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ASSESSMENT OF MORPHOLOGICAL ANALYSIS AND ANTIOXIDANT ALTERATION OF TRIAZOPHOS PESTICIDE ON EARTHWORM *(Eudrilus eugeniae)*

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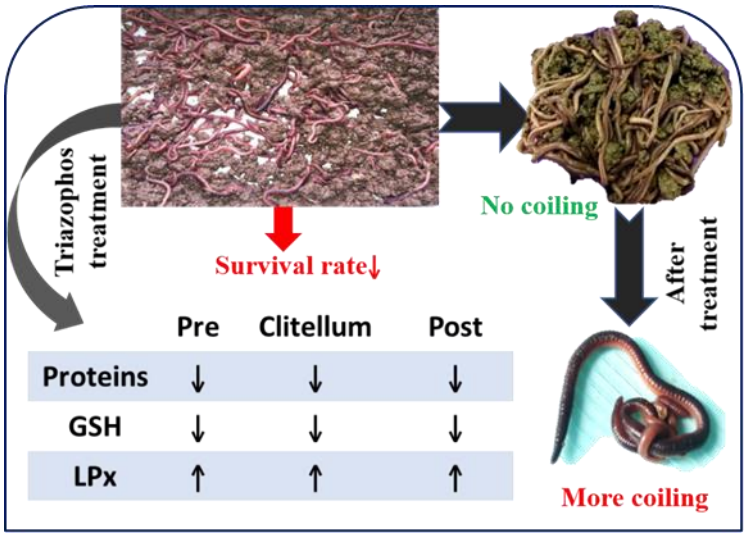
ABSTRACT
The use of pesticides and fertilizers in the agricultural field is increasing tremendously to overcome the food requirement. As it is used in huge quantities, it adversely affects the target and other organisms in terrestrial (soil) and aquatic ecosystems. In this present study, the antagonistic effect of the pesticide Triazophos is compared with the earthworm species called *Eudrilus eugeniae*. *E. eugeniae* are nature's aerator, crusher, composter, and moisture builder of the soil surface. Triazophos is used as an organophosphate insecticide by Indian farmers in crop fields. The study found that the protein quantity, GSH, and LPx content were reduced significantly compared with the non-treated control group by taking tissues of three different regions of earthworm, i.e., pre-clitellum, clitellum, and post-clitellum. The morphological analysis showed that the movement of earthworms was reduced compared to control groups. And a significant reduction in reproduction capability was also observed. These findings indicate that Triazophos causes severe toxic effects even at low doses. Therefore, such antagonistic situations may affect the survival of an eco-friendly non-targeted organism and the earthworm in a pesticide-contaminated environment.

KEYWORDS: *Eudrilus eugeniae*, Triazophos, survival, antioxidant.

Graphical Abstract
Triazophos treatments show more coiling and a lower survival rate than non-treated. A reduction of biochemical parameters such as proteins and GSH was found, and an elevated amount of LPx was observed. All of these definitions show that triazophos is more toxic to animals.



1. INTRODUCTION
Soil is a hybrid combination of biological and inorganic matter, minerals, flora, and fauna. Thus, good soil eminence management is contingent much on animal ecosystem fauna because fauna is the primary consumer and decomposer of the soil ecosystem.[1-3] Over 5 billion predictable pesticides are used in diverse areas such as land management, forests, domestic use, agricultural

lands, disease control, and other regions annually.[4,5] Pesticides are used to control pests such as insects, weeds, and plant diseases. These pesticides may have long-term or short-term toxic effects on non-targeted organisms. Only 2 to 5 percent of the total pesticides are applied to reach the target, and the rest goes to the different biotic and abiotic components. The toxicity of the mixture of pesticides might exhibit stabilizer, synergistic or antagonistic effects due to interaction within them.[6-8] Benzene hexachloride (BHC) and Dichloro diphenyl trichloroethane (DDT) were two pesticides used in India for the first time for controlling malaria and locust.[9, 10] Pesticides come into the soil via spray drift during undergrowth treatment. These purposefully serve as plant protection products, such as protecting plants from fungi, weeds and insects.[11]

Triazophos is an insecticide that has been used extensively in India. Though pest control is done effectively using these insecticides, the world is also facing the adverse effect of synthetic insecticides.[12] Earthworms are present in the soil between the non-targeted organisms and contribute 60-70% of total biomass.[13] Earthworms play an important role in the terrestrial ecosystem. Earthworm helps in various processes like decomposition activity, soil formation, organic substances breakdown, mineralization and nutrient recycling, hence called ecosystem engineers.[14, 15] Therefore, in our study, an earthworm was used to study the various parameter of insecticide toxicity. Earthworm was used to study the non-enzymatic antioxidant system affected by Triazophos insecticide. Various types of biomarkers were developed in earthworms to study the effect of inorganic and organic substances, such as enzymological, behavioral, neurological and histopathological.[16]

Reactive oxygen species (ROS) impede the regular physiological function of the cell in all living organisms. ROS is a destructive molecule in the body. ROS can interact with all the biomolecules, and cells must defend themselves or neutralize the oxygen endogenous metabolic byproduct by mediating antioxidant enzymes, for example, superoxide dismutase (SOD), Catalase (CAT) and glutathione-S-transferase (GST), that act as detoxifying enzyme.[2, 17, 18] However, with the excessive increase of the ROS level inside the body, the cell gets oxidative stress, which ultimately leads to lipid peroxidation increases and causes cellular damage and death of the organism.[19-23] Therefore, the biomarkers mentioned above, such as SOD, CAT, and levels of LPx and GSH, can be studied to evaluate the contaminant's impact on earthworms. The present research aimed to investigate the induction of oxidative stress parameters and genotoxicity by triazophos in earthworms. Thus, hoping for the impact of this research work on a better understanding of agriculture and the environment.

2. MATERIAL AND METHODS

2.1. Chemicals

Triazophos, phosphate buffer, Bovine Serum Albumin, Na_2CO_3, NaOH, Folin & Ciocalteu's phenol, $KNaC_4H_6O_6$, $CuSO_4$, reduced GSH, DTNB, TBA, SDS, BHT, tri-carboxylic acid, ammonium molybdate, ammonium acetate, potassium dichromate, sulphuric acid Milli-Q water. All the chemicals mentioned above were purchased from Himedia Pvt. Ltd India has a molecular and analytical grade.

2.2. Equipments

Spectrophotometer (SystronicVisi scan 167), Remi centrifuge, Systronics pH meter, digital monopan balance (Shimadzu, ELB 300), Elico CL378 Flame Photometer, Systronics Conductivity meter 306, Xiaomi Redmi Note 8-Pro.

2.3. Rearing and treatment

Earthworms (*Eudrilus eugeniae*) were brought from the Human Development Foundation (HDF) Gramin Industrial Training Centre (ITC) horticulture soil, Kuradiha, Mayurbhanj, Odisha. They were taken to the laboratory with mother culture and moist soil having the same age group. Before performing the experiments, earthworms were acclimatized for 20 days in a tub having farmyard and vegetable waste in the soil in a 2:1 ratio (per 1 kg of soil: 250 g of vegetable waste, 250 g of normal soil and 500 g of farmyard manure) with a suitable atmospheric condition, food, proper light, air and humidity. For treatment, three different trays were taken; in each tray, 100 earthworms were placed. Then tray C was used as the control tray, and E1, E2, and E3 as Triazophos treatment trays. The soil in tray EH was spiked with Triazophos as 0.01 g/kg soil (See Table 1). Experiments A, B and C were taken for 24, 48 and 72 hours of earthworms.

Table.1 Experimental set-up of triazophos treatments.

Tray no.	No. of earthworm	Type of dose given	Amount of dose (g/kg soil)	Time interval (hours)
C	100	Nil	Nil	0
E1	100	Triazophos	0.01	24
E2	100	Triazophos	0.01	48
E3	100	Triazophos	0.01	72

2.4. Examination of the proper soil

The soil sample was tested at the local soil testing office, Takatpur of Mayurbhanj district, Odisha. The soil pH level, percentage of oxidizable carbon, phosphorous, potassium content, and electric conductance were examined for quality testing. Briefly, 40 ml of distilled

water was taken in a beaker to which 20 g of dried soil sample was added and stirred for half an hour. Then the beaker was kept at rest to allow sedimentation of the soil. Then the beaker was subjected to the Systronics pH meter in which the reading pH was taken. For oxidizing organic carbon testing, 100 ml of a beaker and 1 g of dried soil sample were taken to which 2 ml of potassium dichromate and 2.5 ml of sulphuric acid were added. Then 44.5 ml of tap water was added to the beaker making the total volume 50 ml. The beaker was kept for 24 hours. After the color solution developed, a reading was taken in a spectrophotometer.

2.5. Inorganic content determination

For the inorganic chemical determination of the soil, 1 g of dried soil sample was taken in a 100 ml beaker, to which 10 ml of Bray's solution was added to check phosphorous (P_2O_5) contents. Then it was kept on a shaker plate for 15 minutes. Then the solution was filtered using filter paper. The 2 ml of filtered was taken to which 2 ml of ammonium molybdate was added. Again, 1 ml of working solution of stannous chloride was added to the solution. Then 5ml of distilled was added, and a reading was taken in a spectrophotometer. To determine potassium (K_2O), 77g of ammonium acetate was taken in a 1litr of distilled water. Then, 5gm of dried soil sample was taken in a beaker to which 25ml of ammonium acetate solution was added. Then it was kept for 5 minutes on a shaker plate. After shaking solution was filtered. Then reading of the filtrate was taken in an Elico CL378 Flame Photometer. For electric conductance determination, 20g of dried soil sample was taken to which 40 ml of distilled water was added. Then the solution was stirred for 30 minutes. After that, the reading was taken in a Systronics Conductivity meter 306.

2.6. Morphological study

Various morphological changes were observed in the triazophos pesticide exposed to earthworms and compared with the non-treatment one. These changes, such as coiling, clitellar swelling and body constriction, were checked. All the observed morphology was captured on the Xiaomi Redmi Note 8-Pro mobile camera with 48 Megapixels (f/1.9) of the real camera.

2.7. Sample preparation

For experiment A, three numbers of *E. eugenia* were taken up from each tray. At first, earthworms were kept in the icebox to restrict their movement. Then, the animals were dissected under digital monopan balance (Shimadzu, ELB 300) by separating their body parts into pre-clitellum, clitellum, and post-clitellum. A 10% homogenate was prepared in ice-cold 50 mM phosphate buffer with pH 7.4 using pre-chilled porcelain mortar and pestle by grinding at 4°C. Then the homogenate was centrifuged at 4000 rpm for 10 minutes at 4°C. Then the supernatant was collected and kept at -20°C freezer for biochemical assays.

2.8. Determination of protein contents

Protein concentration was measured by following the method given by Lowry et al., 1951 using BSA (Bovine Serum Albumin) as a standard.[24] Briefly, 0.1 ml of 10% homogenate, 0.4 ml of Milli-Q water, 5 ml of biuret reagent containing 2% Na_2CO_3 in 0.1N NaOH, 1% sodium potassium tartrate, and 0.5% copper sulphate pentahydrate solution in 100:2:2 ratio and vortex well. Incubate for 10 minutes, then 0.5 ml of Folin & Ciocalteu's phenol reagent was added, vortex well, and kept for 30 minutes in the dark at room temperature. Then absorbance was taken at 700 nm by spectrophotometer (SystronicVisi scan 167). Protein content was expressed in the tissue's mg/g wet weight from the calculated BSA standard curve.

2.9. Reduced glutathione assay

The reduced glutathione assay was performed from Ellman's reagent (5,5'-Dithiobis(2-nitrobenzoic acid), DTNB) given by Ellman et al. 1961 by taking glutathione as standard.[25] Briefly, 0.8 ml of supernatant was precipitated in ice-cold tri-carboxylic acid (TCA, 10%) and centrifuged at 4500 rpm for 10 minutes at 4°C, and the supernatant was used to determine the GSH content. 0.5 ml of supernatant was mixed with 2.5 ml of DTNB, and the samples were incubated at room temperature. Absorbance was taken at 412 nm in a spectrophotometer. The GSH content was expressed as mg/g tissue.

2.10. Lipid peroxidation assay

The Lipid peroxidation assay was followed by Ohkawa et al., 1979 TBA-RS (Thiobarbituric acid reacting substance) method.[26] Briefly, 1.9 ml of Thiobarbituric acid (TBA) reagent containing 8.1% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) acetic acid pH 3.5, 0.5 ml of 0.8% (w/v) aqueous solution of Butylatedhydrotoluene (BHT), TBA and Milli-Q water was added to 0.1 ml of supernatant and mixed carefully. All the test substances were boiled in a water bath for 45 minutes. Then the test tubes were cooled and centrifuged at 2000 rpm for 10 minutes at room temperature. The supernatant was measured at 532 nm in a spectrophotometer.

2.11. Statistical analysis

All the above-used experimental data were performed at least thrice. The MS-Excel program calculated the arithmetic mean±standard deviation from the mean and standard deviation. One-way ANOVA and post hoc analysis were conducted to determine the significance level between-group data. A difference was taken as significant when *P* was less than or equal to 0.05. All the statistical data were done with the help of the software Statistical Package for Social Sciences (SPSS) version 2.5 and GraphPad Prism 5.0 software.

3. RESULTS AND DISCUSSIONS

3.1. Morphological study

The morphological changes showed more coiling and constriction of the body were observed in triazophos treatment groups. It was also observed that the clitellar region was more swollen than the other body parts (Fig. 1). Hence, it was confirmed that the triazophos is toxic to the earthworm. Therefore, after seeing these morphological changes, we are more curious to check the oxidative stress parameters. We also performed earthworm's protein, GSH and LPx content with different treatment of time points. This study shows many changes in earthworms' morphological, behavioral and reproduction when exposed to the insecticide triazophos. The growth rate has declined drastically, reproduction has become slow, and the swelling of the reproductive organ clitellum has been seen (Fig. 1). Biochemical parameters such as GSH, LPx and protein content have been changed in triazophos treatment. The growth and reproduction of earthworms have been important endpoints used in environmental eco-toxicity. It was confirmed that juvenile earthworms may be more sensitive to pollutants and chemicals than adults.[27] Earthworms are affected by toxicants through skin contact or feeding in contaminated soil. The toxicants reach the coelomic fluid through the skin and are transported throughout the body.

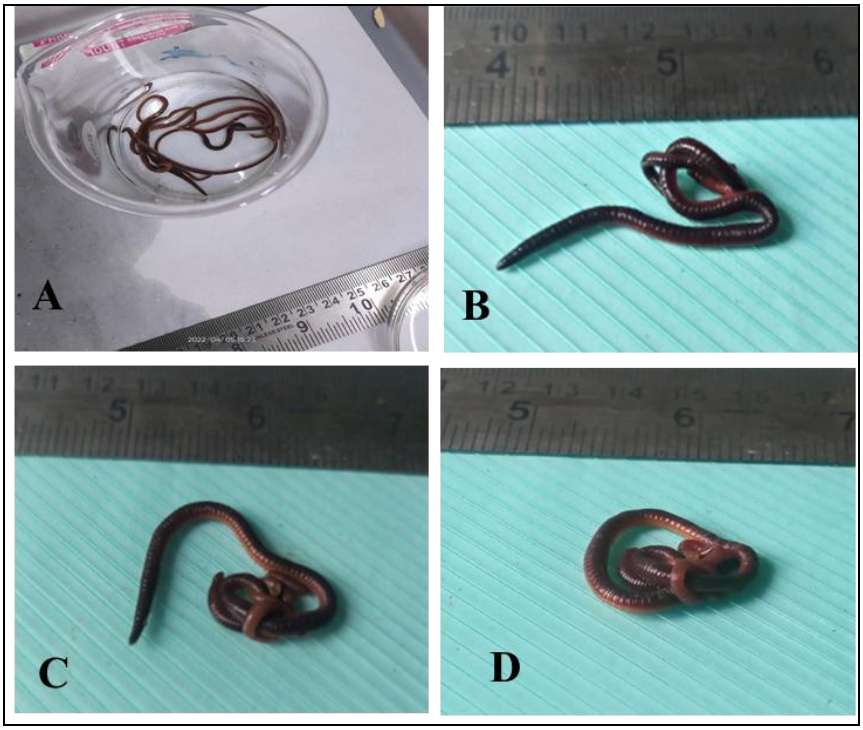


Fig. 1. Morphological observation in earthworm *E. eugeniae* exposed to different concentrations of triazophos, A) Not treated with triazophos (Control), B) Triazophos treated with 24 hours, no coiling observed, C) 48 hours of triazophos treatment at 48 hours, seen less coiling, D) more coiling was observed at 72 hours of triazophos treatment.

3.2. Examination of the proper soil

The soil was tested in a Takhatpur, Baripada, Mayurbhanj testing center. The 1 kg of soil sample was found to be 85-90% moisturization, sand 35-55%, silt 21-45%, and clay 15-25%, respectively. The pH of the soil was measured to be 6.03-8.21, OC from 1.25-8.56%, N from 0.20-3.2 g/kg, K from 0.8-8.56 g/kg, Ca from 1.58-80.76 g/kg, Na from 0.68-1.63 g/kg, EC from 56.3-630.42 µS, and TDS from 58.2-153.5 mg/L. These parameters were suitable for the growth and development of earthworms in suitable soil conditions.

3.3. Determination of protein contents

From protein assay, protein content from the different body parts was reduced in triazophos treatment groups compared to the control. It was found that less protein content was observed in the meantime of treatment, i.e., 48 hours of treatment. But, at 48 hours of triazophos treatment groups, a little more amount of protein content was observed as compared with the 24 and 72-treatment-hour groups in the pre-and post-clitellum region (Fig. 2). Individually, in the pre-clitellum region, at 24 and 48 hours of treatment reduced protein content as compared with the control and a very less amount of protein content at 72 hours of treatment (Fig. 2 (A)). In the clitellum region, a small protein concentration decreased in 24 hours compared to the control and decreased in 48 hours of treatment. But high protein content is reduced within 72 hours of treatment (Fig. 2 (B)). In the post-clitellum region, all the treatment group's protein content decreased compared to the control groups (Fig. 2 (C)).

The above outcomes showed that the reduction of protein content showed that the triazophos had altered the

biomolecule content and were toxic to the animals. The protein content of the pre-clitellar region of earthworm gets slightly decreased at all the treatment hours. However, a fall to a more amount was observed at 72 hours of exposure at all regions of the earthworm. It may be due to the triazophos' oxidative stress, which reduced the protein content at long exposure.

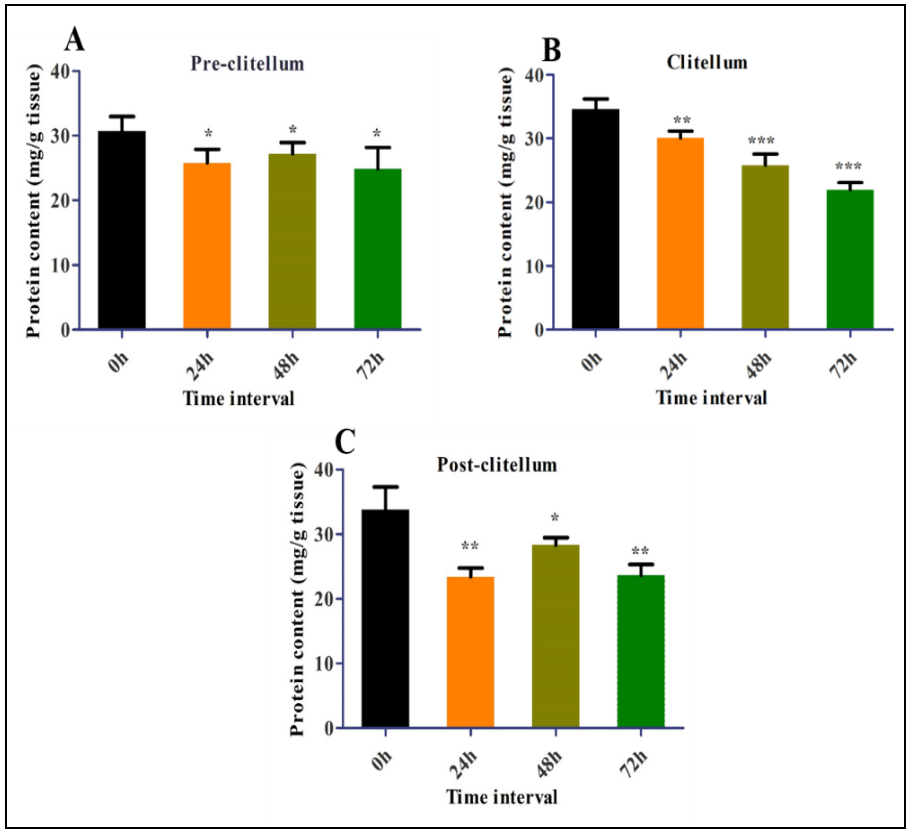


Fig. 2. Comparison of protein content (mg/g tissue) of *E. eugeniae* treated with Triazophos, A) pre-clitellar region, B) clitellar region, C) post-clitellar region *(ns: non-significant, *P <0.05, **P<0.01, and ***P<0.001)*.

3.4. Reduced glutathione assay

From the GSH assay, GSH content from the different body parts was found to be downregulated as well as at some point, it was elevated in triazophos treatment groups compared to the control. It was found that at the meantime of treatment, i.e., 48 hours of treatment, little elevated amount of GSH content was observed as compared with the other treatment hour groups (Fig. 3). In the pre-clitellum region, 24 and 72 hours of treatment reduction of GSH content was observed as compared with the control and a very less amount of GSH level at 72 hours of treatment (Fig. 3 (A)). In the clitellum region, a small amount of GSH level was decreased in 48 hours compared to control and decreased in 24 and 48 hours of treatment. But high amount of GSH content is reduced within 72 hours of treatment (Fig. 3 (B)). In the post-clitellum region, all the treatment group's GSH content decreased compared to the control groups, and a high amount of GSH level reduction was observed within 72 hours of treatment (Fig. 3 (C)). The above results showed that the reduction of GSH level showed the triazophos had altered the GSH level and is toxic to the animals.

GSH was performed to know the toxicity effect on earthworm body ions exposed to triazophos and agreed with Sigh et al., 2019. Sigh et al., 2019 it was observed that the application of a sub-lethal dose of combined chemical substances (Triazophos and Deltamethrin) has a noticeable decrease in the pre-clitellar region (67%), clitellar region (52%) and post-clitellar region (67%) concerning the control on 48 hours of exposer.[16] It was also reported that when *E. eugeniae* was treated with triazophos, insecticides showed various ups and down-regulations of the antioxidant system. The maximum decline was observed for pesticide triazophos in post-clitellar, pre- clitellar and clitellar regions of earthworms exposed for 72 hours in a concentration-dependent manner. A maximum decrease in the GSH level was recorded in 48 and 72 hours of the post-clitellar region exposed group followed in comparison to the control. Our result also corroborates the results reported by Valavanidis et al. and Marcano et al., researchers.[28, 29]

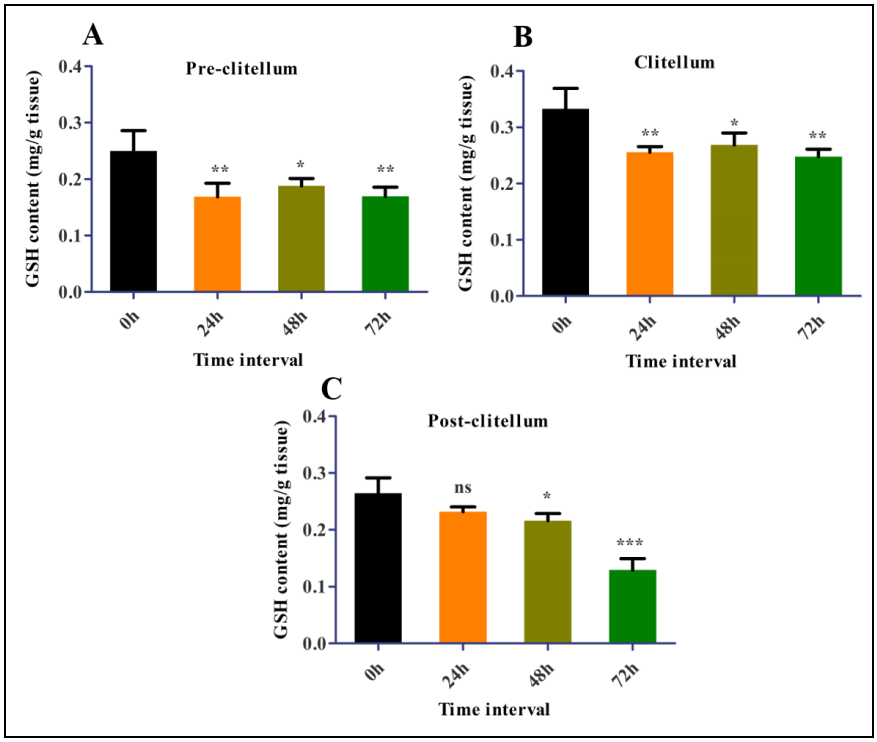


Fig. 3. Comparison of GSH content (mg/g tissue) of *E. eugeniae* treated with Triazophos at a different time interval, A) pre-clitellar region, B) clitellar region, C) post-clitellar region *(ns: non-significant, *P <0.05, **P<0.01, and ***P<0.001)*.

3.5. Lipid peroxidation (LPx) assay

From the LPx assay, LPx content from the different body parts was higher in triazophos treatment groups than in control in all regions. In the meantime of treatment, i.e., 48 hours of treatment, a high amount of LPx content was observed. But, at 24 hours of triazophos treatment groups, less amount of LPx content was observed as compared with the other treatment hour groups (Fig. 4). Individually, in the pre-clitellum region, 24 hours of treatment non-significant amount of LPx content as compared to with the control and more elevated amount of LPx at 48 and 72 hours of treatment (Fig. 4 (A)). In the clitellum region, a heavy amount of LPx content was increased in all hours compared to the control. But high amount of LPx content is less in 24 hours of treatment than 48 and 72 hours (Fig. 4 (B)). In the post-clitellum region, all the treatment groups LPx content significantly increased compared to the other groups (Fig. 4 (C)).

When the earthworm was treated with triazophos, the LPx test was done to check the LPx content. It was noticed that there were changes in LPx content in treatments. In 48 and 72 hours of treatment, a high amount of LPx was elevated significantly as compared to other treatments and control (Fig. 4). The results reflect the connection of organ/tissue in the detoxification of free radicals overproduced via activation of antioxidant enzymes in pesticide exposed earthworms [2]. It may be mainly due to the toxic triazophos depleting the body's GSH level and protein content. Thus, the GSH maintains the internal hydrogen peroxide as it works like catalase. But, when treated with triazophos, it reduces the selenocysteine-containing lipid membranes peroxides.[30] When the GSH level is depleted, the lipid membrane breaks down, leading to membrane damage.[15, 31, 32] Alterations of lipid membranes may further result in damage to cellular-lipid membranes. Thus, LPx content or MDA formation can indicate pesticide pollution in earthworms, as earlier research reports corroborate.[33, 34] The biomarkers, such as biochemical, molecular, and stress parameters, may serve as an alarming signal for soil pollution and stress conditions of organisms living in the soil, such as earthworms.[35]

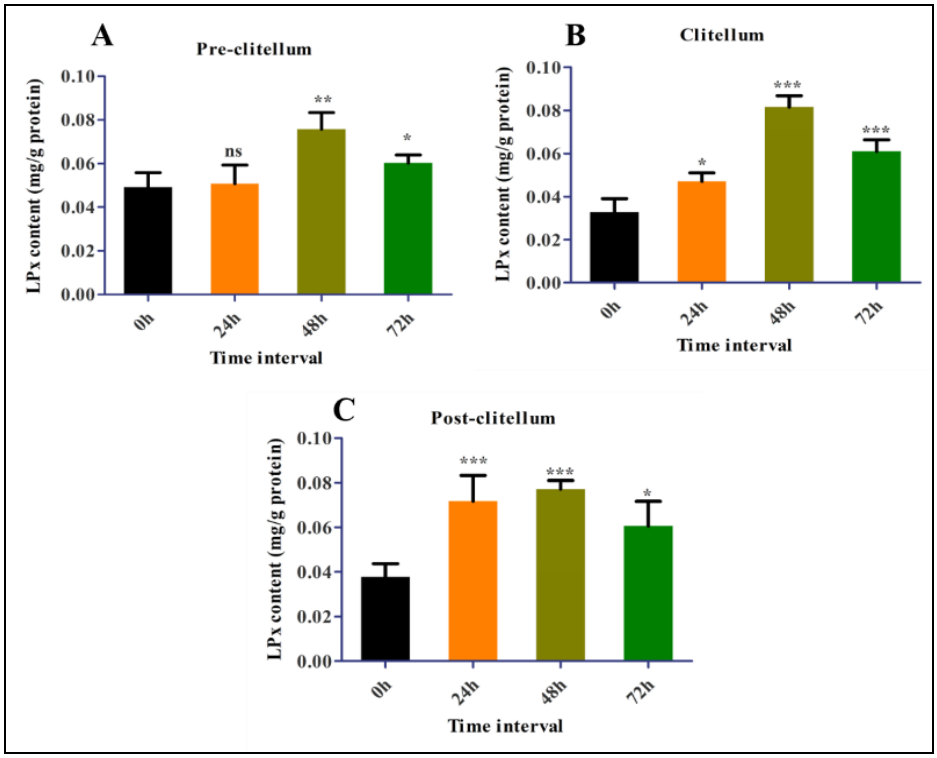


Fig.4. Comparison of LPx content (mg/g tissue) of *E. eugeniae* treated with Triazophos at different time intervals in, A) pre-clitellar region, B) clitellar region, C) post-clitellar region *(ns: non-significant, *P <0.05, **P<0.01, and ***P<0.001)*.

4. CONCLUSION

Farmers use Triazophos, a very common and widely used chemical, and earthworms (antioxidant systems) get exposed to this chemical. These alterations indicate that the chemicals used adversely affect the soil ecosystem. Instead of using these chemicals, natural substances like vermicast can improve crop production and natural substances can be used for pests. In the present study, the triazophos insecticide has altered the activities of oxidative stress-related enzymes, LPx and GSH content, protein concentration, and morphological changes. This variation indicates that the toxic potential of these pesticides for the tested organism is greater when present in a mixture and an individual. Thus, a pesticide-contaminated environment may adversely affect the survival of an eco-friendly non-target organism and the earthworm under such circumstances.

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Conflict of interest

The authors report there are no competing interests to declare.

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