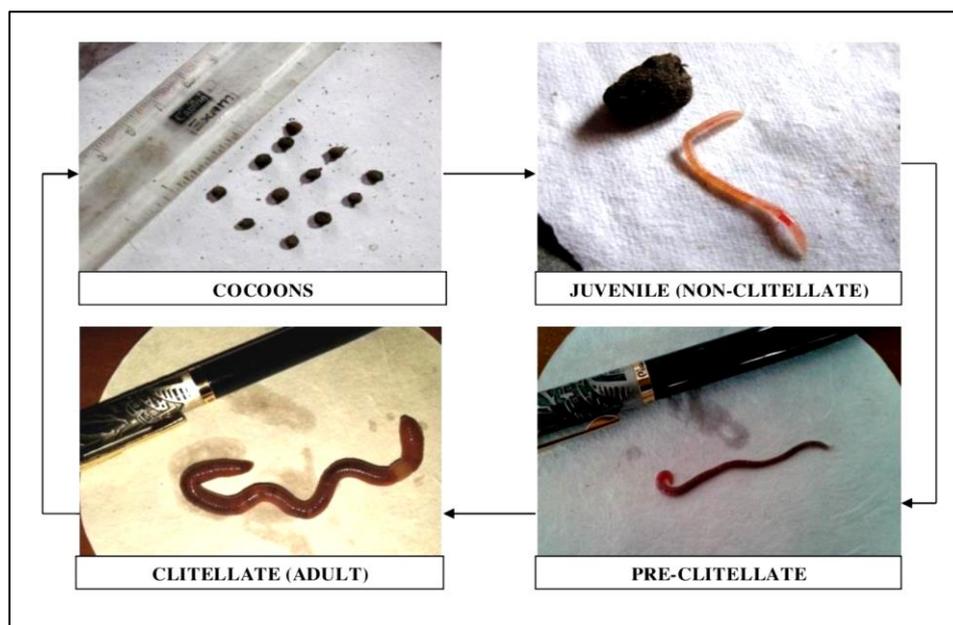


Figure 1: Stages of the life history of *Perionyx excavatus*

2.1.1 Collection, culture and maintenance

The test specimens were collected from the grasslands of Midnapore district (Figure 2) that do not have any history of direct pesticide application. Cool, moist and shady areas were

chosen for collection of specimens. Soil was dug up to a depth of at least 12-16 cm with the help of a hand spade and the specimens were collected in plastic bags with little amount of moist soil and they were brought to the laboratory for identification and culture (18).

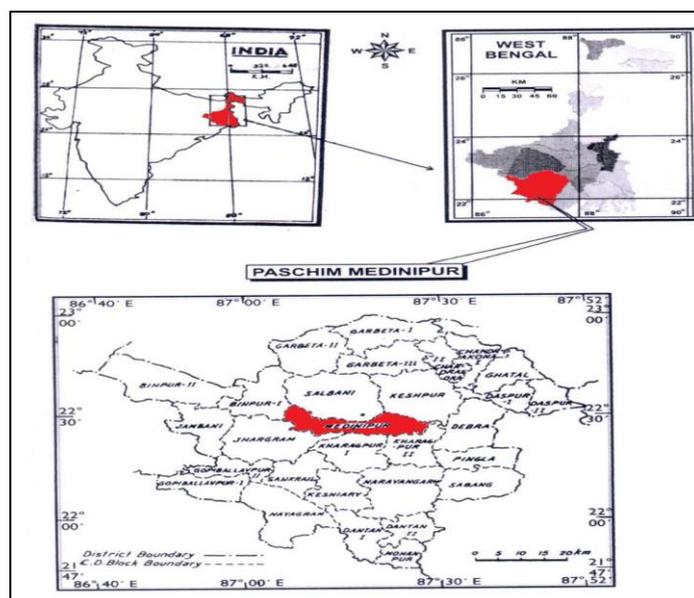


Figure 2: Site of specimen collection

In the laboratory, large cement vats (Figure 3) were used for the culture of earthworms. Soil used for the medium was collected from the same grasslands as of the specimens. A mixture was prepared with finely ground soil and farmyard manure in the ratio 1:1 and was used as the culture medium for the specimens (19). The vats were covered with fine meshed iron nets. An approximate level of 50-60 % moisture was maintained in the medium by adding distilled water. Dried and ground farmyard manure was added as food for the earthworms every week during the entire culture period. Cocoons were then hand sorted and examined, placed in separate experimental pots for use as test specimens in the experiments following

OECD guidelines (14, 15).

2.2 Test Media

Grassland soil was collected from the same grasslands from where the mother earthworm specimens of *Perionyx excavatus* were collected.

2.2.1 Determination of physicochemical parameters of the test media

Soil texture, pH and organic carbon of the test media were determined and were recorded in Table 1.

Table 1: Physicochemical parameters of the soil media used

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

2.3 Pesticide Used

Commercial grade pesticide namely Ustaad (10% EC cypermethrin, United Phosphorus Limited, Gujarat, India), was used for acute as well as chronic toxicological study.

2.4 Experimental procedure

2.4.1 Acute toxicity test

The median lethal concentration of cypermethrin on *Perionyx excavatus* was evaluated following the 96-hr acute toxicity test method according to OECD guidelines (14, 15).

2.4.2 Chronic toxicity test

Separate bioassays were made to determine the effects of the sublethal doses (25% of LC₅₀-T1 and 50% of LC₅₀-T2) of cypermethrin on the biomass, cocoon production and specific activity of cellular enzymes acid phosphatase and alkaline phosphatase under laboratory conditions in natural garden soil. Ten pre-

clitellate (for biomass change and cocoon production) and clitellate (for specific activity of enzymes) earthworms were released inside separate inert (192 cm²) area polyethylene boxes (Figure 4) each containing 500g of sieved garden soil with 60-70% moisture content contaminated with the sublethal doses of cypermethrin. Separate replicates were made for T1 and T2 doses. The experiment was set following the procedure of OECD guidelines (14, 15). The whole set up was kept inside an environmental chamber (Figure 5) and the temperature (28±0.5°C) and relative humidity (67%) was maintained. The evaluation of change in biomass, cocoon production was performed as per OECD guidelines (14, 15), and change in specific activity of acid and alkaline phosphatase was evaluated following the spectrophotometric process of (20) on 28th day from the setting of the experiment. The number of cocoons was monitored at the end of each week for 4 weeks and kept in a separate box.



Figure 3: Culture vat of earthworms



Figure 4: Earthworms in test box



Figure 5: Environmental chamber

2.4.3. Statistical analysis

The lethal concentration value was evaluated using Probit analysis software USEPA version 1.5 and for the evaluation of chronic toxicity results SPSS version 16.0 was used.

3.0 RESULTS

3.1 Acute toxicity test

The 96 hours LC₅₀ values of cypermethrin for *Perionyx excavatus* was 0.012 mg/kg soil in natural garden soil.

3.2 Chronic toxicity test

3.2.1 Life Cycle Toxicity

3.2.1.1 Biomass Change

Control worms showed 71.4 ± 3.67 % increases in biomass whereas a reduction in the

percentage increase of 45 ± 3.00 % of biomass was noted when exposed to T1 dose representing 25% of LC₅₀ and earthworms exposed to T2 dose of cypermethrin (50% of LC₅₀) recorded a further reduced percentage increase, 31.7 ± 2.53 %, in biomass of the test worms from their control value (Figure 6). The values of T1 and T2 doses were significantly different (p<0.05) from control value (N).

3.2.1.2 Cocoon production

Cocoon production was 0.70 ± 0.04 cocoons/worm/week in control (N) and was significantly reduced to 0.15 ± 0.02 (T1) and no cocoon production were observed in T2 (50% of LC₅₀) (Figure 7).

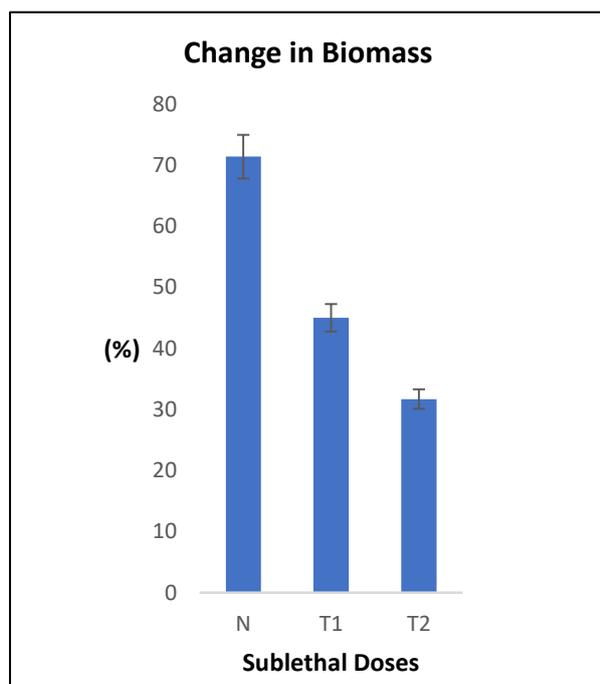


Figure 6: Change in biomass of *P. excavatus* (n=10) after 28 days exposure to sublethal doses (T1 and T2) of cypermethrin. Control (N) has no pesticide.

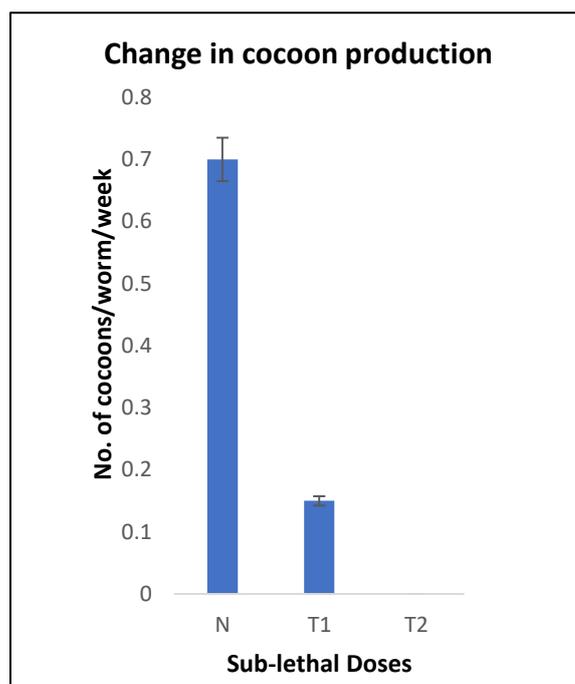


Figure 7: No. of cocoons/worm/week of *P. excavatus* (n=10) exposed to sublethal doses (T1 & T2) of cypermethrin and the control (N) with no pesticide. No cocoons were observed in the T2 replicate of cypermethrin. The values of T1 and T2 doses are significantly different (p<0.05) from control value.

3.2.2 Biochemical Toxicity- Effects on specific activity of enzymes

3.2.2.1 Acid phosphatase

The acid phosphatase value in uncontaminated worms (N) was 5.93 ± 1.75 $\mu\text{gPNP}/\text{mg protein}/30\text{mins}$ whereas the level was suppressed to 5.45 ± 1.80 $\mu\text{gPNP}/\text{mg protein}/30\text{mins}$ when exposed to T1 dose representing 25% of LC_{50} and earthworms exposed to T2 dose of cypermethrin (50% of LC_{50}) showed an elevated enzyme level of 6.57 ± 1.90 $\mu\text{gPNP}/\text{mg protein}/30\text{mins}$ (Figure 8). The reduced and elevated activities of enzymes in the treated replicates were not

significantly different from the control replicate.

3.2.2.2 Alkaline phosphatase

The alkaline phosphatase value in uncontaminated worms (N) was 15.4 ± 2.90 $\mu\text{gPNP}/\text{mg protein}/30\text{mins}$ whereas the level was elevated to 21.8 ± 1.05 $\mu\text{gPNP}/\text{mg protein}/30\text{mins}$ when exposed to T1 dose representing 25% of LC_{50} and earthworms exposed to T2 dose of cypermethrin (50% of LC_{50}) showed a considerably elevated enzyme level of 41.6 ± 1.10 $\mu\text{gPNP}/\text{mg protein}/30\text{mins}$ (Figure 8). The values of T1 and T2 doses were significantly different ($p < 0.05$) from control value (N).

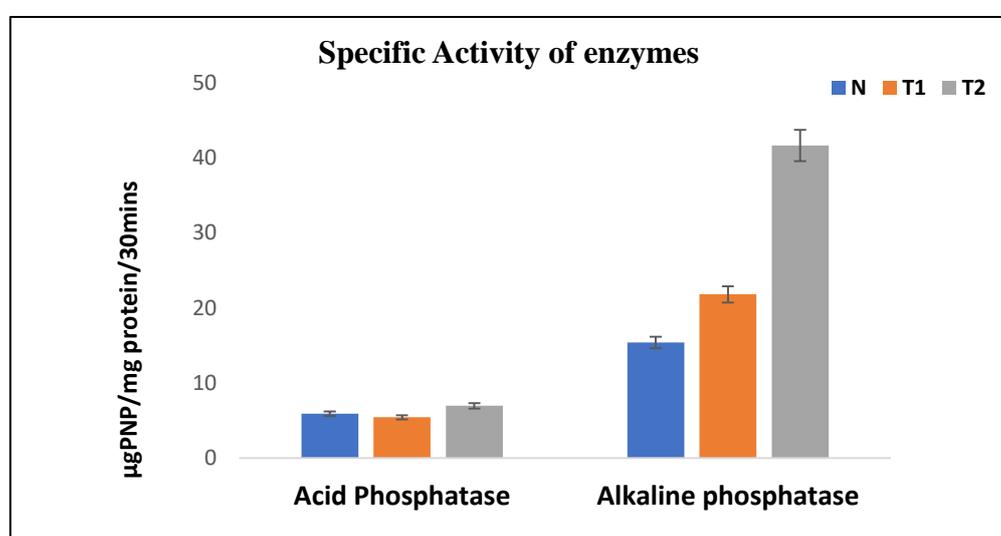


Figure 8: Specific activity of acid and alkaline phosphatase enzymes of *Perionyx excavatus* (n=10) exposed to sub lethal doses (T1 & T2) of cypermethrin and the control (N) with no pesticide.

4.0 DISCUSSION

4.1 Acute toxicity

Based on LC_{50} value of the pyrethroid pesticide cypermethrin was found toxic to *Perionyx excavatus* in the present study.

Mostert et al. (2002), found duration dependent toxicity of the pyrethroid pesticide cyfluthrin on the earthworms of the Pheretima group as 351 mg/kg and 330 mg/kg for 24 hr & 48 hr respectively (21). Das Gupta et al. (2011) reported that Eisenia fetida in spite of being highly susceptible to cypermethrin (LC_{50} - 0.054 mg kg⁻¹), the acute toxicity value was higher than its Recommended Agricultural Dose (RAD) (22). Rosa et al. (2016) evaluated the toxicity of esfenvalerate, a neurotoxic pyrethroid pesticide,

on the aquatic oligochaete Lumbriculus variegatus, the organisms showing visible stress symptoms but LC_{50} value could not be established (23). Cang et al. (2017) carried out 14-day soil toxicity test on the earthworm Eisenia fetida and reported that a relatively less toxicity was found for lambda-cyhalothrin with an LC_{50} value of 560.3 mg a.i. kg⁻¹ dry weight (a.i.- Active ingredient) (24). Dose dependent toxic effects of deltamethrin on mortality of E. fetida have also been reported by (25). Das Gupta et al. (2010) found cypermethrin to be most toxic (LC_{50} - 0.008 mg/kg) than other group of insecticides to the earthworm *Perionyx excavatus* (26). Mosleh et al. (2003) investigated the toxicity of cypermethrin to the earthworm Aporectodea caliginosa and recorded 28 d LC_{50}

value as 72.94 mg/kg (27). Fahreem et al. (2014) found cypermethrin to be most toxic to *Pheretima posthuma*, when compared with other pesticides, LC_{50} 0.14ppm (28). Singh et al. (2019) reported dose dependent toxicity of pyrethroid deltamethrin in earthworm *Eudrilus eugeniae* with 100% mortality (29). Tiwari et al. (2019) have shown similar results of dose dependent toxicity of cypermethrin in earthworm *Eudrilus eugeniae* with 100% mortality. Accordingly, the LC_{50} for the cypermethrin was found to be 0.066 $\mu\text{g}/\text{cm}^2$ (30).

4.2 Chronic toxicity test

4.2.1 Life Cycle Toxicity

4.2.1.1 Biomass Change

In the present study, *P. excavatus* exposed to sub-lethal doses of the pyrethroid pesticide showed significant alteration in biomass compared to its control values. Due to the high rate of soil persistence of cypermethrin or to the rapid degradation of cypermethrin in the earthworms we may conclude that the significant reduction in biomass may happen as a result of subsequent elimination of the metabolites. It could also be assumed that feeding inhibition with the earthworm due to pesticide contamination leading to reduced consumption and therefore ultimately affecting growth.

Zhou et al. (2011) reported that cypermethrin did not cause any significant reduction in the biomass of the earthworm *Eisenia fetida andrei* when exposed to 5mg/kg concentration of the pesticide (31). Mosleh et al. (2003b) investigated the toxicity of cypermethrin to the earthworm *Aporrectodea caliginosa* and observed significant reduction in growth in all insecticide-treated worms during the 4-week test period (27). A dose dependent toxic effect of deltamethrin on growth of *E. fetida* was reported by (25) from sub-chronic exposures with a 42d exposure period. The biomass was significantly reduced with increased pyrethroid concentrations. Rosa et al. (2016) studied a significant decrease in biomass per worm of the aquatic oligochaete *Lumbriculus variegatus*, with a no observed effect concentration (NOEC) value of 2.34 $\mu\text{g}/\text{kg}$ and a lowest observed effect concentrations (LOEC) value of 36.36 $\mu\text{g}/\text{kg}$ when exposed to the neurotoxic pyrethroid insecticide

esfenvalerate (23). Gopi et al. (2014) reported a significant reduction in biomass of *Eisenia fetida*, when exposed to lambda-cyhalothrin. The increase in concentration of the pesticide resulted in increased biomass reduction (32). Dasgupta et al. (2015) exposed *Eisenia fetida* to different sub-lethal doses of cypermethrin (12.5%, 25% and 50% of LC_{50} values) and reported that the worms did not show any significant alteration in change of biomass as compared to control (33).

4.2.1.2 Cocoon production

Results of the present study indicated that for *Perionyx excavatus* cocoon production were significantly reduced in case of cypermethrin at the lowest sublethal dose (T1) tested, while increased sublethal dose of pesticide led to no cocoon production. This may happen as a result of decreased viability of sperms in the spermatheca, alteration in cell proliferation genetic changes in spermatogonia.

Zhou et al. (2008) found that application of cypermethrin on *E. fetida* at rates of 5, 10, 20, 40, 60 mg/kg in OECD soil produced significant reduction in cocoon and juvenile production. Juveniles were more sensitive than the adults (34). Rosa et al. (2016) reported that in the 28-day chronic test on the aquatic oligochaete *Lumbriculus variegatus*, a significant decrease in reproduction was observed with a NOEC value of 0.25 $\mu\text{g}/\text{kg}$ and a LOEC value of 2.34 $\mu\text{g}/\text{kg}$, when exposed to the neurotoxic pyrethroid esfenvalerate (23). Dasgupta et al. (2015) exposed *Eisenia fetida* to different sub-lethal doses of cypermethrin (12.5%, 25% and 50% of LC_{50} values) and reported that cypermethrin did not significantly affect the cocoon production except in the highest dose where the number of cocoons was significantly reduced to 0.47 ± 0.04 cocoons/worm/week than the control value of 1.04 ± 0.05 cocoons/worm/week (33).

4.2.2 Biochemical Toxicity- Effects on specific activity of enzymes

4.2.2.1 Acid phosphatase

In case of Cypermethrin, the acid phosphatase activity was decreased in i.e. T1 (25% of LC_{50} value) but was elevated in T2 (50% of LC_{50} value) compared to the control set i.e. N. Mosleh et al. (2003c) explained the increase in activity of

acid phosphatase in earthworms exposed to lower doses of pesticides. The presence of phosphoric esters may elevate the enzyme activity which compensates the energy requirement of the organism to counter stress situation (35). Ahmed et al. (2004) noticed that cypermethrin treated beetles also showed an increase in acid phosphatase activity (36). Banerjee et al. (2003) found that *Schizodactylus monstrosus* treated with pyrethrum showed significant increased acid phosphatase activity after pyrethrum treatment in both brain and ventral nerve cord with ganglia compared with the controls (37).

4.2.2.2 Alkaline phosphatase

In case of cypermethrin, the alkaline phosphatase activity was significantly elevated in both the sublethal doses, i.e. T1 (25% of LC₅₀ value) and T2 (50% of LC₅₀ value) compared to the control set i.e. N. Ahmed et al. (2004) noticed that cypermethrin treated beetles showed no changes in alkaline phosphatase activity (36). Dasgupta et al. (2015) exposed *Eisenia fetida* to different sub-lethal doses of cypermethrin (12.5%, 25% and 50% of LC₅₀ values) and reported that activities of the enzyme alkaline phosphatase of *Eisenia fetida* were significantly elevated in all the three treatments except in 12.5% of LC₅₀ values where the elevation was statistically insignificant in comparison to control (33). Murphy and Porter, (1966) explained that, the observed rise in alkaline phosphatase activity seen in the present study may be the indication of an adaptive rise in the enzyme activity to persistent stress (38). It has also been reported that the rise in the alkaline phosphatase level may be related to resistance of the organism towards the insecticide and the level of pathological and physiological damage caused to the particular organism [39, 40].

5.0 CONCLUSION

In natural environment, cypermethrin exist in different forms and therefore its impact on the organisms specially earthworms may be significant depending on their chemical nature, structure and mechanism of action imposing a serious threat to the life of these organisms. In the present study, cypermethrin has altered the activity of acid and alkaline phosphatase along

with changes in life cycle parameters. This indicates the toxic potential of cypermethrin for the tested organism. The effects observed were tissue specific and in dose dependent manner. Such changes in pesticide contaminated environment may adversely affect the survival of an eco-friendly non target organism, earthworm. At the same time these changed parameters can be used as an essential tool to detect pesticide pollution in agro-ecosystems.

6.0 ACKNOWLEDGEMENTS

We thank the Principal, Raja N.L. Khan Women's College, Midnapore for providing necessary laboratory facilities and the teaching and non-teaching staffs for their support and thankfully acknowledge University Grants Commission, New Delhi for their financial support.

7.0 CONFLICT OF INTEREST

The authors have no conflict of interest regarding the research content of the article.

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