TOXICOLOGICAL AND HISTOPATHOLOGICAL EFFECTS OF LEAD AND ZINC ON JUVENILES OF *APORRECTODEA GIARDIA* IN NORTHEAST OF ALEGRIA

[1,2]HOUDA, Berrouk, [2]KAOUTER, Moukhtel and [2]KHADIJA, Touahria

[1]Laboratory of Terrestrial and Aquatic Ecosystem, Mohamed El-Cherif Messaadia University, Souk-Ahras, Algeria.

[2]Faculty of Life and Natural Science, Mohamed El-Cherif Messaadia University, Souk-Ahras, Algeria.

Corresponding Author: Houda, B. Laboratory of Terrestrial and Aquatic Ecosystem, Mohamed El-Cherif Messaadia University, Souk-Ahras, Algeria. **Email:** houda.berrouk@yahoo.fr **Phone:** +213 (0)664871285

Received *April 22, 2022;* ***Revised*** *June 27, 2022;* ***Accepted*** *July 05, 2022*

ABSTRACT

Most studies investigating the effects of heavy metals on soil communities have focused on using earthworms such as Lumbricus terrestris and Aporrectodea giardia as efficient bioindicators of soil health. Therefore, the present study was aimed to assess the toxicological and histopathological effects of four different concentrations of lead and zinc (0, 100, 200, 500 and 1000 ppm) on Juveniles of A. giardia for four exposure periods (24, 48, 72 and 96 hours). The physicochemical analysis proved the soil as an appropriate living environment for earthworms due to its richness in organic matter (OM = 8.93 > 4), neutral pH, and the sandy-silty texture owed to the capacity in the field (19.37) and in the interval (12 – 14). Moreover, a reduction in earthworms survival rate confirmed by a proportional increase of lethality rate with the increase concentrations of lead and zinc (Lead: LC_{50} = 893.27; LC_{90} = 12394.42; Zinc: LC_{50} = 785.22; LC_{90} = 92348.13). The histological findings of earthworms exposed to 100 and 200 ppm of Zn and Pb for 96 hours showed a dispersion or loss of junctions and cohesions of the chloragogen cells, while serious alterations at the level of the epidermal and intestinal cells (chloragogen) characterized by the formation of vacuoles and detachment of cellular fibres were noticed in the concentrations of 500 and 1000 ppm of the two metals.

Keywords: *Aporrectodea giardia,* Lead, Zinc, LC_{50}, LC_{90}, Lethality, Histopathology

INTRODUCTION

Most of the trace metallic elements (heavy metals) are ubiquitous in the environment, since others are introduced in the natural ecosystems by the anthropogenic human activities that lead to increasing contamination of soil, water and air (Tchounwou *et al*., 2012). Additionally, trace elements cannot be destroyed in the soil, but they accumulate in soils, and hence their persistence in the soil and their potential adverse effects on human health presents current and future concerns (Wuana and Okieimen, 2011). It is well established that the bioavailability and toxicity of heavy metals to living organisms are strongly determined by the forms, in which they occur in the environment (Allen *et al*., 2001). Lead and zinc occur naturally in the environment, and found at various concentrations in nature as a result of human activities. Furthermore, some fishes living in zinc-contaminated waters can accumulate zinc in their cells, as well as crustaceans can equally accumulate small concentrations of lead in their cells (Maaboodi *et al*., 2011). Lead and zinc in aquatic ecosystem causes disruption of phytoplankton functions (Gheorghe *et al*., 2017). Soil

invertebrate animals are vulnerable to heavy metal attacks. The life, reproduction and success of earthworms that can be compromised by the direct action of any contaminant that makes them as bioindicators of contaminated terrestrial ecosystem (Suthar *et al.*, 2008). As reported by Wu et *al.* (2005) and Figueira *et al.* (2009), bioindicators are organisms that present measurable changes at different levels (morphological, physiological, cellular and even molecular) when exposed to toxicants in the environment. In this regard, the present study was intended to assess the toxic effects of lead and zinc, worldwide used metals by human on a biological model *Aporrectodea giardia*, owing to its bioindicator sensitivity through toxicological and histopathological studies.

MATERIALS AND METHODS

Sampling Site: The earthworms were obtained from a home garden located in the Ouled-Driss municipality, Souk-Ahras City Northeast, Algeria at the border with Tunisia. The site is characterized by a continental climate with both Mediterranean and desert influence with a rainfall varying between 300 and 1000 mm/year (Khoualdia and Hammar, 2017) (Figure 1).

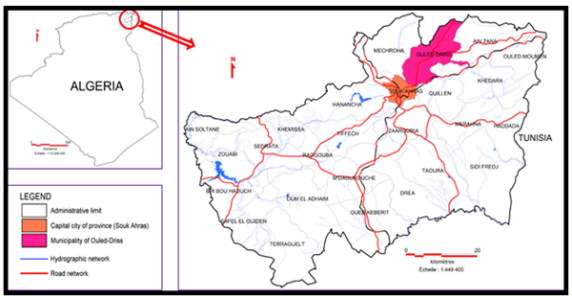

Figure 1: Geographical location of the sampling site of juveniles *Aporrectodea giardia* (modified from Barour and Taouarfia, 2022)

Physicochemical Analysis of Soil: To determine the physicochemical parameters of soil, a soil sample was taken randomly from the study site, subjected to separation process to extract the fine soil from the gravels, and then dried in the open air for a week.

The soil's pH was measured according to the method of Winterkorn (2008). A soil solution containing 10 g of fine soil and 50 ml of distilled water was prepared and shacked to homogenize the solution, then kept standing for 15 – 30 minutes, and afterward, pH value was measured by introducing the electrode of the probe into the solution. In addition, a soil solution containing 20 g of fine soil, 100 ml, and 50 ml of KCl was stirred for 5 mm, left standing for 30 minutes, and then used to determine pH value of KCl by introducing the probe electrode into the solution.

The electrical conductivity (CE) of soil was determined according to the method of Okalebo *et al.* (2002). Briefly, a soil solution containing 10 g of fine soil and 50 ml of distilled water was shacked, kept stand for 15 – 30 minutes and then used to record the EC value indicated on the digital screen by introducing the electrode of the probe into the suspension.

The hygroscopic water or the non-usable water was measured according to the method of Winterkorn (2008). Here, 20 g of fine soil sample was placed in aluminum foil, and dried in an oven at 105°C for 24 hours, to enable the calculation the hydroscopic water percentage according to the following formula: Hygroscopic water (%) = weight of water / weight of dry soil × 100, where: weight of water = sample before drying - sample after drying, and weight of dry soil = dry soil + aluminium foil.

The field capacity and saturation capacity were measured based on the preparation of the saturated dough by adding water drop by drop until a shiny and slippery dough was obtained, which was kept to rest for 1 hour and put into an oven at 105°C for 24 hours. Then, the following indices were determined: Y = weight of dry soil, SR (saturation rate) = X/Y × 100, and FC (field capacity) = saturation rate ÷ 2.

The soil texture per soil humidity or field capacity was determined as described elsewhere (Winterkorn, 2008). Herein, the method consists in measuring the percentage of FC and comparing it to a scale determining the corresponding texture. The texture scales used were: 0 – 10 % sandy soil, 11 – 20 % sandy-loamy, 21 – 40 % loam-clayey-sandy, 41 – 50 % clay-sandy and 60 – 100 % clay (Duchaufour, 1977).

The soil carbonate was determined according to the method of FAO (2020) in which carbonates decompose and carbon dioxide is released under the action of a strong acid (HCl). In this method, a solution containing 10 ml of diluted HCl and 30 ml of distilled water was prepared, then 10g of fine soil was added into a beaker containing HCl, shacked and kept stand for few minutes. The carbonate is calculated as follows: Weight of CO_2 released = $P_2 - P_3$, Percentage of $CaCO_3$ (%) = weight of CO_2 released $\times 2.274 \times 100$ / weight of soil, where, P_1 = weight of the beaker + acid, $P_2 = P_1 + 10$ g of soil, and P_3 = weight of the beaker+ acid + soil.

The soil organic matter (OM) was determined using the Muffle furnace method (Okalebo *et al.*, 2002). 10 g of well mixed air dry soil sample of known moisture content was weighed into a dry crucible, and heated slowly in a furnace at 550°C for 8 hour. The crucible containing a greyish white ash was removed from the furnace, cooled in a desiccator and weighed. The percentage ash and organic matter were calculated by the differences in weight of the crucibles before and after combustion as follows: ash (%) = [(W3 – W1) / (W2 – W1)] x 100 and organic matter (%) = 100 – ash % where W1 = the weight of the empty, dry crucible; W2 = the weight of the dry crucible containing soil; and W3 = the weight of the dry crucible containing soil following ignition. Note that the weight of the ash = W3 – W1.

Biological Material

Breeding and Adaptation of Earthworms: The earthworm (*A. giardia*), is the omnipresent annelid in the East Algerian region, and play a crucial role in the structuring and evolution of organic matter in no-tilled soils. Interestingly, earthworms are efficient biological model for the terrestrial ecotoxicological studies owed to their low cost, availability and maintenance. In this study, earthworm samples were collected by digging the superficial layer of the soil to a depth of 10 – 15 cm which is the most inhabited zone by earthworms.

The collected earthworm samples were placed in plastic containers at a rate of 45 individuals per container, whose dimensions were 12 cm width, 48 cm length and 15 cm height. Thereafter, the plastic containers were filled with 10 cm of soil from the same garden, in order for the earthworms to adapt and acclimatize in their new environment for 14 – 21 days; they were fed with leaf debris and decaying plant materials during the whole period of the experiment.

Description of the Used Chemicals: Lead is a soft metal possessing two unpaired electrons on the last layer and two major oxidation states Pb^{+2} and Pb^{+4} in addition to the metallic form. Zinc is a moderately reactive metal, can combine with oxygen and react with dilute acids by releasing hydrogen. Its best known oxidation state is Zn^{+2}.

Toxicological Study

Acute Toxicity: The two metals used in this study (lead and zinc) were obtained from Sigma-Aldrich, St. Louis, Missouri, USA. Twelve earthworms were used for LC_{50} determination, and were grouped into four groups of three earthworms each. The first three groups were administered with 10, 100 and 1000 ppm body weight of each metal respectively and mortality monitored, while the last group was subdivided into three groups of one earthworm each and were administered with 2500, 3500 and 5000 ppm body weight of the each metal respectively and mortality monitored. LC_{50} was calculated as: $LC_{50} = [M_0 + M_1] \div 2$, where M_0 = highest dose of test substance that gave no mortality and M_1 = lowest dose of test substance that gave mortality (Lorke, 1983).

Sub lethal toxicity: The sub lethality study of lead and zinc on earthworm (90 individuals) was done using sets of two completely randomized design of five treatments replicated thrice with three worms per replicate, respectively, 45 earthworms per set. Earthworms in treatments A, B, C, D and E were exposed to 0.0, 100, 200, 500 and 1000 ppm of zinc or lead in soil, respectively. 0, 100, 100, 200, 500 and 1000

ppm of each of the chemical (either zinc or lead) was dissolve in one litre of distilled water and used for treatments A, B, C, D and E respectively. The A group served as the control experiment. All the soils used were (inert) sieved using 0.3 mm fine mesh sieve, and autoclaved at 100 ^{o}C for two hour.

Mortality monitoring: The mortality rate was determined by counting the number of dead earthworms per replicate at 24, 48, 72 and 96 hours according to Oluah *et al*. (2010). The earthworms were confirmed dead when they remained immobile and motionless when pricked or touched with an object. The percentage mortality was calculated using the formula: % mortality = [(TM – CM) ÷ (N – CM)] x 100, where TM = total mortality, CM = natural (control) mortality, N = number in the treatments.

Histopathological Evaluation: At the end of the experiment, one earthworms from each replicate (three per treatment) were recovered from the soil, washed with distilled water, transferred to five Petri dishes (labelled A – E corresponding with the treatments) containing 1 % agar gel, kept standing for 96 hours to remove soil from the digestive tract, and then cut into 2 parts (Oluah *et al*., 2010). The earthworms were placed in specimen bottles and fixed with Bouin's fluid for 12 hours before subjecting it to histological procedures of embedding in paraffin wax, sectioning and staining with Haematoxylin-Eosin for microscopic observation. The photomicrography of the histological sections was photographed using Leica EZ4 HD microscopes at x 25 magnification.

Statistical Analysis of Data: Mortality data obtained were analysed using a two-criterion analysis of variance (ANOVA) (concentration and duration) at $p<0.05$ probability.

RESULTS AND DISCUSSION

Soil Physicochemical Composition: The levels of pH (H_2O) and pH (KCl) are presented in Table 1. The physicochemical analysis of the soil showed that the earthworm can live in a neutral soil, because the soil pH (H_2O) and pH (KCl) are in the interval [7.14 – 6.88].

Table 1: Physicochemical characteristics of a home garden soil from Ouled-Driss municipality, Souk-Ahras City Northeast, Algeria

Physicochemical Parameters	Levels
pH (H_2O)	7.14
pH (KCl)	6.88
Electrical conductivity (mmhos/cm)	0.15
Organic matter (%)	8.93
Carbonate (%)	52.99
Saturation level (%)	38.73
Field capacity (%)	19.37

In a similar study, Bennour *et al.* (2020) reported that the pH 7.80 in El Tarf (garden) sandy-loam soil and pH 7.76 in Sidi Amar (field) loamy-clay soil all in Northeast Algeria supported the growth and survival of the earthworm *Lumbricus terrestris* Linnaeus, 1758 (Opisthopora: Lumbricidae). In addition, the study of Römbke *et al.* (2005) proposed a classification of earthworms according to soil pH values, suggesting that majority of the earthworm species in temperate regions are found in soils with a pH between 5 and 7.4. Also, Singh *et al*. (2016) have reported an optimal pH for each species, where worms are absent in very acidic soils (pH<3.5) and scarce in medium acidic soils (pH<4.5).

The electrical conductivity (EC) of the soil was 0.15 mmhos/cm (Table 1). Soil electrical conductivity is a measure of the quantity of salts in soil (soil salinity). It is an excellent indicator of nutrient availability and loss, soil texture, and available water capacity (USDA-NRCS, 2014). As the EC value in this study was lower than 2, the soil was not salty.

The organic matter level was 8.93 % (higher than 3) (Table 1). According to the classification of Huang *et al.* (2009), the soil was rich in organic matter. Peres (2003) highlighted the influence of pedoclimatic conditions on the density and biomass of earthworms, and reported that endemic species prefer soil rich in organic matter.

The level of $CaCO_3$ in the soil was 52.99 % (above 15 %) (Table 1). According to the

classification of FAO (2016), the soil was very strongly calcareous. Lower range values of 0.8 – 28.1 % have been reported for soils in the Annaba plain, North-East Algeria (Fekrache, 2018) indicating that soils from this region tends to be mild – strongly calcareous.

With regards to the soil texture, the value of the field capacity was 19.37 %. This value was within the range of 12 – 24 %, which according to Winterkorn (2008) is of the sandy-silty soil texture. As reported, soil type, depth and texture influence the abundance of earthworm populations (Singh *et al.*, 2016), as well as earthworms are sensitive to soil texture because of the size and roughness of the soil elements, and the physicochemical properties generated by the texture, and in particular, the water retention capacity which is low in a sandy soil environment (Bertrand *et al.*, 2015). Furthermore, a significant positive correlation between earthworm abundance and soil clay content has been established by Chauhan *et al.* (2015).

Acute and Sublethal Toxicities: The medium lethal dose (LC_{50}) of lead (y = 0.6375x + 3.2087 R^2 = 0.6193) and zinc (y = 1.1223x + 1.6893 R^2 = 0.7647) indicated that both chemical were highly toxic to *A. giardia* juveniles (Figures 2 and 3).

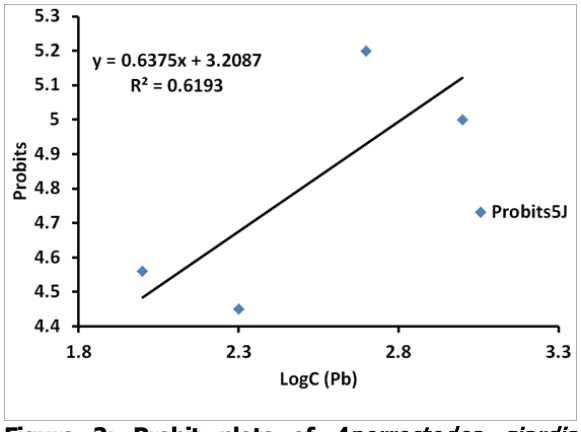

Figure 2: Probit plots of *Aporrectodea giardia* juveniles exposed to varied concentrations of lead in a home garden soil from Ouled-Driss municipality, Souk-Ahras City Northeast, Algeria

The lethal concentrations of lead and zinc that can kill 16 – 90 % of *A. giardia* juveniles increased with increasing mortalities (Table 2). The sub lethal toxicity of lead and zinc on *A. giardia* juveniles was concentration and duration dependent (Table 3).

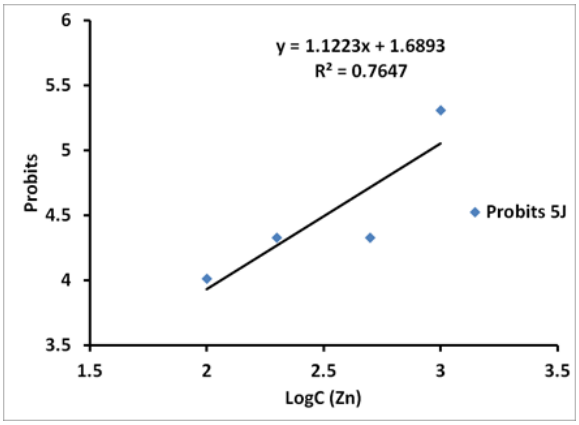

Figure 3: Probit plots of *Aporrectodea giardia* juveniles exposed to varied concentrations of zinc in a home garden soil from Ouled-Driss municipality, Souk-Ahras City Northeast, Algeria

Table 2: Lethal concentrations of lead and zinc to *Aporrectodea giardia* juveniles in a home garden soil from Ouled-Driss municipality, Souk-Ahras City Northeast, Algeria

Lethal Concentrations	Lead	Zinc
LC_{16}	19.43	116.04
LC_{50}	785.22	893.27
LC_{84}	31740.63	6876.13
LC_{90}	92348.13	12394.42

Table 3: Mortality of *Aporrectodea giardia* juveniles due to duration of exposure to varied concentrations of lead and zinc in a home garden soil from Ouled-Driss municipality, Souk-Ahras City Northeast, Algeria

Concentration (ppm)	Mortality of earthworms due to duration of exposure (Hours)			
	24	48	72	96
	Lead			
Control	0	0	0	0
100	3	5	7	8
200	2	6	6	7
500	5	9	9	14
1000	3	10	10	12
	Zinc			
Control	0	0	0	0
100	1	2	4	4
200	2	6	6	6
500	1	2	2	6
1000	7	10	13	15

More deaths were recorded for animals exposed to 1000 ppm lead or zinc for 96 hours than for 24 hours.

Pesticides, heavy metals and other chemical pollutants result in poisoning and disruption of the natural habitat of animals, and hence the ecological imbalance (Ibtissem, 2014). In this context, our findings revealed an increase mortality rate with an increase of the concentrations of lead and zinc treated earthworms. According to Žaltauskaitė and Sodienė (2010), the increase in soils heavy metal content above a certain threshold causes mortality and reduces the density of earthworms by negatively influencing weight, growth, sexual development and cocoon production. Similarly, Scott-Fordsmand *et al.* (2000) and Maboeta *et al.* (2004) have shown that zinc, nickel and copper can be toxic on earthworms at high concentrations and have adverse effects, such as reducing biomass and reducing cocoon production. Additionally, Spurgeon *et al.* (2003), Langdon *et al.* (2005) and Karbowska (2016) reported that lead and thallium are toxic to biological organisms even at low concentrations. Similarly, Pelosi *et al.* (2013) showed that pesticides decrease growth and fertility and increase mortality of earthworms. Moreover, Leveque *et al.* (2015) reported that soil trace metal elements (TMEs) significantly influence the survival of earthworms, and Toure *et al.* (2017) reported that zinc, lead, copper, nickel and thallium were the main pollutants influencing the survival of earthworms along the edges of the highway. Houda *et al.* (2021) have shown that insecticide (Acetamiprid) decrease growth and increase mortality in *A. giardia*, with LC_{50} value varying from 89.03 – 240.23 ppm^{-1}

Effect of Lead and Zinc on *A. giardia* Histology: The histological section of *A. giardia* exposed to 0.0 ppm of lead (control) showed normal tissue structure, in which the chloragogen cells (ch), epithelial cells (SE) and muscle cells (SM) had normal histological architecture (Figure 4). Exposure of *A. giardia* to 100 and 200 ppm of lead caused mild degeneration of the vacuole (V), disruption of the muscles (SM) and the epithelial (SE) (Figures 5 and 6).

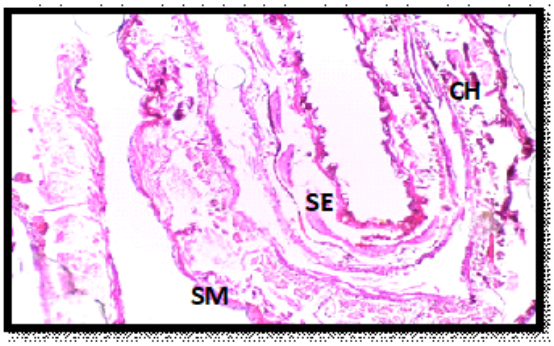

Figure 4: Histological section of *Aporrectodea giardia* exposed to 0.0 ppm of lead (control) Key: Chloragogen cells (ch), epithelial cells (SE), muscle cells (SM) (Magnification x 25, H&E)

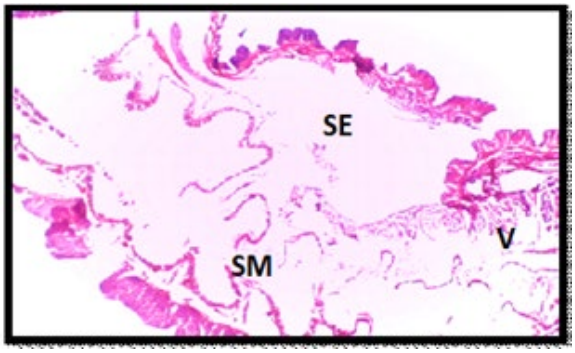

Figure 5: A longitudinal section of *Aporrectodea giardia* exposed to 100 ppm of lead for 96 hours Key: Vacuole (V), epithelial cells (SE), muscle cells (SM) (Magnification x 25, H&E)

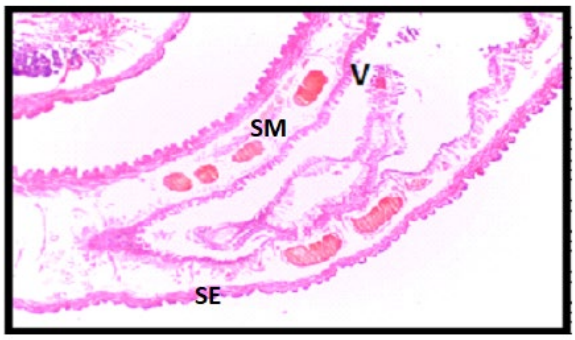

Figure 6: A longitudinal section of *Aporrectodea giardia* exposed to 200 ppm of lead for 96 hours Key: Vacuole (V), epithelial cells (SE), muscle cells (SM) (Magnification x 25, H&E)

Exposure of *A. giardia* to 500 ppm of lead caused moderate degeneration of the vacuole (V), disruption of the muscles (SM) and the epithelial (SE) (Figure 7), while the exposure of *A. giardia* to 1000 ppm of lead caused serve degeneration of the vacuole (V), disruption of the muscles (SM) and the epithelial (SE) (Figure 8).

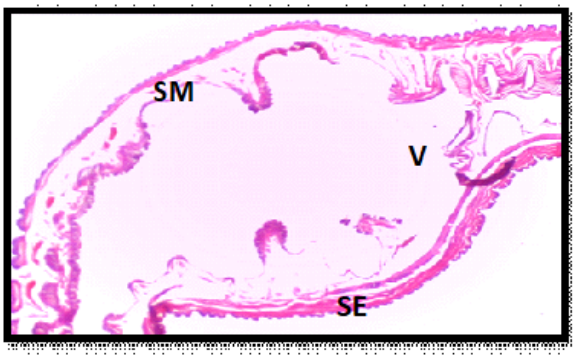

Figure 7: A longitudinal section of *Aporrectodea giardia* exposed to 500 ppm of lead for 96 hours Key: Vacuole (V), epithelial cells (SE), muscle cells (SM) (Magnification x 25, H&E)

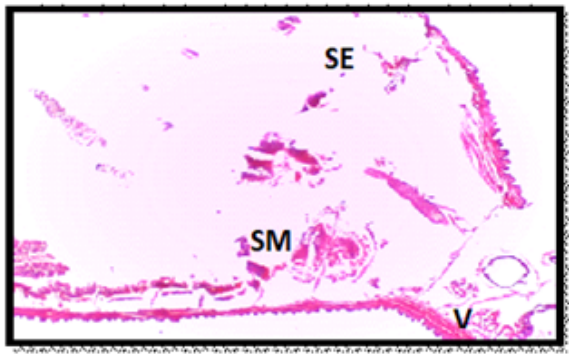

Figure 8: A longitudinal section of *Aporrectodea giardia* exposed to 1000 ppm of lead for 96 hours Key: Vacuole (V), epithelial cells (SE), muscle cells (SM) (Magnification x 25, H&E)

Histological section of *A. giardia* exposed to 0.0 ppm of zinc (control) showed normal tissue structure, in which the chloragogen cells (ch), epithelial cells (SE) and muscle cells (SM) had normal histological architecture (Figure 9).

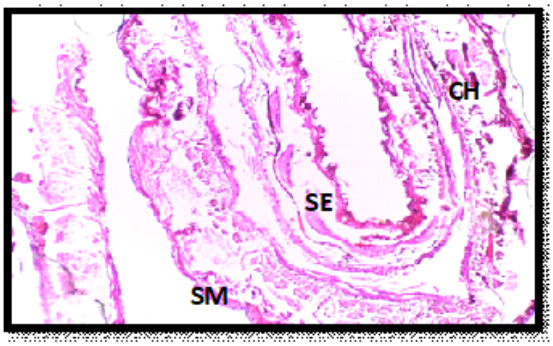

Figure 9: Histological section of *Aporrectodea giardia* exposed to 0.0 ppm of zinc (control) Key: Chloragogen cells (CH), epithelial cells (SE), muscle cells (SM) (Magnification x 25, H&E)

Exposure of *A. giardia* to 100 and 200 ppm of zinc caused mild degeneration of the vacuole (V), disruption of the muscles (SM) and the epithelial (SE) (Figures 10 and 11).

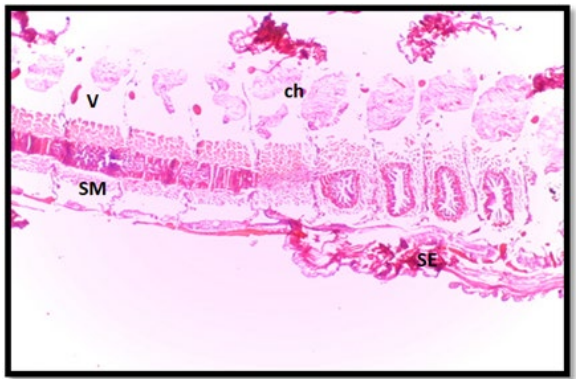

Figure 10: A longitudinal section of *Aporrectodea giardia* exposed to 100 ppm of zinc for 96 hours Key: Chloragogen cells (ch), Vacuole (V), epithelial cells (SE), muscle cells (SM) (Magnification x 25, H&E)

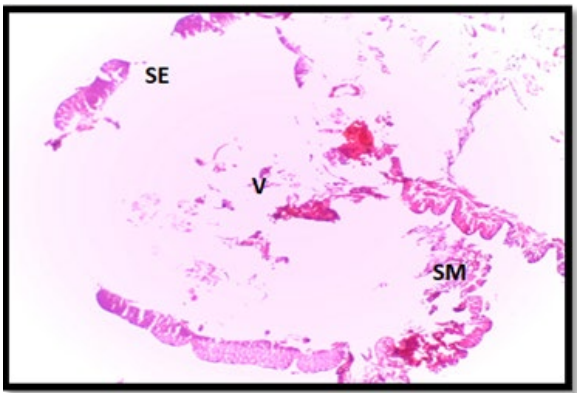

Figure 11: A longitudinal section of *Aporrectodea giardia* exposed to 200 ppm of zinc for 96 hours Key: Vacuole (V), epithelial cells (SE), muscle cells (SM) (Magnification x 25, H&E)

Exposure of *A. giardia* to 500 ppm of zinc caused moderate degeneration of the vacuole (V), disruption of the muscles (SM) and the epithelial (SE) (Figure 12), while the exposure of *A. giardia* to 1000 ppm of zinc caused serve degeneration of the vacuole (V), disruption of the muscles (SM) and the epithelial (SE) (Figure 13).

The study of heavy metal accumulation in earthworm tissues could be a good indicator of the of soil contamination. The findings of this study showed that both heavy metals caused severe alterations in the circular muscles, as evidenced by vacuole formation and cell detachment and separation.

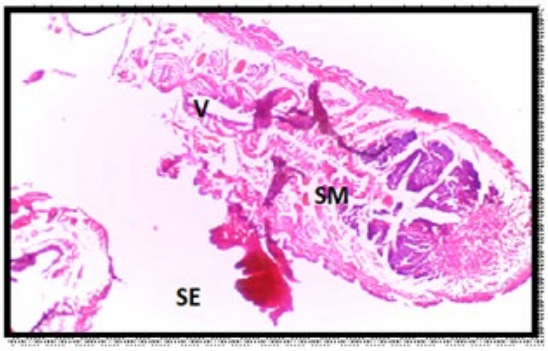

Figure 12: A longitudinal section of *Aporrectodea giardia* exposed to 500 ppm of zinc for 96 hours Key: Vacuole (V), epithelial cells (SE), muscle cells (SM) (Magnification x 25, H&E)

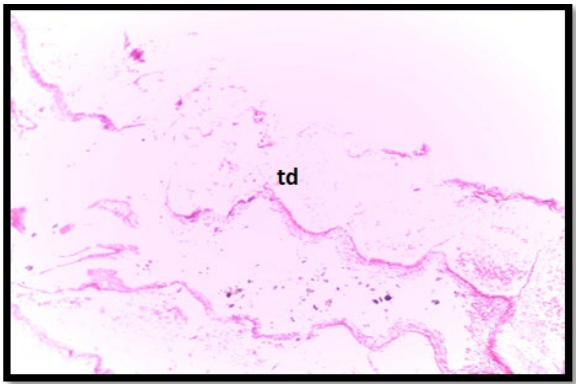

Figure 13: A longitudinal section of *Aporrectodea giardia* exposed to 1000 ppm of zinc for 96 hours Key: tissue destruction (Magnification x 25, H&E)

According to Morgan and Turner (2005) and Oluah *et al.* (2010), histological observations of tissues and cells are valuable tools for assessing the toxic effects of contaminants, such as heavy metals on animal species including earthworms. Thus, heavy metals can accumulate in the digestive tissues, either orally after soil ingestion, or dermally in earthworms that do not have a protective cuticle and live in permanent and direct contact with the contaminants (Conder and Lanno, 2000; Lanno *et al.*, 2004). In this regard, Morowati (2000) noticed marked alterations in the epithelial tissues of the earthworm *Pheretima elongata* Perrier, 1872 (Opisthopora: Megascolecidae) following treatment with glyphosate. Also, Kılıç (2011) reported that xenobiotic damage and accumulation occur primarily in the circular muscles of earthworms exposed to environmental contaminates. In addition, Ibtissem *et al.* (2012) and Ibtissem (2014) reported severe alterations evidenced by formation of epithelial vacuoles in the circular muscles of the earthworm *Octodrilus complanatus* Dugès, 1828 (Crassiclitellata: Lumbricidae), while these alterations can lead to tissue destruction at the higher concentrations of the insecticide (Methomyl). Houda *et al.* (2021) equally reported that histological studies revealed marked alterations in epidermal and intestinal cells of *A. giardia* exposed to Acetamiprid insecticide.

Conclusion: In accordance to the findings of this study, conclusive highlights includes: (i) Several earthworms' species are reported as organism's model for ecological and toxicological research. (ii) *A. giardia* with the other annelidian species are the engineers of the terrestrial ecosystems due to their crucial role in the decomposition of organic matter, the structuring and the aeration of the soil. (iii) *A. giardia* with other earthworm species are bio-indicators of contamination by various pollutants, including insecticides, herbicides, fungicides and heavy metals. (iv) The soil physicochemical analysis showed that the soil is of sandy-silty texture, rich in organic matter and with a neutral pH which explains its suitability for the *A. giardia* survival. (v) The toxicological studies showed that lead and zinc cause increasing mortality rates in relation to increasing used doses. The histological study has shown alterations in epidermal cells, muscle cells and chloragogen cells following metals (lead and zinc) treatment.

ACKNOWLEDGEMENTS

The first author thanks the Laboratory Technicians of Chemistry and Biology Laboratories, without forgetting those of Anatomical Pathology Department. All authors thank Dr. Moundji Taouarfia for the provision of the map of the study area.

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