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Effect of Copper and Iron on Earthworms *Eudrilus eugeniae* (Kinberg 1967) (Oligochaeta, Eudrilidae), under Laboratory Conditions

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ABSTRACT

Earthworms are invertebrate organisms and act as engineers of the soil environment, whose main activity is involved in the decomposition and mixing of organic matter on the structuring and water functioning of the soil, in addition to the effective metal accumulation. As a result, earthworms were reported to be valuable bioindicator organisms of metals-contaminated soils, as well as the sanitary state of the soil. In this regard, the present work was undertaken to study *in vitro* the effect of copper and iron, trace elements, on the body weight growth, survival and tissue histology of *Eudrilus eugeniae* using six increasing concentrations: 250 ppm, 500 ppm, 1000 ppm and 1500 ppm, 2000 ppm and 2500 ppm for 24, 48, 72 and 96 h. The physico-chemical analysis of the soil proved its suitability to support the earthworms' life, owing to its richness in organic matter (OM =11%), sandy-loam texture, and neutral pH. Both copper and iron caused a significant decrease in body weight and no marked mortality rate in treated animals during the entire experimental period, since the mortality of animals was noticed after 7 days of treatment. Moreover, results showed a decrease in the mobility activity of earthworms exposed to low and median doses, but the higher doses of copper and iron-induced a loss of pigmentation and solidification of the body after 7 days of treatment. Further, the no mortality observed after 96 h of copper and iron-treated earthworms was strongly proved by the histopathological observations of tissue sections, showing no signs of alteration or damage in the epidermal cells or the muscle fibers.

Keywords *Eudrilus eugeniae*, Iron, Cu, Growth, Mortality, Histopathology.

INTRODUCTION

Several chemical elements, including heavy metals, are found naturally even, at low quantities in the soil environment and hence can become hazardous inorganic contaminants for non-target organisms. Heavy metals such as lead, cadmium, copper, zinc, and mercury cannot be destroyed and therefore can persist in the environment for long periods. Furthermore, earthworms effectively contribute to the soil nutrient dynamic by changing their physical, chemical, and biological properties (Kooch and Jalilvand 2008). Also, earthworms are sensitive to various

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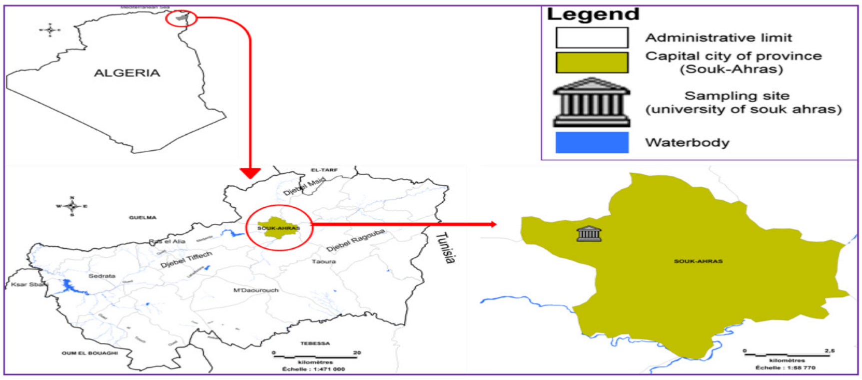

Fig. 1. Sampling site of *Eudrilus eugeniae* (Berrouk *et al.* 2023).

contaminants, and consequently, they are selected as bio-indicators in the biological monitoring of modifications, alterations, and instability of the environment quality, the toxicological assessment of pollutants on earthworms include the *in vivo* or *in vitro* study of their survival, growth, reproduction and behavior via chronic or subchronic exposure.(Cemagref 2010, Piren-Seine 2011). Thus, the performed study was to assess the effects of copper and iron, trace heavy metals, on the growth and survival of adult earthworms *Eudrilus eugeniae.*

Sampling site

Earthworms were obtained from the forest of Mohamed El-Cherif Messaadia University of Souk-Ahras city (Northeast of Algeria) near the Tunisian-Algerian border). It is surrounded by El-taref city from the northeast, Guelma city from the northwest, Tébessa city from the south and Oum El-Bouaghi city from the southwest (Fig. 1).

Soil physico-chemical analysis

Soil samples collected randomly from the study site were physico-chemical analyzed, where the major soil parameters were determined. The pH of the soil was measured as previously described (Baize and Jabiole 1995). In brief, a mixture of 10 g of fine earth was mixed with 50 ml of distilled water, shaken for 10 minutes, left to stand for 30 minutes and then the soil pH value was measured by introducing the electrode of the pH meter into the soil solution. While the pH-KCl was measured by mixing 50 ml of KCl with 20 g of fine earth, stirring for 5 minutes, letting stand for 30 minutes and then the mixture was calibrated by pH meters. Further, the electrical conductivity was determined by the method of Bonneau and Sauchier (1996), in which a solution composed of 10 g of fine earth and 50 ml of distilled water was agitated, left to stand for 30 minutes, and then used to read the EC value displayed on the digital screen, after rinsing the electrode in the solution. The measurement of hygroscopic water or unusable water was performed according to the method described by Baize (1988) which consists of drying the soil sample in an oven at 105 C° for 24 hrs up to get a constant weight and hence the difference in weight before and after drying corresponds to the amount of hygroscopic water. In addition, the field capacity and the saturation capacity were measured according to the experimental protocol of Baize (1988) based on adding water drop by drop to 50 g of fine soil to have a saturated paste, followed by one hour of standing, and then heating step in an oven at 105 C for 24 hrs. The field saturation and the field capacity can be determined by the following equations :

Y=Weight of dry soils (Saturation rate)

FC (Field capacity) = Saturation rate ÷2

Soil texture can be determined using the method of Baize (1988), based on the measurement of the percentage of humidity and comparing it to a scale determining the corresponding textures: 12% (sandy

Table 1. Results of water pH and KCl pH values.

pHWater	pHKCl
8.05	7.72

soil), 12%–24% (sandy-loamy), 24%–45% (sandy-loamy); 45%–75% (clay-loamy), 75% (clayey). The determination of carbonate content in the soil was carried out according to the method of Dermeche *et al.* (1982), where the decomposition of carbonates releases CO_2 in the presence of HCl. On top of that, the organic matter content in the soil was determined by the method described by Bonneau and Sauchier (1996), where the loss of ignition makes it possible to directly measure the organic matter in the soil. In this method, the soil samples were placed for 16 hrs in an oven at 400 C° and the loss of weight after calcination leads to determining the proportions of organic matter.

Biological materials

Sampling and acclimatization conditions of earthworms

The sampling procedure of adult earthworms was performed according to the method previously reported (Bouché 1972). In brief, earthworms were collected from a soil depth of 20 to 30 cm, placed in plastic bins (17 cm wide and 45 cm long) containing soil and plant residues, and then placed in a cool place for 10-14 days under standard acclimatization conditions (air, temperature, and natural photoperiod).

Description of the selected heavy metals (iron and copper)

Iron is a metal transition element with an atomic number of 26 and a symbol Fe, having similar chemical properties as cobalt.

Copper is a metal element with an atomic number of 29 and the symbol Cu, and considered toxic to many aquatic and terrestrial organisms.

Table 2. Organic matter content in the soil samples.

Dry weight of soil (g)	Weight of incinerated soil (g)
91.80	80.80

Table 3. Results of hygroscopic water.

Saturation rate %	Field capacity %
49.43	24.71

Both heavy metals are brought back from the chemistry laboratory of Mohamed El-Cherif Messaadia University.

The toxicological study

Treatments

Six increasing concentrations of each metal separately were chosen (250, 500, 1000, 1500, 2000, 2500 ppm/500 mg^{-1} sol) and three repetitions (5 individuals/repetition) for each concentration were used. The concentrations were prepared by dissolving the test metals in one liter of distilled water, 300 ml of solutions were sprayed on 500 mg of soil and then the earthworms were placed for 24 h, 48 h, 72 h and 96 h. The control untreated earthworms were sprayed with distilled water.

Growth evolution

The growth rate of earthworms was determined by a weight weighing every day from 0 hours to 96 hours using a precision scale.

Determination of mortality rate

The mortality rate was determined by counting the

Table 4. Cumulative mortality of earthworms treated with increasing iron concentrations. Number of concentrations= 6, Number of repetitions per concentration =3, Size of each repetition =5.

	24 h	48 h	72 h	96 h	Cumulative mortality
250 ppm	00	00	00	00	00
500 ppm	00	00	00	1	1
1000 ppm	00	00	00	5	5
1500 ppm	00	00	00	3	3
2000 ppm	00	00	00	1	1
2500 ppm	00	00	00	5	5

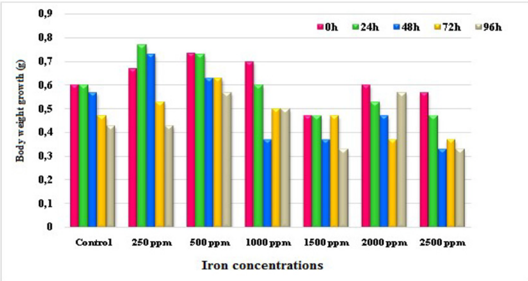

Fig. 2. Effect of increasing iron concentrations on body weight growth of *Eudrilus eugeniae*.

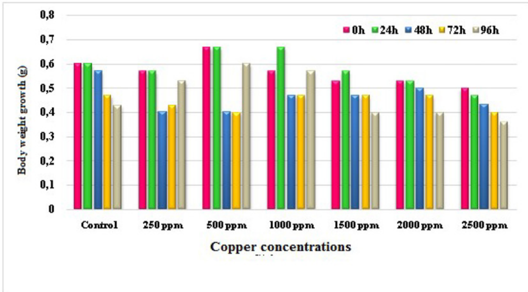

Fig. 3. Effect of increasing copper concentrations on the body weight growth of *Eudrilus eugeniae*.

number of dead earthworms per repetition during the 4 chosen periods, of note the mortality is confirmed by the immobility of the treated earthworms.

Statistical analysis

Mortality results were obtained by two-way analysis of variance (ANOVA) (Concentrations and durations), with a probability of $p < 0.05$.

Histopathological evaluation

The histopathological study of the earthworm tissues was performed in the laboratory of anatomy pathology of Souk- Ahras hospital (Algeria) according to the classical method of histology (Oluah *et al.* 2010, Berrouk *et al.* 2021, Berrouk *et al.* 2022). Briefly, three worms from control and each metal concentration were washed with distilled water, transferred to boxes containing 1% jelly agar, leaf to stand for 96 hrs to release the soil from the digestive tract, and then cut into two parts. After that, the samples were placed in cassettes containing formalin and passed for 18 hrs in the automaton apparatus consisting of 12 stations containing formalin, and xylene before passing them through the paraffin molds. The samples were afterward passed through the microtome to get rid of the extra paraffin using a razor blade and the paraffinic samples were fine-cut by the microtome and spread the sections on slides by putting a drop of water to lay them well. Stain the samples and fix them on slides covered with coverslips and then observe the histological sections under the optical microscope (x40).

Table 5. Cumulative mortality of earthworms treated with increasing copper concentrations. Number of concentrations= 6, Number of repetitions per concentration =3, Size of each repetition =5.

	24h	48h	72h	96h	Mortalité cumulée
250 ppm	00	00	00	00	00
500 ppm	00	00	1	00	1
1000 ppm	00	00	1	00	1
1500 ppm	00	00	00	3	3
2000 ppm	00	1	00	2	2
2500 ppm	00	00	00	9	9

RESULTS

Soil analysis

Water pH and KCl pH values

As deduced from Table 1, the soil is neutral, where the water and KCl pH values are between 8.05 and 7.72.

Electrical conductivity and salinity of the soil

The electrical conductivity (EC) is 194.4 µs/cm, which is inferior to 2 and thus the soil is unsalted.

Soil organic matter contents

As indicated in Table 2, the organic matter rate equals 11% which is higher than 4 and according to the soil classification system provided by Bonneau and Souchier (1996) based on the level of organic matter, the soil is rich in organic matter.

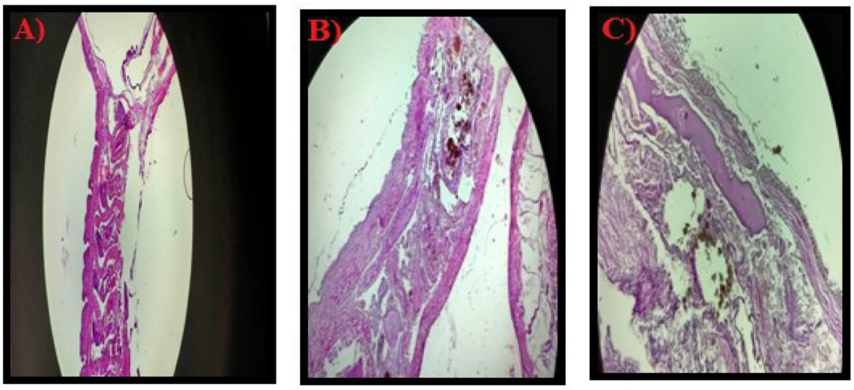

Fig. 4. (A, B, C) Longitudinal sections of *E. eugeniae*, after 96 h (Optical microscope: x40). A) Control untreated earthworms, B) Iron-treated earthworms and C) Copper-treated earthworms, showing normal histological structure.

Hygroscopic water: Field capacitance and saturation capacitance

Table 3 shows that the field capacity equals 24.71, which is approximately located in the interval (12-24) and according to Baize and Jabiol (1995), the soil has a loam-sandy texture.

Effect of Cu and Fe on the body weight growth of earthworms

As shown in Fig. 2–3, the evolution of the body weight decreased markedly in earthworms treated with the different concentrations of copper and iron during the four treatment periods as compared with controls. This decrease was more pronounced in earthworms that received the highest concentrations of chemicals over time.

Mortality

As indicated in Tables 4–5, both metals treated earthworms did not cause intense mortalities during the four treatment periods, even with the high concentrations. Whilst, the recorded mortality rate increases with an increase of concentrations after 96 hrs (from 500 to 1000ppm and from 1500 to 2500 ppm).

Histopathology

As shown in Fig. 4, No marked histological and cellular alterations, including the epidermis, the essential body part of contaminants penetration and the fiber muscles were observed in metals-treated earthworms.

DISCUSSION

As previously reported that the earthworms can be present in all types of soil (Forests, prairies, savannahs, pastures), excluding soils of extreme environments (Glaciers, deserts, acid soils, or very salty soils). In this context, the performed study proved that the earthworms live in moderately alkaline soil, because the soil has KCl pH water pH values between 8.05 and 7.72 and a sandy-loam texture with a saturation rate of 49.43%, and richness level in organic matter (OM=11%), which is suitable for the survival and activity of *Eudrilus eugeniae*. According to the literature, neutral and alkaline soils are favorable to the proliferation of earthworms compared to acid soils characterized by the absence of earthworms. Also, Bachelier (1978) reported that earthworms are more abundant in loam, sandy-clay and loam-clay soils than in gravel and clay soils. The distribution of earthworms was reported to be limited by the richness of the soil in organic matter, and also Aoun and Guerfi (2023) has found that crop residues returned to the soil are essential for the development and growth of all earthworm species. In addition, Hamaizia and Chabi (2021) found that *Eudrilus eugeniae* sampled from the Fatouma El-Souda region in Souk-Ahras city lives in strongly alkaline, sandy-loam, unsalted soil rich in organic matter. *Aporrectodea giardi* collected from the region of Ouled –Idriss in Souk-Ahras city was reported to live in the soil of sandy-loam texture, rich in organic matter, and a neutral pH soil (Berrouk *et al.* 2021, 2022a) and *Lumbricus terresteris* sampled from the Sidi –Fredj in Souk-Ahras city was found to prefer alkaline soil with a silty-clay texture (Berrouk *et al.* 2022b).

Effect of copper and iron on the growth of *E. eugeniae*

Both iron and copper caused body weight decrease in the earthworms treated with the different concentrations during the four treatment periods, especially with the highest concentrations and after 96 hrs. Zhou *et al.* (2006) reported that body weight is the most sensitive index after exposure of earthworms to different toxic chemicals, and similarly, Moukhtel and Touahria (2015) reported negative effects on the body weight of zinc and lead-exposed juvenile *Aporrectodea giardi*. Also, Otmani (2018) has reported a marked decrease in body weight and growth rate in *Allolobophora caliginosa* after exposure to high concentrations of cadmium and/or zinc. According to the literature, all copper-based pesticides caused very remarkable body weight drops in earthworms found near contaminated sites, since iron remains as a metal with no toxic effect on soil invertebrates. Aoun and Guerfi (2023) reported that mercury and cadmium cause a significant drops in *Aporrectodea trapezoides* weight after 96 hrs with 2500 ppm concentration.

Effect of Cu and iron on the survival of *E. eugeniae*

Heavy metals are chemical pollutants (e.g. arsenic, cadmium, mercury and lead) poisoning and disturbing the natural habitat of all animals and can lead to ecological imbalance and hence can be toxic to the environment. Our results revealed no marked mortalities in copper and iron-treated earthworms for the four control periods, even with the high concentrations. This can be explained by the short period of exposure (96 h) and that the effect of the two heavy metals can appear after a long period of exposure where the earthworms exposed to the different concentrations of metals in soils could absorb these metals via the epidermis or digestive tract. This agrees with the results of other authors, who showed no effect on the survival *A. caliginosa* exposed to different concentrations of copper, cadmium, and zinc for 8 weeks and likewise, Otmani (2018) has reported no mortality percentage of *Allolobophora caliginosa* exposed to cadmium and/or zinc. As reported in the literature, heavy metals affect differently living organisms, and their environment and accordingly, iron was reported to be a less toxic metal to human and environmental health (Karimi *et al.* 2021) since copper-contaminated soil was found to be toxic only on young earthworms (juveniles) (Leveque 2014). Further, the number of earthworms could be reduced after treatment for 3 years in a row (40 kg of copper / 1 hectare) and currently, the dose of 4 kg per hectare represents the authorized limit in Europe (Karimi *et al.* 2021). Bart *et al.* (2017) have reported no effect on the survival of earthworms treated with copper, up to 20,000 kg/Cu/hectare. According to other authors the increase in heavy metal content beyond a certain threshold reduces the density of earthworms by negatively influencing body weight growth and sexual development and the production of cocoons. Additionally, Mercury showed a remarkable effect on the survival of *Aporrectodea trapezoides* sub-adult treated with different concentrations for 14 days (Aoun and Guerfi 2023), as well as lead and thallium were found to be quite toxic even at low concentrations to biological organisms Langdon *et al*. (2005). According to Pelosi *et al.* (2014), Toure *et al* (2017), reported that zinc, lead, copper, nickel and thallium were the main pollutants influencing the survival of earthworms, copper-based fungicides have a long-term impact and hence earthworms respond in delay toward this metal. Berrouk *et al.* (2022) have proved that zinc and lead are toxic metals to *Aporrectodea giardi* juveniles since iron was reported to be less toxic to humans and their environment.

Histopathology

The histological evaluation of tissue and cellular alterations in earthworms treated with metallic trace elements is important to support the physiological and biochemical alterations. Our findings revealed no histological alterations and tissue damage was observed in the epidermis and muscle fibers as well as intestinal cells. Other authors have reported that metal trace elements can deteriorate the dynamics of cells and damage their cytoplasmic membranes. In this regard, Amaral *et al.* (2004), Goven *et al.* (2005), have suggested that exposure to heavy metals can cause very significant cytological and ultra-structural alterations. Mincereli *et al.* (2019) have found 20% of damaged DNA of *Eisenia fetida* exposed to copper-contaminated soil for 6 days, and also, Otmani (2018) has reported serious alterations in chlorogenic tissues in Cd and Zn-exposed *Allolobophora caliginosa* for 14 days, since zinc alone did not affect the histological tissues of animals. Another study has reported marked histological alterations in epidermal cells and muscle fibers of *A.giardi* earthworms (Berrouk *et al.* 2022).

CONCLUSION

Earthworms effectively contribute to the development and maintenance of soil fertility, in addition to the transformation of organic waste into nutrients, and as a result, they are considered the most important invertebrates of the soil fauna. Moreover, the intense use of pesticides and the contamination of ecosystems by heavy metals directly or indirectly influence all living organisms, including earthworms. Our study resulted in :

Iron and copper markedly affected the body weight growth of *Eudrilus eugeniae*.

Iron and copper did not cause intense mortalities, and noteworthy, the recorded mortality rate was proportionally related to the increased concentrations. Iron and copper did not cause histological alterations in epidermal and intestinal cells and muscle fibers.

Use another animal species to better assess the sensitivity and bio-indicator potential of earthworms.

Test the effect of other more toxic heavy metals, such as cadmium and mercury.

Test other biochemical biomarkers like total protein, total lipids, and total carbohydrates, and antioxidant parameters, including MDA, CAT, AChE, GST.

Test the reproductive effect of heavy metals in earthworms.

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