Evaluation Histopathologique Après Exposition A Un Cocktail Toxique Chez Un Coelomate « Aporrectodea Caliginosa (Savigny, 1826) »

Abstract :

The objective of this study was to investigate the physiological consequences of a toxic mixture of cadmium chlorite ($CdCl_2$) and the herbicide 2,4-dichlorophenoxyacetic (2,4-D)on the histological appearance of the earthworm "Aporrectodea caliginosa", a prevalent species in the Annaba area. We took four worms, one from each of the treatments. The first was a control. The other batches, one batch exposed to Cd, one batch exposed to pesticides, and a combination of the two, were exposed for 14 days. Histological sections were obtained to conduct a qualitative and quantitative evaluation of the tissue lesions resulting from the treatments. The findings demonstrated tissue damage, necrosis, and the presence of vacuoles in clusters, demonstrating the application of an unspecified defense strategy that resulted in the accumulation of minerals. Furthermore, the rates of intestinal and gonadal lesions were highly significant compared with controls in all treatments, underlining the harmful effect that these xenobiotics can have on the growth and reproduction of earthworms.

Keywords: Aporrectodea caliginosa, pesticide, ETM, cocktail, toxicity, study, histology

1. Introduction :

Agricultural soils are exposed to various contaminants such as the use of agrochemicals (pesticides), sewage sludge (pharmaceutical and industrial residue), the introduction of metals, etc. However, these pollutants continuously accumulate in the form of complex mixtures in natural system**s (Altenburger et al., 2004).** Known for their large surface area and adsorption capacities, they eventually modify the behavior of other elements. However, the synthesis of chemical products forms complex molecules, and once in contact with the cells of a living organism, they are called xenobiotics, toxic, and can modify the behavior of an entire population **(Delfosse V et al., 2015).**

Until now, scientists only considered individual compounds to assess the risks of environmental contaminants. Nevertheless, the joint assessment of the effects of chemical mixtures is based on a single chemical toxicity. This tends to overestimate or underestimate the level of toxicity of joint exposure. While a few studies have shown that the mixture of toxic substances can be more harmful than a single substance **(Carvalho et al., 2014; Schnug et al., 2014; Ji et al., 2017; Cai et al., 2017; Rillig et al., 2019).**

Exposure to toxic mixtures can have a similar effect (additive), greater (synergistic), or less significant (antagonistic) than the sum of the effects of individual exposures **(Warne and Hawker, 1995).** Pollutants are generally the same in water and soil systems. However, they can vary depending on their fate and effects on living organisms in both environments. Soils are considered complex ecosystems composed of organisms, mineral particles, and organic matter. The binding of certain toxic compounds to certain fractions of minerals and humus can reduce

their mobility and bioavailability, thus modulating their toxicity. Furthermore, the availability and toxicity of soil pollutants are also influenced by the time and age of the soil. This makes the assessment and extrapolation of toxic concentrations and their effects on terrestrial organisms more challenging than for aquatic animals **(Warne and Hawker, 1995).** Approaches that allow for on-site studies or experiments on natural or artificial soils are recommended to explain the availability of toxic substances and their relative exposure conditions **(Spurgeon et al., 2003).** The most challenging issue regarding the assessment of ecological risks is that exposure and determination to soil contaminants involve determining the contamination level of living organisms and assessing the toxic effects of these contaminants at individual, population, and community levels.

The intensive use of pesticides in the agricultural sector improves annual agricultural production, but their residues can lead to soil contamination directly or indirectly, which can be directly or indirectly stressful for soil organisms **(Choung et al., 2013).** In addition to pesticides, the global production of heavy metals has seen an alarming increase and has been widely dispersed in the environment since the industrial revolution **(Nriagu and Pacyna, 1988).** Significant contamination of soils, water, and air with metals and pesticides, as well as their transfer to higher organisms along the food chain, still pose an environmental problem that can present various risks to the health of future generations.

To date, very few studies have been conducted on the cross-contamination of pesticides and heavy metals. However, they have reported that their interactions are likely to produce a synergistic mixture in the terrestrial environment **(Wang et al., 2012; Cedergreen, 2014).**

Earthworms are organisms present in soil ecosystems and play a crucial role in improving soil quality. A reduction in the number of earthworms in the soil ecosystem can lead to a decrease in the nutrient cycle and their availability for absorption by plants **(Rizhiya et al., 2007).** Earthworms, as bio-indicators of soil pollution, have been considered a key organism for assessing ecological risks **(Song et al., 2009).** Pesticides and heavy metals can accumulate in earthworms and then be transferred to other organisms through the food chain.

Due to the widespread application of chemicals in the agricultural ecosystem, the study of earthworm ecotoxicology has become increasingly widespread in recent years, and study methods have been regulated **(OECD; 1984).** Different studies on the ecotoxicological mechanisms of earthworms towards pollutants, such as heavy metals, pesticides **(Stenersen; 1979),** and the combined pollution of different heavy metals **(Spurgeon et al.; 2003),** metals, and pesticide mixtures **(Xu et al.; 2006),** have been reported worldwide. However, studies on the interactions between the toxic effects of pesticides and heavy metals are still very limited. Therefore, it is preferable to work on earthworms that represent the ecosystems of a given region to obtain a good representation of ecotoxicological tests, as in the case of Aporrectodea caliginosa, a species representative of agricultural lands **(Bastardie et al., 2005).**

As a result, in this study, we investigated the effect of a toxic mixture combining a metal and a pesticide on tissue damage in earthworms following exposure to xenobiotics (histological study).

Materials and Methods

2.1. Study Area:

The Chetaibi Forest is a municipality in the city of Annaba, located 62 km northwest of the Annaba province.

2.2. Sampling Methods:

We employed a physical method for earthworm sampling using a shovel and manual sorting according to the Bouché method (1972). Soil and earthworm samples were collected during the winter, a favorable period (in terms of temperature and precipitation) for earthworm proliferation in the Annaba region. The collected earthworms were identified in the Ecology Laboratory (Constantine) using keys provided by Alvarez (1971) and Bouchet (1972).

2.3. Biological Material:

a) Species Selection:

We chose the species "Aporrectodea caliginosa (Savigny, 1826)," representative of the study area. The individuals selected for the study were adults with a clitellum of identical size and weight (150-200 mm and 0.8 to 0.9 g), respectively. The individuals are kept in terrariums in the laboratory.

2.4. Chemical Material:

Our xenobiotics are a toxic cocktail composed of a metallic element "Cadmium Chloride" and a pesticide "2,4-dichlorophenoxyacetic acid," both chosen due to their common use in the region and globally. The concentrations were chosen from the literature at 1/4 of the LD50 (see Annex 2).

a) Pesticide:

The treatment was carried out by spraying Desormond lourd D (C.H. No: 19 58 39). This is the commercial form of 2,4-dichlorophenoxyacetic acid commonly known as (2,4-D). In terrariums, at the following concentrations: 43.9 µg/1 kg of wet soil.

b) Trace Metal Element (TME):

Cadmium chloride ($CdCl_2$), (C.A.S: 7790-78-5). This is the soluble form of cadmium. We used cadmium chloride as a chemical product in our study. Deionized water was used to enrich the soil with cadmium. The solutions were prepared to obtain the following nominal concentrations in the soil: 0 (control), 14.28 mg/kg of wet soil.

2.5. Treatment Mode:

The experiment was conducted in vitro under controlled conditions. First, the pots were filled with 1 kg of wet soil. Then, 10 mature earthworms with well-developed clitella of identical size and weight were incorporated into each pot. For 14 days, 1/4 of the LC50 (LC50: found in the literature) was chosen for this experiment. Soils and ambient temperature were maintained, successively, at 60% field capacity and 22°C throughout the experiment. Each condition was repeated in 3 repetitions plus a control.

2.6. Toxicological Tests:

2.5.1 Dissection and Tissue Sampling:

After each treatment period, the earthworms were fasted for 48 hours in Petri dishes to empty their digestive tract. For the histological study, earthworms from each batch were randomly selected. After dissection, a piece of tissue just behind the clitellum region was placed in a 10% formaldehyde solution for histological study following the method of **(Martoja and Martoja, 1967).** The sections were observed using a Leica DM 1000 photomicroscope.

2.6.2 Histological Sectioning: A Comprehensive Methodology

Histological techniques play a crucial role in preserving the cellular and tissue morphology of samples for precise analysis. The work was conducted at the Anapath service of El Bonni Emergency Hospital in the state of Annaba, with the equipment and reagents.

a) Fixation:

Samples were immersed in a 10% formalin solution. After measuring dimensions (length and width), they were fixed based on their condition (solid or hemorrhagic). A knife suitable for the size of the specimen was used, and once cut, the samples were coded with a number. These fragments were grouped into baskets and successively immersed in 12 baths containing 2 formalins, 6 ethanols, 2 paraffins, and 2 xylenes for 12 hours at a controlled temperature.

b) Embedding (Inclusion):

This step aims to coat the samples with paraffin for easy slicing. Cassettes were immersed in paraffin, placed in custom molds, then frozen and cooled. The result is a solid mold containing the cassette with the paraffin-fixed sample.

c) Sectioning:

After cooling and separating the molds, the mass was worked on with a scalpel. Cutting was done at a specific speed, producing sections of 4 to 5 micrometers, thanks to the previous fixation. These sections were placed on numbered slides according to the installation step, and water was poured to detach them.

d) Deparaffinization:

To remove paraffin from the sections, they were placed in an oven at 60-50 °C for 20 minutes.

e) Staining:

After decaffeination and rewetting, routine staining was performed using a staining station. Sections passed through various chemical solutions, including xylene, two alcohol baths, Mayer's hematoxylin, aqueous leucine, water rinse, two alcohol baths, and xylene acetone, allowing the differentiation of sample components.

f) Mounting:

Once staining was completed, slides were cleaned with xylene, and a new slide was placed with Eukitt glue. For mounting in anhydrous conditions between the slide and cover slip, the previous colored slide was placed on the new slide, also tinted with Eukitt glue. The final mounting was done in xylene, eliminating air bubbles. Slides were numbered, categorized in ascending order, and ready for reading.

This comprehensive methodology provides a rigorous approach for the preparation of histological samples, preserving cellular morphology while facilitating subsequent analysis.

2.7. Statistical Analysis:

In our study, to enhance the visualization of the obtained results, the chosen graphical representation is that of histograms using Microsoft Excel 2017. The results obtained underwent a semi-quantitative analysis using the software (QuPath 3.2).

3. Results and Discussion

The slides illustrate longitudinal sections **(1, 2, 3)** conducted on the anterior, median, and posterior parts, showing the histological aspect of the epidermis in **Slide 1 and 3**, while **Slide 2** depicts the tissue aspect of the intestinal and reproductive organs in earthworms, both control and treated with Cd, 2,4-D, and the toxic cocktail of both xenobiotics, after 14 days of exposure.

The analysis of **sub-slides (A, B)** for **Slide 1** identifies the appearance of the epidermis in the anterior region (segments 1-16) of earthworms after 14 days of treatment. A normal structure of the epidermis is observed in the control group.

Sub-slides (C, D, E, and F) respectively represent those treated with 2,4-D and Cd, causing alterations at the apex, subsequently forming vacuoles, and significant detachment of the scale epithelium (25 to 50 µm).

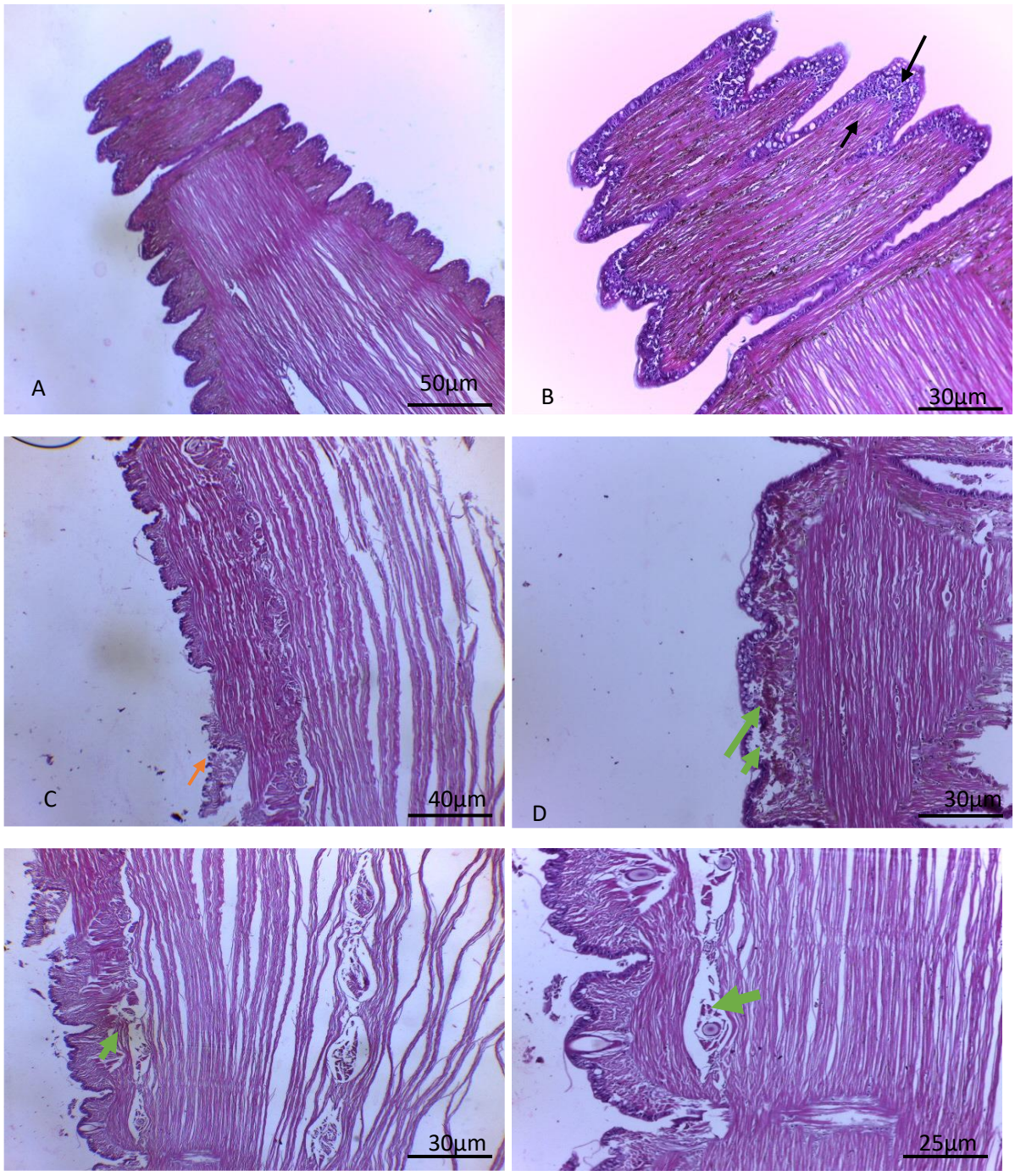
Slides of the posterior part revealed epidermal alterations, including necrosis and detachment, in all treated groups compared to the control.

Sub-slides (2) represent the median parts containing reproductive organs and intestines. Those exposed to 2,4-D and Cd for 14 days **(sub-slides D, E in Slide 2)** showed voluminous epithelium covering the typhlosole compared to control, indicating an ongoing absorption process. The same slides revealed modifications in gonadal organs in all cases compared to controls.

In addition to the previous findings, **Slides (4 and 5)** show cross-sectional cuts on the abdominal part. **Sub-slides (4)** revealed lesions in gonadal tissues compared to controls. Regarding **Slide 5 (A)** in a control, the structure of the Typhlosole with chloragogenous cells of the intestinal epidermis is clearly visible, contrasting with the alteration seen in S**lide (B). Slides (C and D)** show the nerve cord and did not reveal significant differences.

3.1. Microscopic Observations of Sagittal Sections of the Anterior Part:

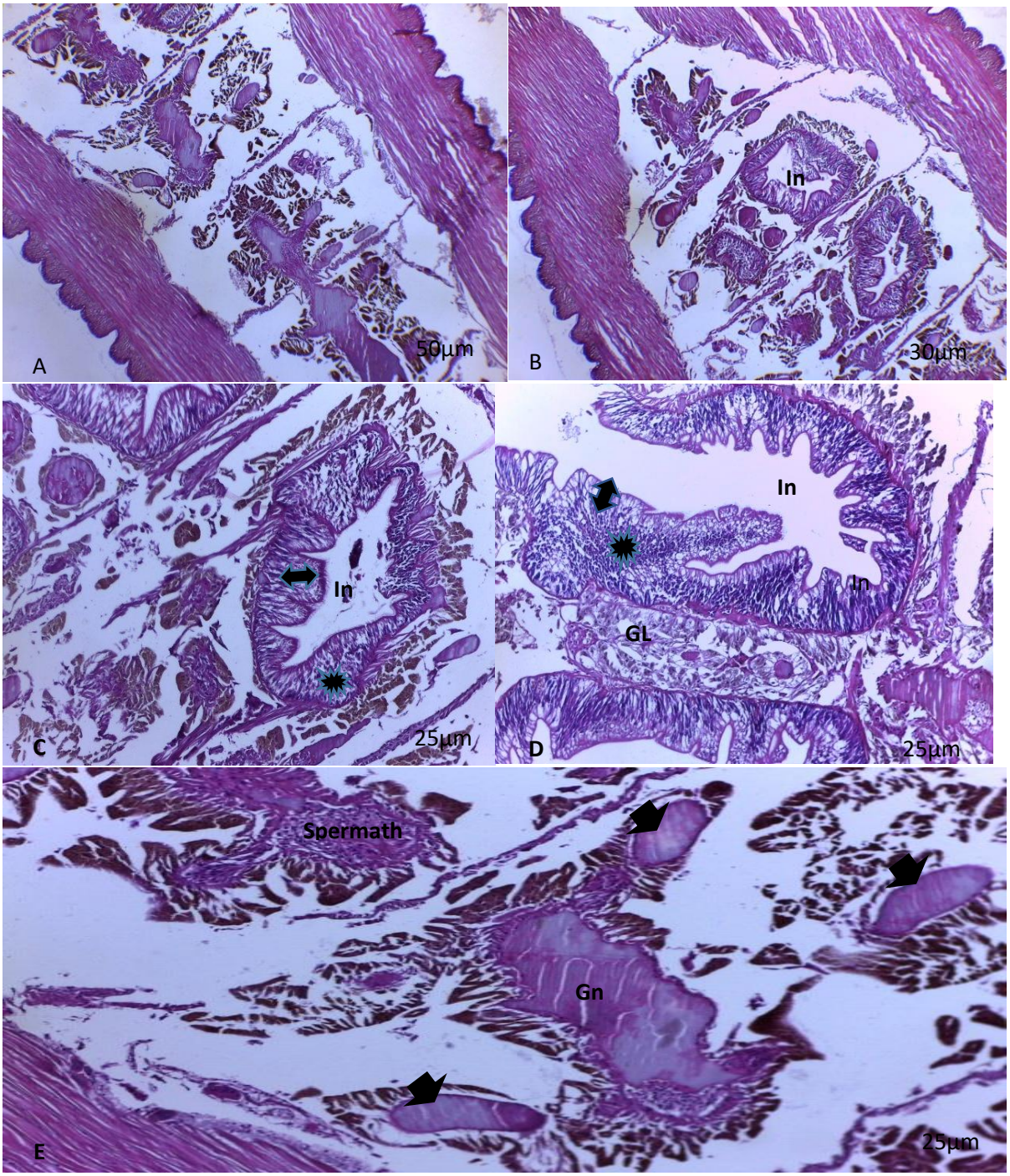
The microscopic observations of sagittal sections of the anterior part reveal distinct features in both control and treated groups. In **Slide 1, sub-slides (A, B)** show the normal structure of the epidermis in the anterior region (**segments 1-16)** of earthworms treated for 14 days. Conversely, **sub-slides (C, D, E, and F)** for the 2,4-D and Cd-treated groups exhibit alterations at the apex, the formation of vacuoles, and significant detachment of the scale epithelium (25 to 50 µm). These observations underscore the impact of the treatments on the epidermal morphology and provide valuable insights into the potential toxic effects on earthworms' anterior segments.



Slide No. 1: Histological Sections of the Earthworm's Anterior Region (Segments 1-16), Details of the Epidermis of the Esophagus (right side); Chloragogenous Cells (left side); Control (A and B) and Treated with 2,4-D and Cd (C, D, E, and F), Hematoxylin/Eosin (x40). Green arrow indicating alteration at the apex and disorganization of the epithelium, scale bar from 25 to 50 µm.

3.2. Microscopic Observations of Sagittal Sections of the Middle Part:

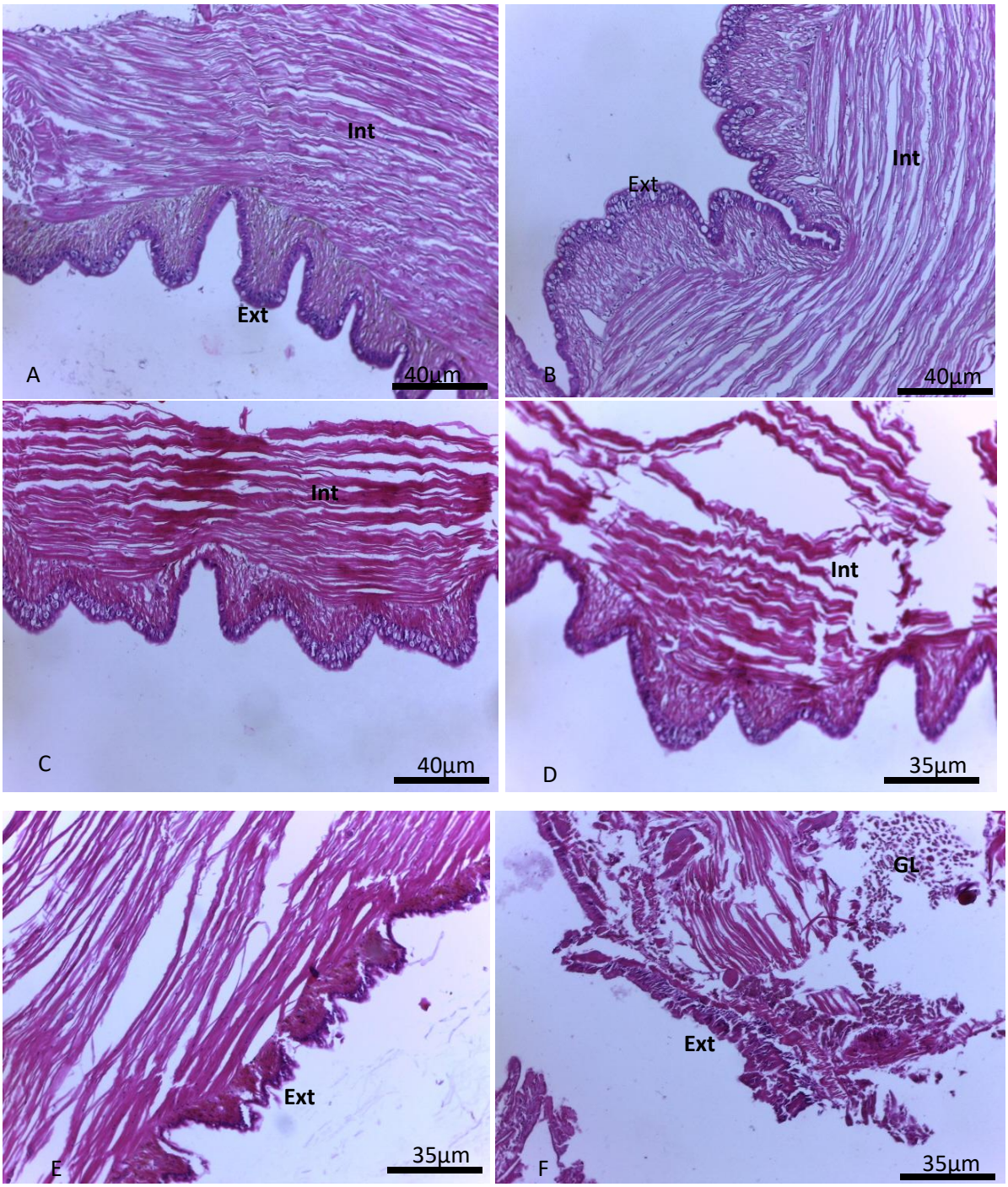
The microscopic observations of sagittal sections in the middle part provide insights into the histological features of both control and treated groups. These observations contribute to understanding the impact of the treatments on the middle segment of the earthworms.



Slide No. 2: Histological Sections of the Middle Region, (A, B, and C Control, and D, E Treated, showing the intestine (In) and reproductive organs (Gn) with blood vessels (black arrow), ganglia (GL), and spermatheca.

3.3- Microscopic Observations of Sagittal Sections of the Posterior Part:

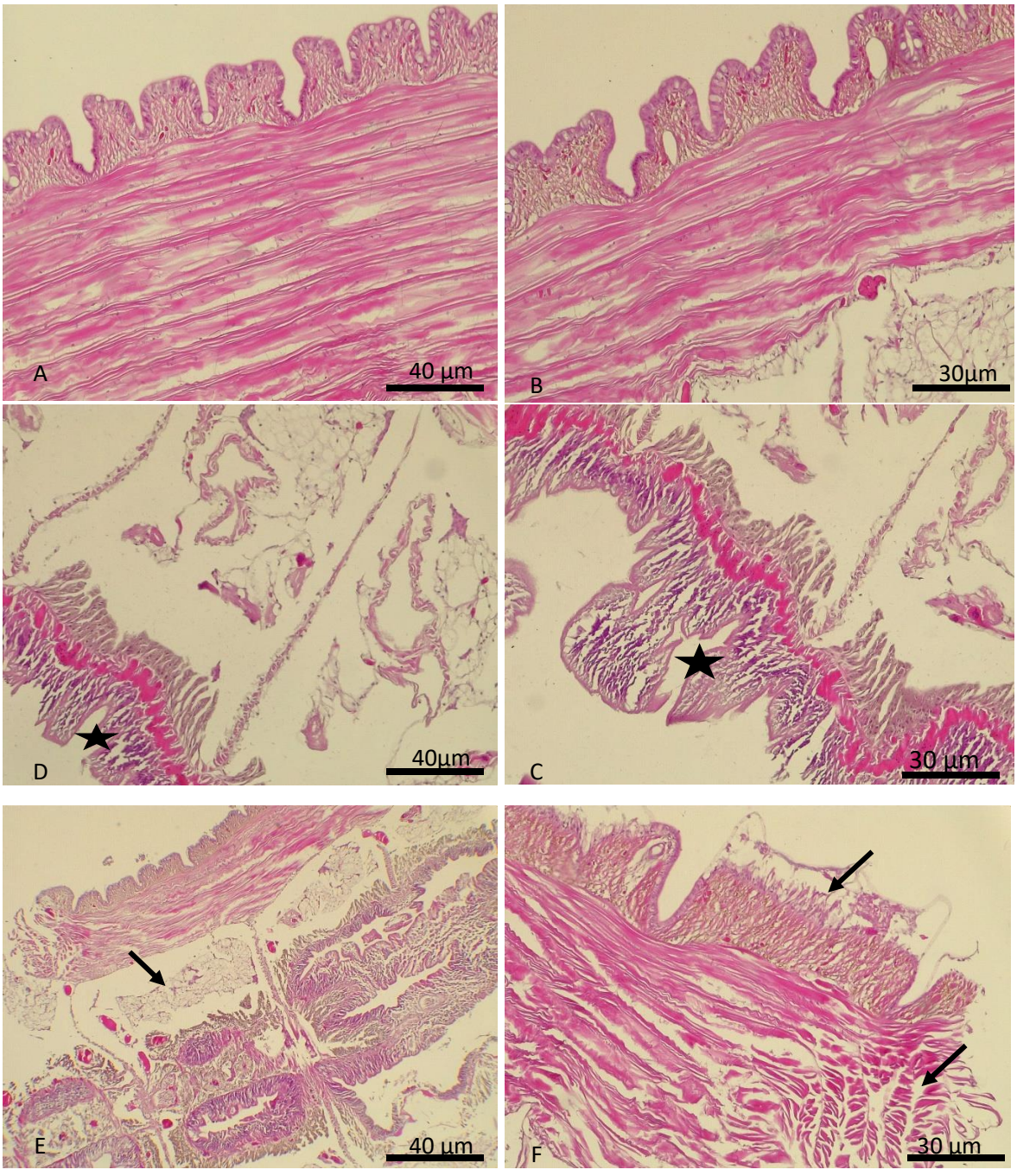
The microscopic observations of sagittal sections of the posterior part provide insights into the histological features, highlighting alterations and abnormalities in both control and treated groups. These observations contribute to understanding the impact of the treatments on the posterior segment of the earthworms.



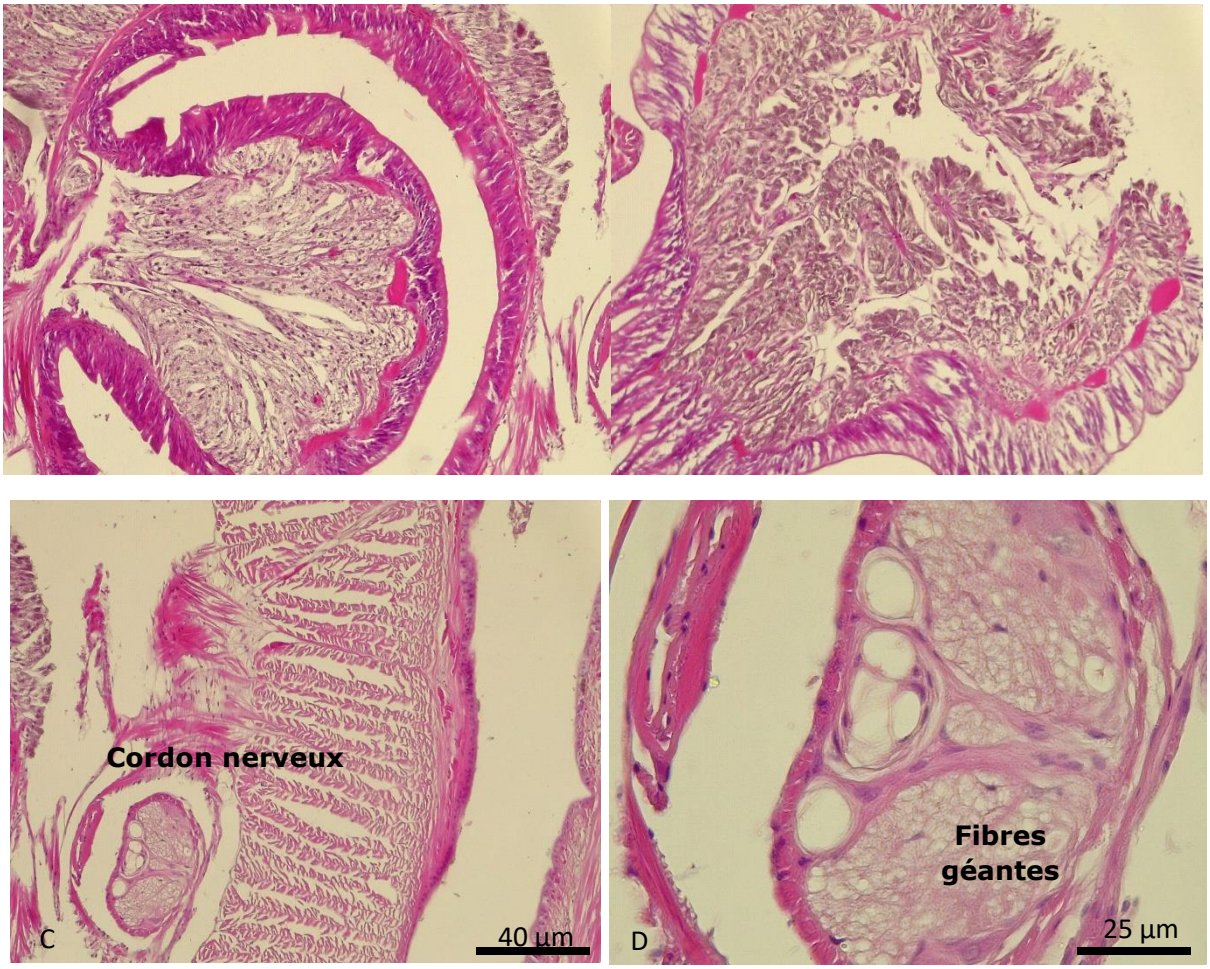
Slide No. 3: Histological Sections showing the posterior part of earthworms. Control A and B exhibit a normal structure of segments, while those treated with Cd (C and D) show alterations on the inner side (Int). However, individuals treated with 2,4-D (E and F) display tissue alterations on both the inner and outer sides. Scale bar from 34 to 40 µm.

3.5- Microscopic Observations of Transverse Sections of the Abdominal Part:

The microscopic observations of transverse sections of the abdominal part provide insights into the histological features, specifically focusing on alterations and abnormalities in both control and treated groups. These observations contribute to understanding the impact of the treatments on the abdominal segment of the earthworms.



Slide No. 4: Sagittal Histological Sections of the Abdomen, Control (A and B); Treated with 2,4-D (C and D), and Treated with Cd (E and F).



Slide No. 5: Transverse Histological Sections, showing the Typhlosole (A) in a control and Typhlosole (B) in a treated specimen. Additionally, the nerve cord (C and D).

3.6- Frequency of Lesions in Gonadal and Intestinal Tissues:

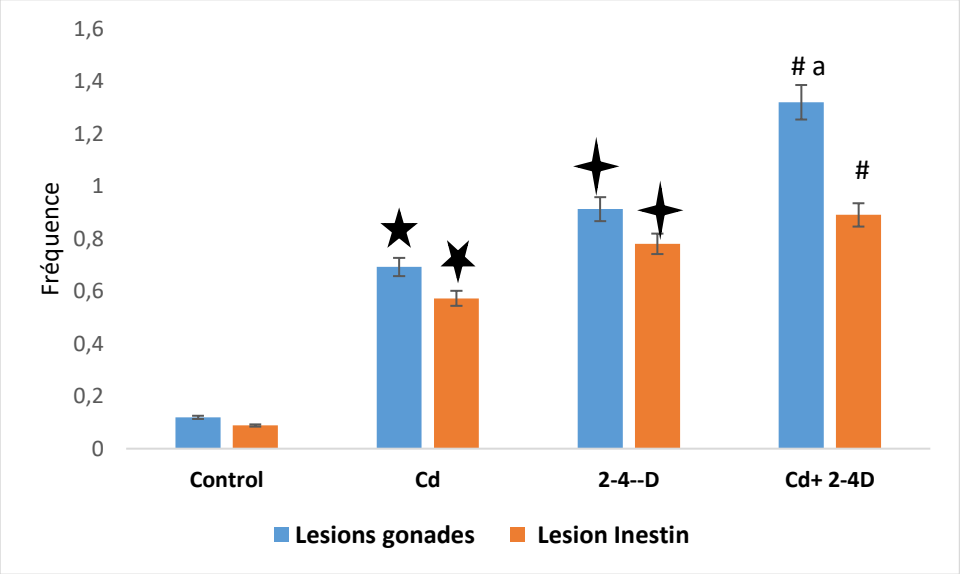


Figure No. 25:

Variation in the Frequency of Lesions in Earthworms Exposed to Cadmium, 2,4-D, and the Cocktail **P < 0.05 vs T and # P < 0.01 vs T,** and **a P < 0.05 vs Cd** and **2-4D (**frequency calculated using image processing **(software QuPath 3.2).**

The results demonstrate a significant increase in intestinal and gonadal lesions in groups treated with Cd and 2,4-D compared to the control. However, this increase is more pronounced in worms treated with the Cd+2,4-D mixture, especially in gonadal lesions confirming the aforementioned observations.

4. Discussion

According to **Morgan and Turner (2005)** and **Oluah et al. (2010),** histological observations of tissues and cells are valuable tools for evaluating the toxic effects of contaminants on various species, including earthworms. This is the framework within which we conducted our study. Earthworms were exposed to D-2,4 acid, Cd, and a combination of both in the soil. These treatments caused significant tissue damage to the epidermis, necrosis, epidermal sloughing, vacuolation, as well as lesions in the intestinal epidermis and gonadal cells.

Our results align with studies on the effects of pesticides on various earthworm species, such as **Zeriri (2013),** who tested the effect of Methomyl on earthworms (Approcteda caliginosa), showing similar signs of tissue necrosis, severe alterations, and sloughing after a 12-day exposure**.** Additionally, **Gao et al. (2013)** studied the effect of Triazole on Eisenia foetida, highlighting tissue necrosis in this organism. Furthermore**, Belmeskine (2016)** revealed alterations in gonadal organs following a chronic 28-day exposure to Desorban.

The observed lack of structural integrity in the circular muscle after a 14-day chronic exposure for all treatments is another sign of toxicity**. Kiliç (2011)** reported that xenobiotics cause damage and primarily accumulate in the circular muscle of earthworms exposed to pollution.

In earthworms, heavy metals can disrupt cell dynamics and damage their membranes, leading to impaired intercellular exchange and fluidity. As a result, the diffusion of heavy metals increases in cells, causing cellular damage. Hence, our results showed the highest rates of lesions in intestinal tissues. Histological examination of the intestinal tissue of earthworms treated with Cd for 14 days, as conducted by **Otmani (2019),** identified the occurrence of cellular necrosis, caryorrhexis (nuclear destruction or lysis by fragmentation), highly eosinophilic cytoplasm, and imprecise cytoplasmic boundaries when mixed with Cu. These

treatments revealed inflammatory infiltrates (eosinophilia) and imprecise cytoplasmic boundaries. We observed the same for our worms exposed to mixtures, explaining the presence of a synergistic effect in our chosen cocktail.

Our results showed a reduced thickening of the chloragogenous tissue, contrary to the results of **Otmani (2019),** where no alterations were reported in those samples. However, they were also reported in L. terrestris inhabiting naturally polluted soils in volcanic areas **(Amaral et al., 2006; Kiliç, 2011; Lourenço et al., 2011).** The chloragogenous tissue is located between the intestinal epithelium and the coelom, where metals accumulate and discharge **(Cancio et al., 1995; Morgan et al., 2002; Giovanetti et al., 2010).** Alterations to the intestinal epithelium, as a manifestation **(Morgan et al., 2002),** are therefore valuable indicators of pollutant toxicity **(Amaral et al., 2006; Giovanetti et al., 2010; Kiliç, 2011; Lourenço et al., 2011).** They either lose their structural integrity, exhibit chloragogenous tissue necrosis and intestinal epithelium atrophy accompanied by fusion of chloragogenous tissues, or show increased mucus production and disorganization of associated long and short muscles **(Amaral et al., 2006; Kiliç, 2011; Lourenço et al., 2011).**

Tissue integrity loss and necrosis were also reported in the chloragene tissue of E. Fetida exposed to polluted soils for 56 days **(Asensio, 2009).** This loss of integrity was interpreted as a defense mechanism against metal pollution by eliminating metals in extruded chloragocytes. Indeed, the chloragene tissue is the main site of metal accumulation and elimination in earthworms **(Morgan et al., 2002; Giovanetti et al., 2010).**

5. Conclusion

Our results demonstrated a significant increase in intestinal and gonadal lesions in groups treated with Cd, as well as those treated with 2,4-D, showing alterations at the apex and disorganization of the epithelium. The combination of Cd and 2,4-D revealed an apparent synergistic effect at the tissue level. The histological study confirms the earthworms' extreme sensitivity to environmental pollutants in general and highlights the toxicity of Cd and 2,4-D, causing severe tissue damage.

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