EGYPTIAN ACADEMIC JOURNAL OF

BIOLOGICAL SCIENCES
ZOOLOGY

B

ISSN
2090-0759

WWW.EAJBS.EG.NET

Vol. 15 No. 2 (2023)

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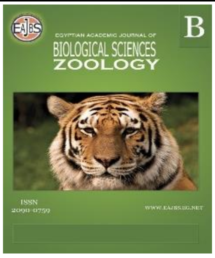
www.eajbs.eg.net

Citation: *Egypt. Acad. J. Biolog. Sci. (B. Zoology) Vol. 15(2) pp:53-64 (2023)*
DOI: 10.21608/EAJBSZ.2023.320322

Egypt. Acad. J. Biolog. Sci., 15(2):53-64(2023)

Egyptian Academic Journal of Biological Sciences
B. Zoology
ISSN: 2090 – 0759
http://eajbsz.journals.ekb.eg/



Assessment of Morphological and Histological Damages in Earthworms (*Aporrectodea caliginosa*) Exposed to Organophosphate Insecticide

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ARTICLE INFO
Article History
Received:29/8/2023
Accepted:7/10/2023
Available:11/10/2023

Keywords:
Organophosphate pesticide, earthworms, *Aporrectodea caliginosa*, Skin toxicity.

ABSTRACT

Earthworms have great environmental importance; they occupy a great place among other soil invertebrates. Further, it contributes greatly to soil fertility and is a main component of the soil fauna. Various chemical pesticides are used that have multiple effects on plants in the soil ecosystem. Earthworms represent a strong bioindicator for the environmental toxicity analysis of soil pollution caused by pesticides. Chlorpyrifos is used in more than 100 countries and is applied annually to approximately more than a million acres of crops. The most beneficial crops include cotton, almonds, corn, fruit trees, oranges, bananas, and apples. In the present work, it was noticed that groups exposed to chlorpyrifos, an organophosphate pesticide, at doses of 0.05, 0.1, and 0.2 µg /cm² when compared with normal earthworms, it significantly affects the behavior of the worms, as it causes many distortions in the external appearance and also affects the stages of the earthworm life cycle, which lead to cocoon, juvenile, and adult death. When examining the histological sections, much damage to the tissues was observed. Even the pesticide led to much mortality among earthworms.

INTRODUCTION

The earthworm is the main individual of the soil fauna and is often responsible for the majority of the biomass of the soil fauna (Fragoso *et al.*, 1999). The earthworm is present in all environments with the exception of Antarctica, but their biomass and abundance vary from site to site due to climate change (Brown & Domnguez 2010; Decans 2010). Only roughly 5,738 of the more than six thousand earthworm species have been described globally (Csuzdi 2012, Misirliolu *et al.*, 2023). Earthworms are important for the health of the soil and consequently for the provision of vital services to humanity. Their advantageous role relates to effects on soil microbial community, carbon and nutrient cycle, and soil structure. They are well known to be incredibly advantageous for soil health and the sustainability of ecosystems (Céline, *et al.*, 2023). Therefore, improving the role of earthworms in agriculture is critical to maintaining or improving soil quality and supporting more sustainable circular agriculture. Here, we provide new insights from earlier decades and review the body of information regarding the function of earthworms in agronomy (Vidal *et al.,* 2023). It can be injected into deteriorated soils to benefit plant growth and soil structure. Additionally, beneficial mycorrhizal fungi can be transported by earthworms into the soil, improving plant nutrition and primary production (Abd El-Aziz & Bashandy, 2019). Coelomocytes, or cells found in the fluids in the worm coelomic cavity, make up the immune system of earthworms (Stein *et al.,* 1997). These cells resemble macrophages, which are immune cells present in vertebrate immune systems (Humphreys and Reinherz, 1994). Eleocytes, free chloragogen cells with nutritive and ancillary functions, and either hyaline or granular amoebocytes, all of which represent effector immunocytes participating in a wide range of defense tasks, including phagocytosis, are the three primary forms of coelomocytes (Engelmann *et al.,* 2002). Galloway and Hany (2003) mentioned that the immune system of earthworms is affected by organophosphate pesticides in a way that lowers immunocyte viability, overall immunological activity, phagocytic activity, and erythrocyte production. Additionally, morphometric coelomocyte and granulocyte changes were observed (Calisi *et al.,* 2011).

Despite the fact that earthworm is extremely important to soil and the ecosystem, widespread pesticide pollution of the environment has grown to be a serious threat to soils for non-target animals like earthworms, whose numbers have been shown to be dropping. However, certain indoor species continue to be common and survive in fields that are heavily farmed, showing that they can eventually tolerate anthropogenic pressure (Audrey *et al.,* 2023). Pesticides are widely produced worldwide and used to manage agricultural pests that threaten the health of both humans and animals, including those that attack crops and vegetables. Synthetic insecticides like organophosphates (OP), pyrethroids, and many others are used in manufacturing, and those who apply them in the field and the general population are exposed to pesticides as a result (Wang *et al.,* 2011). Organophosphate pesticides produce adverse effects on the nervous system by inhibiting the encholinesterase acetylcholinesterase enabsisan at neuromuscular junctions (Wang 2002; Ballesterosetal.2009). Toxic effects of organophosphate pesticides include genotoxicity, liver failure, embryotoxicity, teratogenicity, neurochemical alterations, immune system and neurobehavioral alterations (Goel *et al.,* 2000). The excessive use of pesticides and fertilizers in agriculture has polluted the soil at an alarming rate in recent decades. This leads to changes in the aeration capacity and fertility of the soil leading to an imbalance between the flora and fauna residing in the soil (Ali & Naaz, 2013). Soil is a complex mixture of minerals, organic matter and animals. Therefore, the management of soil quality is highly dependent on the fauna, which are the main consumers and decomposers of the soil ecosystem (Handrix, 2000). Earthworms are an excellent biomarker for assessing the health status of soil ecosystems. Previous reports indicate that pesticide use threatens their lives when they come into contact with pesticide-contaminated soil (Jadhav and David, 2017). They are more fragile and sensitive to soil pollutants than other land dwellers. Therefore, earthworms are the most suitable organism to study the effects of pesticides on stress-related biochemical parameters (Chen *et al.,* 2018). Chlorpyrifos (an organophosphate; OP) is a broad-spectrum insecticide used to control agricultural pests and has been listed as a pesticide by the US National Oceanic and Atmospheric Administration. has the potential to cause concern (Tiwari *et al.,* 2017). A wide range of irreversible/irreversible behavioral and neurotoxic effects as well as physiological and morphological changes have been reported in earthworms, even at very low pesticide concentrations (Gambi *et al.,* 2007).

The aim of this study was to assess the impact of chlorpyrifos (an example of an animal insecticide) on earthworms (*Aporrectodea caliginosa*) (Fig. 1). It was chosen for this study for many reasons: it is very sensitive to any chemicals (Duque *et al.,* 2023), it is cheap, natural fertilizer for the soil, it is widespread in most environments, and it is easy to obtain. Availability of food, which contains a high percentage of protein, and availability of natural medical treatments Its life cycle is short (Pelosi *et al.*, 2014). It also represents a strong model for human skin structure because it is similar to the composition of human skin (Abd Ellah *et al.*, 2019; Abd El-Aziz & Ali, 2021, Abd El-Aziz *et al.*, 2022 a & b; Salem *et al.*, 2022). They are not intermediate hosts for parasites that infect humans, nor are they nuisance pests (Cooper et al. 2012). Earthworms were used in many studies as preclinical in place of higher laboratory animal models because they are simple, inexpensive, and responsive animal models (Misra *et al.*, 2005).

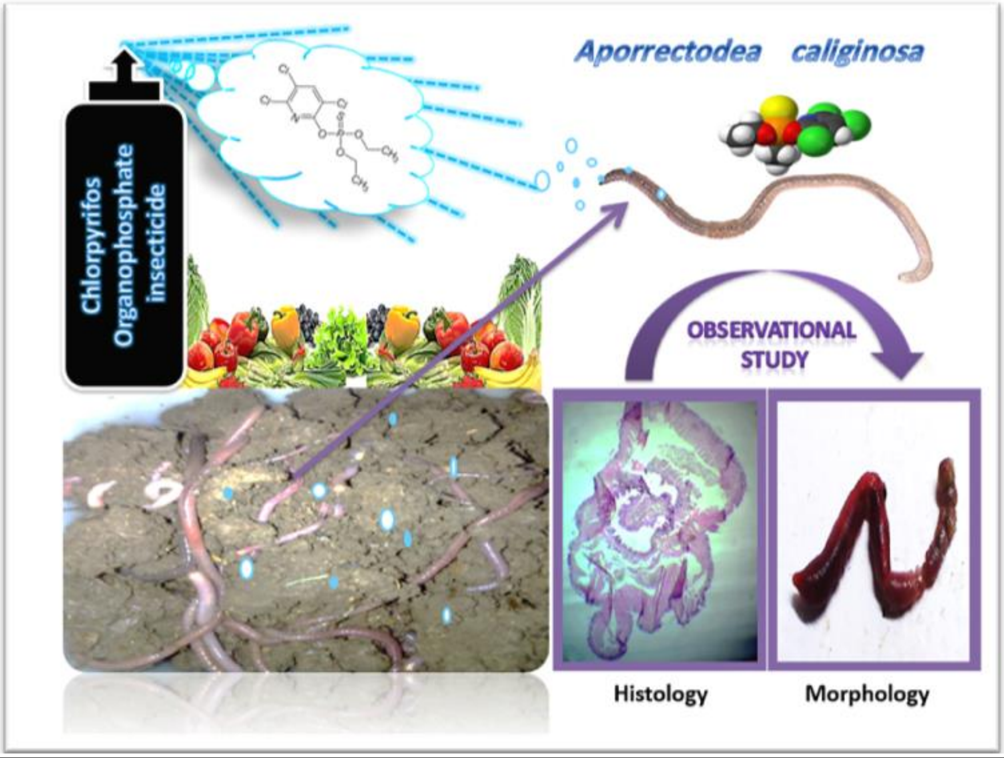

Fig. 1: Diagram showing the Observational study.

MATERIALS AND METHODS

Earthworm's Collection:

Earthworms were collected from the farm of Assiut University and then transmitted to the laboratory of the Department of Zoology, Faculty of Science, Assiut University, where the worms were separated after sorting and isolating the required type after classification. It has dry leaves for feeding. Worms were left in laboratory conditions with 12 hours of light and 12 hours of darkness, and earthworms were acclimatized for 15 days in a breeding tank (95 x 55 x 75 cm3) containing a 10 cm layer of uncontaminated cow manure wet soil (1:1). A thin layer of leaves and dry, wet grass was used for shade and moisture.

Chemicals and Their Preparation:

- Commercial insecticide formulations from the local market used in this study are more harmful than the pesticide compound's pure grades; they incorporate organic solvents, surfactant active ingredients, activity boosters, colors, and stabilizers with subpar toxicity (Dogan *et al.*, 2011; Ezzi *et al.*, 2016). Chlorpyrifos is an O, O-diethyl O-3,5,6-Trichloro-2-Pyridyl Phosphorothioate, CAS 2921-88-2, an organophosphate insecticide, commercially available with the skill label Clorzane 48% EC "Kafre Elzayat KZ CO, Egypt".

- Different concentrations (0.05, 0.1, and 0.2) of the pesticide (chlorpyrifos) were prepared by dissolving them in acetone solvent.

Ethical Statements:

The present investigation was approved in strict accordance with the rules of the National Health and Medical Research Council for the Care and Use of Animals. The Ethical Research No. in Assiut University's Faculty of Veterinary Medicine research number is (06⁄2023⁄0116).

Study Groups:

- **Control group: Group (1)** of 25 earthworms was kept in natural conditions.
- **Exposed groups**: G**roups (2, 3, and 4)** of 25 earthworms each were exposed to the different concentrations (0.05, 0.1 and 0.2) of the chlorpyrifos organophosphate insecticide. Mature adult worms of 2.2 to 3.5 g body weight were used in the study.

Experimental Design:

Acute toxicity experiments were conducted by the 48-hour paper contact toxicity according to Rishikesh *et al*, (2019).

1) Filter paper having a diameter of 8 x 3 cm covers the sides of a glass bottom petri dish of 8 cm in diameter and 3 cm in diameter without overlapping.
2) One (1 ml) of the prepared solutions of the different chlorpyrifos concentrations (0.05, 0.1, and 0.2 g/cm2) was then placed onto filter paper using a flask. To ensure an even dispersion of the poison, flasks were rotated. A gentle stream of compressed air was used to dry the treated paper.
3) Mature adult worms in the exposed groups had been fasted for three hours and placed on paper with damp soil and then were exposed to the various pesticide concentrations for 48 hours.
4) The filter paper was replaced every 12-hour interval, and the same amount of each concentration of chlorpyrifos organophosphate insecticide was reconstituted as above.

Morphological Examination:

The impacts of pesticides on the life history of *A. caliginosa* were observed.

Histological investigations:

Fresh samples of earthworms were collected from all treated groups and fixed with 10% neutral buffered formalin. The tissues were dried in gradients Alcoholic series, purified with methyl benzoate, included in Paraffin wax, sectioned 6 µm thick, and stained with Hematoxylin and eosin (Sakonlaya *et al*., 2014). Histological sections of earthworms were examined by optical microscope and photographed using a digital camera (Bancroft al. 1996).

Mortality:

Mortality of earthworms exposed to Chlorpyrifos. After exposure to organophosphate insecticide, the earthworms were gently transferred petri dish covered with wet filter paper for moisture at 25°C. When the prostomium (a covering for the mouth) revealed no reaction to probe interaction, the earthworm was documented as having died and the time of death was recorded.

Statistical Analysis:

Investigations were conducted in triplicate. Using Prism 8 statistical software and a one-way ANOVA, the significance of variances among the control group and the other three treated groups was evaluated. Significant or very significant data were defined as P 0.05 or 0.01 accordingly.

RESULTS

Toxic Effects of Chlorpyrifos:

- **Morphological findings:**

Earthworms in the control group (Group 1) that were preserved in natural settings and not exposed to organophosphate insecticides showed no signs of harm. Segmented earthworm bodies served important structural purposes. They had segments that were 120:150 mm long, 200:1200 mg of biomass, and roughly 60:85 mm in length. In the front was where the mouth was. The earthworm's clitellum (31-34), which secretes a mucous substance to create its cocoon, was visible on its ventral front (Fig. 2, A). Chlorpyrifos exposure in groups 2, 3, and 4 caused several changes in the body, including dryness of the skin and the observation of body constriction, body coiling, clitellum shrinkage, fragmentation of the body, thinning of the body, and whole-body coiling. Among the abnormal behaviors that appeared in earthworms exposed to the insecticide. At first, the worms showed abnormal S-shaped behavior. When the doses were increased, the worms began to show jumping movements, as the doses increased It was the cumulative percentage of individuals jumping a significant increase. Figure (3) shows the *A. caliginosa* life cycle under laboratory conditions. This species takes about 12 ± 3 weeks to develop from chrysalis to maturity. The biomass of a cocoon is typically 10 to 20 mg, while juveniles typically mature into adults with a biomass of 200 to 1200 mg.

In any stage of their life cycle, from the cocoon, juvenile until the adult stage, worms have been seen to die when exposed to pesticides.

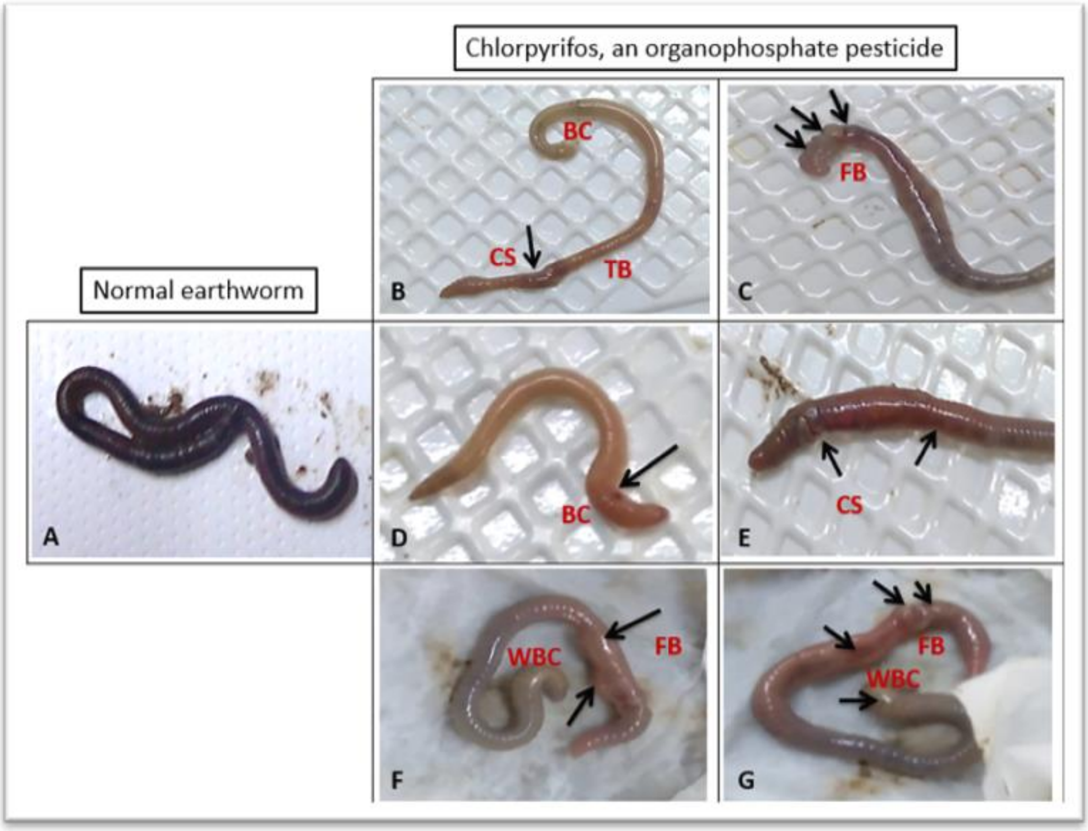

Fig. 2: Morphological changes in earthworm (*Aporrectodea caliginosa*), (A): Control and (B – G) : Morphological changes in earthworm exposed to organophosphate insecticide. Concentrations of organophosphate insecticide 0.05µg /cm²(B,C), 00.1 µg /cm²(D,E) and 0.2 µg /cm²(F,G).(**(BC):** Body Constriction**, (BC):** Body coiling, **(CS):** Clitellum Shrinkage, **(FB):** Fragmentation of Body, **(TB):** Thinning of Body, **(WBC):** Whole Body Coiling).

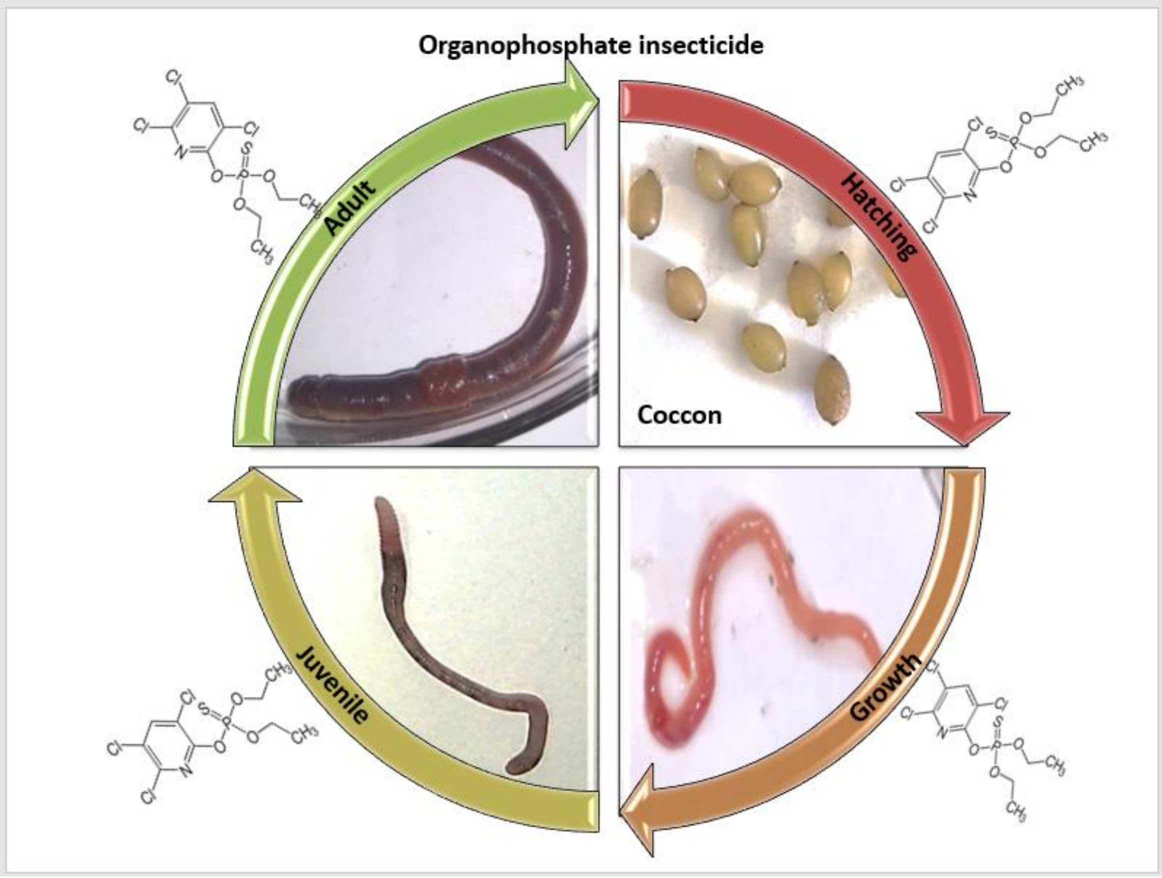

Fig. 3: The life cycle of earthworm (*Aporrectodea caliginosa*) in the laboratory.

- **Histopathological Findings:**

After 48 hours the earthworm was histologically analyzed. Earthworms in the control group (group 1) had a cross-section of their body wall stained with hematoxylin and eosin, which clearly revealed the usual earthworm structure of the epidermis, circular muscle, and longitudinal muscle (Fig. 4, A and C). The histological structure was observed to be changed in groups group (2, 3 and 4) after exposure to organophosphate insecticides. The epithelium has partially shed off and the epidermis is enlarged. Additionally, the epidermis displayed increasing epidermal thickening and turbid edema. The epidermis was entirely destroyed and necrotic, and the epithelium had been somewhat shed off. Total epithelial necrosis and sloughing were found, and circular muscle had epidermal breakdown, loss of muscle fiber integrity, and changed nucleus size and form slight disintegration of the longitudinal muscle (Fig. 4, B and D).

- **Mortality:**

The death rate in the control group was very simple, not exceeding ~5 %,/12 hrs. as earthworms could acclimatize and live in laboratory environments for a long time, while the worms that were exposed to organophosphate the worms that were exposed to organophosphate insecticide revealed highly significant mortality ratio ($P<0.0001$) (Fig.5 and 6).

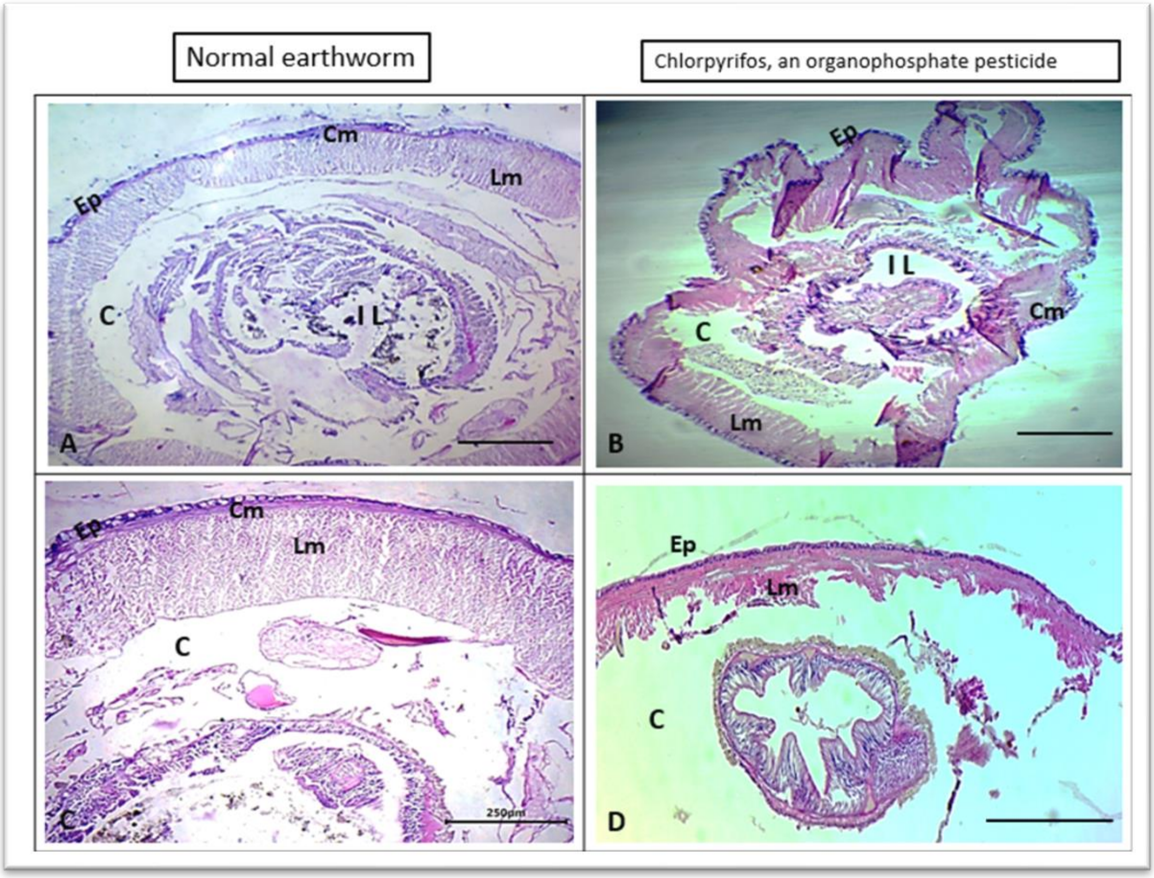

Fig. 4: Photomicrographs of transvers section of the body wall of earthworms (*Aporrectodea caliginosa*) , (A&C): Control and (B &D) : Earthworm exposed to organophosphate insecticide.(**H&E** stain, Scale bar = 250 µm, **(Ep):** epidermis, **(Cm):** circular muscle, **(Lm):** longitudinal muscle, **(IL):** intestinal lumen, **(C):** Coelom).

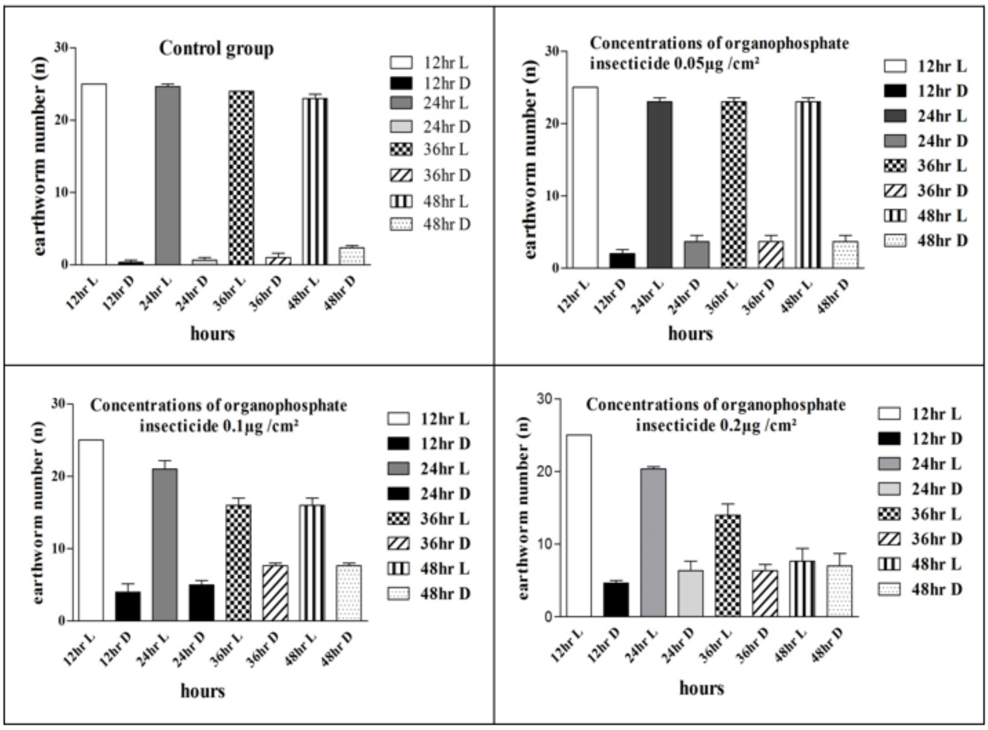

Fig. 5: Mortality of earthworms (*Aporrectodea caliginosa*) after Chlorpyrifos, an organophosphate pesticide exposure .((L): life worms, (D) :dead worms).

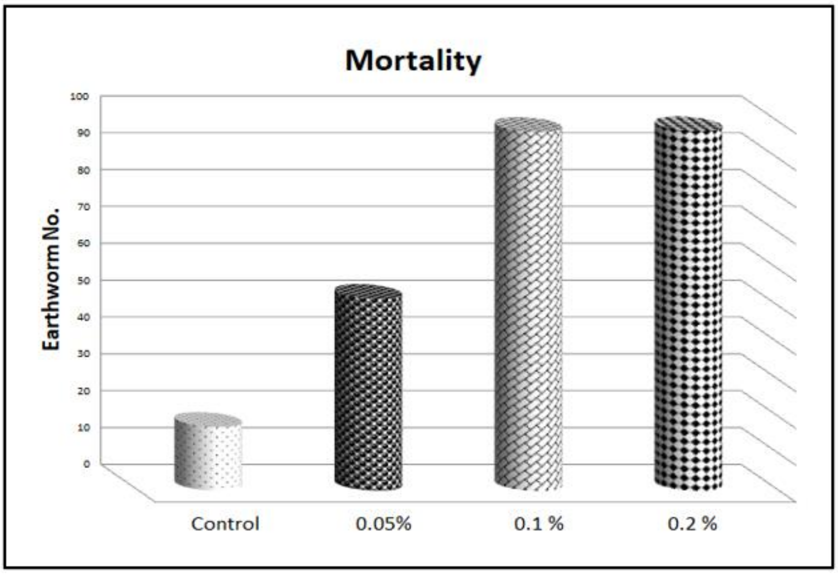

Fig. 6: The percentage of mortality of earthworms (*Aporrectodea caliginosa*) after Chlorpyrifos, an organophosphate pesticide exposure in four groups after 48 hours.

DISCUSSION

Although earthworms occasionally avoid the first layer of soil which contains agrochemicals (Cook *et al.,* 1980), in the experiment, they were resisted by a soil contaminant or an insecticide (Slimak, 1997). This work observed the potential carminative impact of organophosphate insecticides on earthworms (*Aporectodea caliginosa*) and their life cycle (the cocoon, juvenile and adult). When comparing the groups of earthworms that were exposed to different concentrations of the pesticide with the control group, varying degrees of morphological changes such as coiling, swelling of the clitoris, mucus release, and bleeding followed by body segmentation were observed in the earthworm. The present study results showed significant inhibition in the specific activity of earthworms exposed to organophosphate pesticide, especially in high concentrations, with an increase of the time, in their anterior body segments (pre-clitellum). The activity decreased in a dose- and region-dependent manner. Pre-clitellum activity decreased the most, then other locations, which may be explained by the fact that the earthworm's brain and ganglionic structures are located in the prostomium (Rault *et al.,* 2007; Calisi *et al.,* 2011). Organophosphates, on the other hand, render the enzyme inactive by covalently phosphorylating serine residues in the collection of active sites. AChE is irreversibly inhibited, which causes the neuronal and muscular systems to become hyperactive as a result of acetylcholine excess buildup. This outcome is consistent with the findings of (Rault, et al., 2007; Calisi, *et al.,* 2011; and Rishikesh, *et al.,* 2019). While Abd El-Aziz, *et al.,* (2023) exploited the *Sargassum latifolium* hexane invention as a novel foundation of bioavailable organic mixtures for insecticides. It is worthy to mention that (Bart, *et al*.,2018) tested the ecotoxicological with earthworms *Eisenia fetida* and their life cycle for the risk assessment of pesticides prior to registration and commercial use. This is in accordance with the present result.

The histological in the tested earthworm. The epidermal, circular muscle and longitudinal cell layers of control worms were all confirmed to be in perfect health with no changes. However, the earthworm's exposure to organophosphate insecticide caused damage to its epidermis and circular muscle layer. Additionally, it was discovered that exposure to various organophosphate insecticide concentrations caused varying degrees of histopathological alterations in the internal cells, including cellular inflammation, severe lesions, cellular degeneration, necrosis, malignancy, and endothelial degeneration. This result is in accordance with Duque *et al.* (2023).

Conclusion

There are many pesticides in the agricultural environment, and therefore they represent many dangers that affect soil organisms, especially earthworms, which are of great importance to soil and agriculture. In the current study, we noticed the harmful effect of the organophosphate insecticide on the earthworm's morphology, as well as how it affected and harmed their histological structure, and ultimately the death of earthworms after 48 hours of exposure to different concentrations, which confirms the toxicity of pesticides and their harmful effect on earthworms, which results in damage to soil and humans.

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