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Effect of chlorpyrifos and carbofuran on morphology, behavior and acetylcholinesterase activity of earthworm (*Eisenia fetida*)

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ABSTRACT

Organophosphates (OP) and carbamates when applied in agricultural fields are the potential environmental polluters and toxicants for soil flora and fauna. Upon exposure to such chemical pesticides, morphology, behaviour and acetylcholinesterase (AChE) activity of earthworms (*Eisenia fetida*) is altered. The present study dealt with the comparative analysis of toxic effects induced by chlorpyrifos (OP) and carbofuran (carbamate) exposure on morphology, behavior and AChE activity using standard filter paper contact toxicity method. The LC_{50} of chlorpyrifos and carbofuran for 24 hr was 0.25% and 5.13%, respectively, marking chlorpyrifos as more toxic pesticide than carbofuran. The treated worms exhibited abnormal morphological symptoms such as excessive mucus secretion, depigmentation, deformity, loss of metameric segments and damaged clitellum. Variation in behaviour such as reduced activity, sluggish movements and flattened posture marked the stress induced due to pesticide toxicity even at lower doses. Restlessness along with jerky movements was observed on exposure to higher concentrations of chlorpyrifos, whereas lower concentrations resulted in avoidance behavior toward pesticide coated glass vials. Thereafter, worms were exposed to different concentrations of chlorpyrifos (0.13%, 0.25% and 0.38%) and carbofuran (2.57%, 5.13% and 7.70%) for 24h and 48h to assess sub-acute and acute toxicity effects of these pesticides on AChE. The enzyme activity after 48h was 94.14%, 60.21% and 40.44% for the worms exposed to 0.125%, 0.25% and 0.375% chlorpyrifos, respectively. The enzyme activity after 48h was 98.17%, 93.92% and 79.25% for the worms exposed to 2.57%, 5.13% and 7.70% carbofuran, respectively. Time and dose dependent significant ($p<0.05$) decrease in the levels of AChE in pre-clitellar region of earthworms was observed as compared to control when worms were exposed to chlorpyrifos and carbofuran. Alteration in behavioural response of the earthworms may be attributed to the decline in AChE activity of pre-clitellar region. However, chlorpyrifos was more potent inhibitor of AChE activity in *Eisenia fetida* as compared to that of carbofuran. Such alterations indicate the potential health risk of these pesticides' exposure at high concentrations to *E. fetida*.

Key words: Acetylcholinesterase, Biomarker, Carbofuran, Chlorpyrifos, *Eisenia fetida,* Toxicity

Various irreversible/reversible morphological, neurological and neurotoxic alterations have been previously reported in earthworms (*Eisenia fetida*) even at the slightest exposure to pesticides (Gambi *et al*. 2007, Tiwari *et al*. 2019). The impact of pesticide exposure on non-target organisms can be assessed by analyzing the changes caused at physiological, biochemical and molecular levels (Ibtissem *et al*. 2012). Both OPs and carbamates are systemic pesticides that inhibit the acetylcholinesterase enzyme irreversibly and reversibly, respectively (Lionetto *et al*. 2013). As the molecular biomarkers are able to detect the alterations induced in the physiology of an organism due to exposure to toxicant, they can be used as early warnings for assessing environmental risk caused by pollution (Gastaldi *et al*. 2007, Velki and Hackenberger, 2013b, Wang *et al*. 2015). Hence, AChE acts as perfect biomarker for analyzing the presence of pollutants (Calisi *et al*. 2011).

Characterized by rapid biomass gain, fast sexual maturity, extensive reproduction and fast rate of feeding (Sandeep *et al*. 2017), *Eisenia fetida* is the most widely used species for waste management especially in north India (Yadav *et al*. 2017a). Pedestaled upon the facts mentioned above, the present study was planned to assess the impact of chlorpyrifos and carbofuran on the morphology, behavior and acetylcholinesterase activity of earthworm, *E. fetida*.

MATERIALS AND METHODS

Chemicals: The technical grades of chlorpyrifos (20 EC) and carbofuran (3G) were obtained from the Department

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of Entomology, College of Agriculture, CCS Haryana Agricultural University, Hisar. Phosphate buffer solution (PBS) was obtained from Sigma-Aldrich (USA). DTNB (5, 5′- dithiobis-2-nitro benzoic acid; PubChem CID: 6254) and acetylthiocholine iodide (PubChem CID: 74629) were procured from Hi-media® (India) and stored at -20 °C (as per manufacturer's instructions). All the chemicals used for biochemical analysis were of analytical grade.

Earthworms: For achieving the above mentioned objective, the fully clitellated healthy earthworms (with average weight of 500-800 g) were procured from the Vermiculture Unit of Department of Zoology, CCS Haryana Agricultural University, Hisar. Third generation of earthworms was used as test organism to prevent the incidence of pre exposure of pesticides. The worms were then acclimatized on soil type recommended by OECD (which consists of 70% (w/w) sand, 20% (w/w) kaolinite clay and 10% (w/w) sphagnum peat); while 1% (w/w) calcium carbonate was used for setting pH 6.5 (OECD 1984). The earthworm culture was maintained at 20 ± 2°C with normal daylight hours.

Determination of toxicity of insecticides (LC_{50}) against adult E. fetida in vitro: The LC_{50} of insecticides, *viz.* chlorpyrifos and carbofuran was estimated by standard paper contact toxicity test in accordance with OECD guidelines (2000). The standard filter paper test involves the direct exposure of earthworms to the test chemical on moist filter paper to analyze the toxicity of that chemical. The earthworms were cleaned with distilled water and kept on moist filter paper for three hours to ensure the voiding of gut contents. The sides of flat bottomed glass vials (8cm × 3cm) were lined with the medium grade (0.2 mm thick) filter paper of proper size so that the overlapping of sides can be avoided. Different concentrations of chlorpyrifos (0.002%, 0.04%, 0.06%, 0.08%, 0.1%, 0.12%, 0.14%, 0.16%, 0.18% and 0.20%) and carbofuran (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, 6%, 6.5%, 7% and 7.5%) were prepared in water and used fresh. The filter paper coated glass vials were poured with one ml of each concentration and then rotated horizontally to ensure the homogenous distribution of insecticide on filter paper along with the control (having 1 ml deionized water only). After that, one earthworm per vial per worm species was released and glass vials were covered with muslin cloth to avoid the escaping of earthworms. Proper aeration, temperature (20± 2° C) and moisture was ensured during the experiment to minimize the control mortality. Vials were laid horizontally and exposure to light was minimized to increase the efficiency of experiment. After assessing the percent mortality, the corrected mortality was computed by the formula given by Abbott (1925) and the data reported during experiment was subjected to Probit analysis (Finney 1971) as recommended by OECD. Eight replicates for each concentration along with the control were maintained. LC_{50}, one dose higher and one dose lower were used for further determination of AChE activity. The morpho-behavioural alterations during the experimentation were keenly observed.

$$\text{Percent mortality} = \frac{\text{Total number of earthworms died}}{\text{Total number of earthworms released initially}} \times 100$$

$$\text{Corrected morality} = \frac{\text{Morality in treatment-Morality in control}}{\text{100-Morality in Control}} \times 100$$

Preparation of tissue homogenates for AChE enzyme estimation: The worms (n=10) exposed to T1, T2, T3, T4, T5 and T6 (Table 1) for 24h and 48h. Thereafter, they were sacrificed and anterior 10 pre-clitellar segments (that consists of supra-oesophageal ganglion in pair/brain) were processed for enzymatic estimation. The segments were homogenized (10% w/v) in phosphate buffer (0.1M, pH 7.5) using pestle mortar under ice-cold condition. The homogenates were centrifuged at 10000 rpm for 10 min and supernatant was collected. Thereafter, the supernatant collected was again subjected to centrifugation at 10000g for 10 min at 4°C. The supernatant was used for further enzymatic estimation

AChE enzyme assay: AChE enzyme assay was done as per the method described by Ellman *et al*. (1961). 3 ml phosphate buffer (0.1 M, pH 8.0) was added to 10 µl aliquot. To this, 100 µl 5mM DTNB (5, 5′- dithiobis-2-nitro benzoic acid) was added and incubated for 2 min at 37° C. 75 mM acetylthiocholine iodide (50 µl) was used as substrate for the enzyme. The assessment of enzyme activity was done by recording change in absorbance per min for 4 min at 410 nm. One unit of AChE activity was expressed as 1×10^{-6}mole substrate hydrolyzed/min/g under specified experimental conditions.

All the experiments were carried out in triplicates and CRD was used as statistical tool for carrying out research. The data collected were subjected to one way ANOVA at 0.05% was the level of significance to analyze the significant differences among various treatments using OPSTAT software developed at CCS Haryana Agricultural University, Hisar.

RESULTS AND DISCUSSION

LC_{50} values of insecticides for E. fetida: The observations recorded for the bioassays clearly depict the rigorous damage potential of insecticides on earthworms. Dose dependent increase in mortality due to insecticide exposure through skin was recorded during the present paper

Table 1 Description of various treatments given to *E. fetida* during experiment

Treatment	Description
T1	Worms exposed to 0.13% of chlorpyrifos
T2	Worms exposed to 0.25% of chlorpyrifos
T3	Worms exposed to 0.38% of chlorpyrifos
T4	Worms exposed to 2.57% of carbofuran
T5	Worms exposed to 5.13% of carbofuran
T6	Worms exposed to 7.70% of carbofuran

contact toxicity test. Exposure to both the insecticides proved detrimental for worm health. Decrease in survival rate of earthworms with increase in concentrations of insecticides was also recorded. Almost similar results have also been documented by Tiwari *et al*. 2019. However, optimum aeration, temperature and moisture in control resulted in zero mortality but lethargic body movements due to starvation were also observed in control worms. The percent mortality in earthworm, *E. fetida* exposed to chlorpyrifos was found to be 12.5%, 25%, 50% and 100% at 0.08%, 0.14%, 0.18% and 0.8%, respectively. Similarly, The percent mortality in earthworm, *E. fetida* exposed to carbofuran was found to be 12.5%, 25%, 50%, 75% and 100% at 2.5%, 3.5%, 5.5%, 7.5% and 9.5%, respectively. Accordingly, the LC_{50} of chlorpyrifos and carbofuran for 24 hr was 0.25% and 5.13%, respectively (Table 2). On the contrary, Rao *et al*. (2003) reported slightly higher LC_{50} which may be attributed to individual variability (Nusair *et al*. 2017); considering the fact that the exposure conditions were similar. Lower LC_{50} value for chlorpyrifos in comparison to carbofuran marks the greater susceptibility of adult worms towards chlorpyrifos, which is more harmful to worms even at lower concentrations when compared with carbofuran. Reportedly organophosphates are the most toxic insecticides amongst all the agrochemicals (Yuguda *et al*. 2015) which support the findings of present study. However, information regarding comparative toxicity assessments of OPs and carbamates is scanty and needed to explore more.

Morphological observations: Alterations in morphology and behavior, mortality (Schaefer 2003, Ellis *et al*. 2010) and reproduction rate (Calisi *et al*. 2011) may also aid in preliminary detection of toxicity caused due to pesticides' exposure. Clinical symptoms of toxicity like coiling and curling were not observed in control worms. However, the subtle signs of starvation like slightly lethargic body movements were observed in control worms. Progressive symptoms of toxicity were observed with the increased doses of insecticides. Enhanced thick mucus secretion , body lesions, damaged clitellum, depigmentation and loosening of metameric segments were observed in worms exposed to chlorpyrifos. Similar symptoms of toxicity have been earlier reported by Ramsey *et al*. (2011) and Tiwari *et al.* (2019). In addition, beaded appearance of posterior segments marking the toxicity of chlorpyrifos, that further leads to loss of posterior segments was also recorded (0.18% and above), that marks acute toxicity induced by chlorpyrifos. The findings of present investigation on morphological and behavioral changes are in correspondence with the findings of Rao *et al*. (2003). Likewise, dose and duration dependent increase in toxicity levels of carbofuran were observed in worms exposed to it. Worms exposed to carbofuran at higher levels led to intense curling, swollen clitellum, tapered posterior ends with viscous bodily discharge was observed; that points towards carbofuran induced toxicity. It is worth noticing that earthworms treated with chlorpyrifos showed more prominent morphological alterations as compared to carbofuran. Symptoms like coiling and curling due to ridomil exposure, excessive mucus secretion and lethargic activity due to triplen, body upliftment, and coelomic fluid extrusion due to cyren exposure, globular swelling and constriction of segments due to Mamba exposure have previously been reported (Chakravorty and Kaviraj 2010). However, it may also be noted that the impact of pesticides also depends upon the age, species and existing environmental conditions (Uwizeyimana *et al*. 2017).

Table 2 LC_{50} of chlorpyrifos and carbofuran for earthworm, *Eisenia fetida* (identified by well developed clitellum, n=10)

Pesticide	Paper contact toxicity method	
	LC_{50}	$\chi 2$
Chlorpyrifos	0.25%	10.43
Carbofuran	5.13%	0.89
P <0.05		

Note: Control group (Theoretical spontaneous response rate) = 0.000

Behavioral observations: Even the slightest exposure to chlorpyrifos resulted in lethargic and reduced activity. Sluggish movements and supercoiled posterior segments marks the stress induced due to pesticide toxicity. On the contrary, restlessness along with jerky movements was observed on exposure to higher concentrations of chlorpyrifos, whereas lower concentrations resulted in avoidance behavior toward pesticide coated glass vials. Nerve impulses mediated by acetylcholine are responsible for, muscle contraction in worms; the concentration of this neurotransmitter is regulated by acetyl cholinesterase enzyme at nerve synaptic terminals (Yadav *et al*. 2017b). AChE inhibition due to chlorpyrifos and carbofuran disturbed the coordination between nervous tissue and muscular system which resulted in lesser activities and jerky movements (Rao *et al.* 2003). Even after loss of posterior segments on prolonged exposure to higher doses, avoidance behavior was noteworthy. Initial restlessness followed by the lethargic movements at the late hours of experimentation was observed in worms exposed to carbofuran. Reduction in body surface area by extensive coiling may be seen as strategy of worms to escape exposure to pesticides. The present findings can be justified with the observations by Santos *et al*. (2011) and Ferreira *et al*. (2015). Mudiam (2013) stated the decrease in sugar amount in earthworm, *Metaphire posthuma* exposed to carbofuran-treated soil, which also stresses upon the increased energy demands due to toxicity induced stress.

Impact of chlorpyrifos exposure on AChE activity in adult E. fetida: AChE (EC 3.1.1.7) is one of the most widely found cholinesterase that acts as a biomarker for assessment of organophosphate induced toxicity in earthworms. AChE activity is necessary for the functioning of cholinergic synapses present at neuromuscular junctions. It is considered as one of the important biomarker for studying the pesticide induced toxicity especially organophosphates and carbamates (Stepic *et al*. 2013, Nusair *et al*. 2017). For this study, the anterior part of worm was taken into consideration

for enzymatic analysis as the enzyme activity is highest in pre clitellar region (Caselli *et al*. 2006). The presence of ganglionic structures in prostomium of earthworm makes the AChE enzyme assay of pre- clitellar region more reliable (Calisi *et al*. 2011, Tiwari *et al*. 2019). Rault *et al*. (2007) stated the presence of maximum cholinesterase activity in nervous tissue. The mean± S E of acetylcholinesterase activity in worms exposed to 0.13%, 0.25% and 0.38% chlorpyrifos was 52.57± 0.120, 40.00± 4.01 and 35.93± 0.24×10^{-6}mole/min/g, respectively after 24h exposure (Fig 1). After 24h, the enzyme activity was 91.96%, 69.98% and 62.86% of the control in worms exposed to 0.125%, 0.25% and 0.375% chlorpyrifos, respectively. The enzyme activity after 48h was 94.14%, 60.21% and 40.44% for the worms exposed to 0.125%, 0.25% and 0.375% chlorpyrifos, respectively. Time and dose dependent decrease in AChE activity due to chlorpyrifos was observed that can be justified by the findings of Rao and Kavitha (2004). The decrease in enzyme activity points towards the stress induced by toxicant and dysfunction at cellular level. Our results corroborate with the findings of Singh *et al.* (2019) who observed inhibition of AChE due to triazophos exposure in *Eudrilus eugeniae*. AChE enzyme irreversible inhibition up to 97% due to chlorpyrifos exposure in *L. terrestris* has been reported by Hernandez *et al*. (2017). Covalent phosphorylation of serine residues within active site by chlorpyrifos, leads to irreversible inhibition of AChE and thereby accumulation of acetylcholine occurs. Accumulation of acetylcholine leads to hyperactivity, neuronal and muscular system impairment (Yadav *et al*. 2017b). The difference AChE level in control may be due to modifications done by researchers in original method of Ellman *et al*. (1961). Study conducted by Velki and Hackenberger (2013a) also concludes the AChE inhibition due to OP exposure. Inhibition of AChE activity due to sekator herbicide in *L. terrestris* has also been stated by Mekahlia *et al*. (2015). Likewise, reduction in AChE activity due to endosulfan exposure has been reported by Stepic *et al*. (2013).

The mean± S E of acetylcholinesterase activity in worms exposed to 2.57%,5.13% and 7.70% carbofuran was 54.47± 0.24, 51.13± 0.35 and 43.03± 0.2324×10^{-6}mole/min/g, respectively after 24h exposure (Fig 2). After 24h, the enzyme activity was 96.41%, 90.49% and 76.16% of the control in worms exposed to 2.57%, 5.13% and 7.70% carbofuran, respectively. The enzyme activity after 48h was 98.17%, 93.92% and 79.25% for the worms exposed to 2.57%, 5.13% and 7.70% carbofuran, respectively. The decrease in AChE activity due to methiocarb (carbamate) exposure has also been stated by Calisi *et al*. (2009). It is noteworthy that, when treated with chlorpyrifos, the level of enzyme decreased gradually even upto 48h of exposure. While the worms tried to recover AChE activity when exposed to carbofuran for prolonged duration which may be attributed to the reversible inhibition of AChE activity of carbamates. Inhibition in AChE activity can be justified by previous studies and the behavioral changes observed in present study. The inhibition of AChE activity was as expected as the inhibition of AChE activity is the primary mechanism of action of OPs and carbamates (Tiwari *et al*. 2016). The decrease in enzyme activity points towards the stress induced by toxicant and dysfuntion at cellular level. The role of AChE in axonal extension (Bigbee *et al*. 2000), synapsis formation (Sternfeld *et al*. 1998), cell connections (Bigbee and Sharma 2004), hemopoietic stress reactions (Grisaru *et al*. 2006), enzymatic capacity to hydrolyze acetylcholine (Meshorer and Sore 2006) and apoptosis

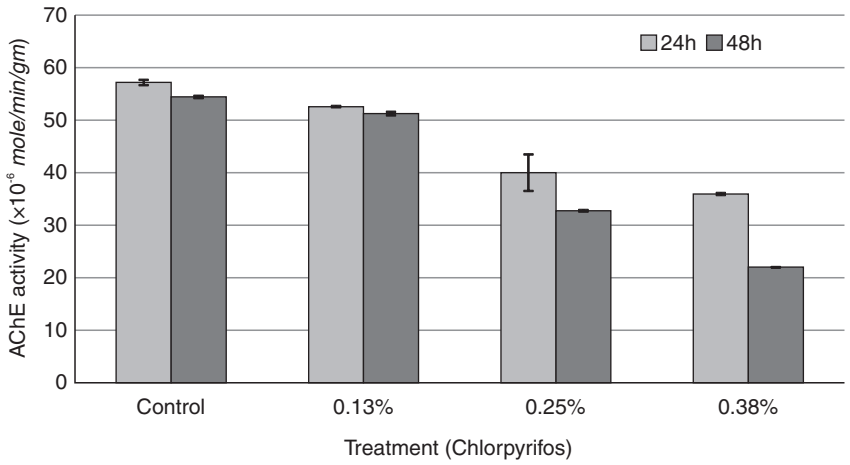

Fig 1 Acetylcholinesterase activity of E. fetida post 24h and 48h of exposure to chlorpyrifos with standard error bars. The figure was produced by using Microsoft Excel 2010.

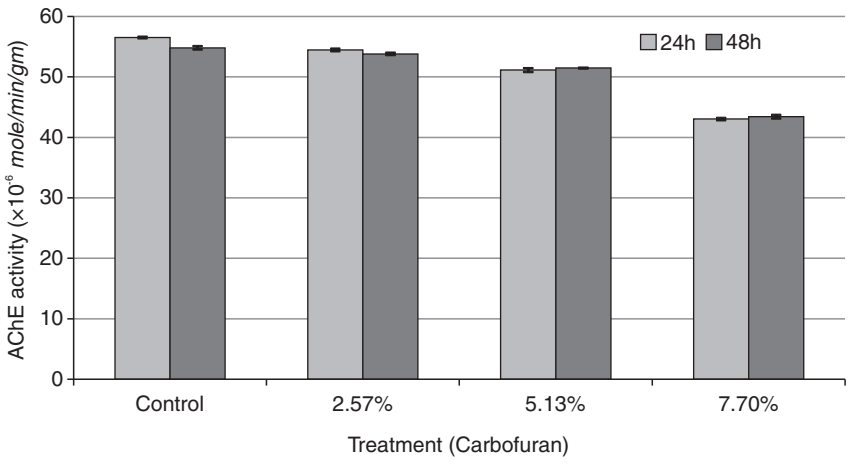

Fig 2 Acetylcholinesterase activity of *E. fetida* post 24h and 48h of exposure to carbofuran with standard error bars. The figure was produced by using Microsoft Excel 2010.

(Soreq and Seidman 2001) has been stated previously. Inhibition in AChE activity can be justified by previous studies and the behavioral changes observed in present study. Chlorpyrifos and carbofuran both are the agonist of post-synaptic acetylcholine receptors. Their toxicity leads to the overstimulation that further lead to impairment and even death. As the molecular biomarkers are able to detect the alterations induced in the physiology of an organism due to exposure to toxicant, they can be used as early warnings for assessing environmental risk caused by pollution (Gastaldi *et al*. 2007). However, it becomes mandatory to include biomarkers for toxicity analysis in ecotoxicological studies (Velki and Hackenberger 2013b).

Conclusion

It can be concluded that 48h of exposure of chlorpyrifos and carbofuran resulted in alterations of morphological characters like thick mucus discharge, bodily deformity, body lesions and loss in metameric segmentation. Behavioral alterations like intense curling and lethargic activity was also observed in worms exposed to pesticides. The higher LC_{50} value of carbofuran as compared to that of chlorpyrifos marks its lower toxicity to the earthworms. In addition, exposure to chlorpyrifos resulted in dose and time dependent AChE inhibition, while carbofuran exposure led to significant inhibition in AChE activity in dose dependent manner levels.

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