

## **Biochemical responses of the earthworm *Allolobophora caliginosa* exposed to cadmium contaminated soil in the Northeast of Algeria.**

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### **Résumé**

Les réponses de vers de terre *Allolobophora caliginosa* aux expositions sub-létales de cadmium (Cd) (25, 50, 100, 150 mg/500g d.w), ont été examinées dans cette étude. L'Enquête a été faite sur le taux de protéines totales, le stress oxydatif et la réponse antioxydante à travers un ensemble de biomarqueurs enzymatiques ((catalase (CAT), glutathione (GSH), glutathione S-transferase (GST) et malondialdéhyde (MDA)), qui ont été choisie pour évaluer les réponses des vers de terre dans une période jusqu'à deux semaines. Les vers de terre exposés au cadmium ont démontré différentes réponses des biomarqueurs enzymatiques. Le cadmium a augmenté le taux de protéines totales. De plus, la surveillance des biomarqueurs de stress oxydatif a montré un épuisement significatif au niveau du glutathione (GSH) accompagné d'une forte augmentation de glutathione S-transferase (GST) et de la stimulation de l'activité catalase (CAT) et malondialdéhyde (MDA). Nos résultats indiquent les effets toxiques du cadmium sur les vers de terre *Allolobophora caliginosa*.

**Mots clés :** Cadmium, *Allolobophora caliginosa*, protéines totales, stress oxydatif, Biomarqueurs.

### **Abstract**

The responses of earthworms *Allolobophora caliginosa* to sub-lethal exposures of cadmium (Cd) (25, 50, 100, 150 mg/500g d.w), were investigated in this study. Investigation was done on the total proteins rate, oxidative stress, and antioxidative response through a set of enzymatic biomarkers ((catalase (CAT), glutathione (GSH), glutathione S-transferase (GST) and malondialdehyde (MDA)), which were selected to evaluate the responses of the earthworms in a period up to two weeks. The earthworms exposed to the cadmium stress demonstrated different responses of the enzymatic biomarkers. Cadmium stress increased the total proteins rate. Furthermore, monitoring biomarkers of oxidative stress showed a significant depletion in glutathione level (GSH) accompanied with a strong increase of glutathione S-transferase (GST) and stimulation of catalase activitie (CAT) and

malondialdehyde (MDA). Our results indicated the toxic effects of cadmium on *Allolobophora caliginosa* earthworms.

**Keywords :** Cadmium, *Allolobophora caliginosa*, total proteins, Oxidative stress, Biomarkers.

## **1. Introduction**

Widely spread in the environment from anthropogenic sources, Heavy metals have caused damages to ecosystems close to emission sources (Derome and Nieminen, 1998). Although they are essential trace elements, metals above certain concentrations and exposure times are toxic to soil animals and affect the abundance, diversity, and distribution of the animals (Hopkin, 1989). In many ecosystems, earthworms play a major role in the development and maintenance of soil structure. They have a great impact on soil decomposition activity, on nutrient mineralization, and hence on primary productivity (Edwards and Bohlen, 1996). Earthworms play a key role in many soil processes and are in full contact with the soil solution, thus they are relevant indicators of environmental change and have been included in a group of five key indicators for ecotoxicological testing of industrial chemicals determined by the Organization de Cooperation et de Developpement Economiques and the European Economic Community (Capowiez *et al.*, 2003). Earthworms are more susceptible to metal pollution than many other groups of soil invertebrates (Bengtsson *et al.*, 1992). Considering their relative sensitivity, toxicity data from earthworms are important in determining safe levels for metals in soil (Spurgeon and Hopkin, 1996). This species accumulate high concentrations of metals from contaminated soil (Morgan and Morgan, 1999). Cadmium is a non-essential heavy metal is very toxic and have particularly harmful effects on organisms (CEPA, 1994). It is effectively lethal to earthworms and particularly affects earthworms growth and reproduction (Matthias, 2010). Many previous studies focused on Cd effects on earthworms *E. fetida*' survival, reproduction and even the responses of detoxification system as well. While the effects of high-level Cd on earthworms *Allolobophora caliginosa* (an abundant species in the region of Seraïdi-Annaba) are rarely reported, that's what the present work carried to investigate. The main aim of this study was to evaluate the effects of cadmium on the growth, oxidative stress, and antioxidative response through a set of enzymatic biomarkers (CAT, GSH, GST and MDA) of earthworms *Allolobophora caliginosa* upto two weeks, and to provide valuable information for ecological risk assessment of the cadmium effects at environmentally relevant levels.

## **2. Materials and methods**

### **2.1. Biological material**

Adult (clitellate) earthworms (*Allolobophora caliginosa*), weight subsequently found to average 1.5 g, were collected from Séraïdi region near Annaba (East of Algeria). They were randomly assigned to treatment groups, either their native substrate (soil) under laboratory conditions ( $20 \pm 1^\circ\text{C}$ ; 12 h dark/12 h light).

## **2.2. Chemical material and soil amendment**

Cadmium chloride ( $\text{CdCl}_2$  pentahydrate, Fluka) was the chemical product used in our experiment. Deionized water was used as vehicle to spike soil with cadmium. Solutions were prepared to obtain the following nominal concentration in soil: 0 (control), 25, 50, 100, 150 mg/500g d.w. Soils were kept at 20°C for 14 days after spiking and before the beginning of the assays. Deionized water and Cd solutions were added to get 75 % of the soil water holding capacity (WHC).

## **2.3. Exposure and measurements**

The worms were weighed and exposed at different concentrations of Cd-spiked soil (500 g d.w.) in terrariums, with 5 adults per terrarium and for each concentration. Humidity in terrariums was adjusted by adding deionized water if necessary (12-15 % humidity). Worms were fed with 2 grams of dried cereals, every week. After 7 and 14 days earthworms were then collected and purged during 24 h, then weighted. The worms were cut into five portions for analysis of various parameters. Total proteins content was measured according to the method of Bradford (1976). The determination of glutathione rate (GSH) was realized according to the method of Weckberker and Croy (1988). The measurement of glutathion-S-transferase (GST) activity was realized according to the method of Habig *et al.* (1974). The catalase activity measurement (CAT) was followed according to the method of Regoli and Principato (1995). The rate of malondialdehyde (MDA) was measured according to the method of Draper and Hadley (1990).

## **2.3. Stastical analysis**

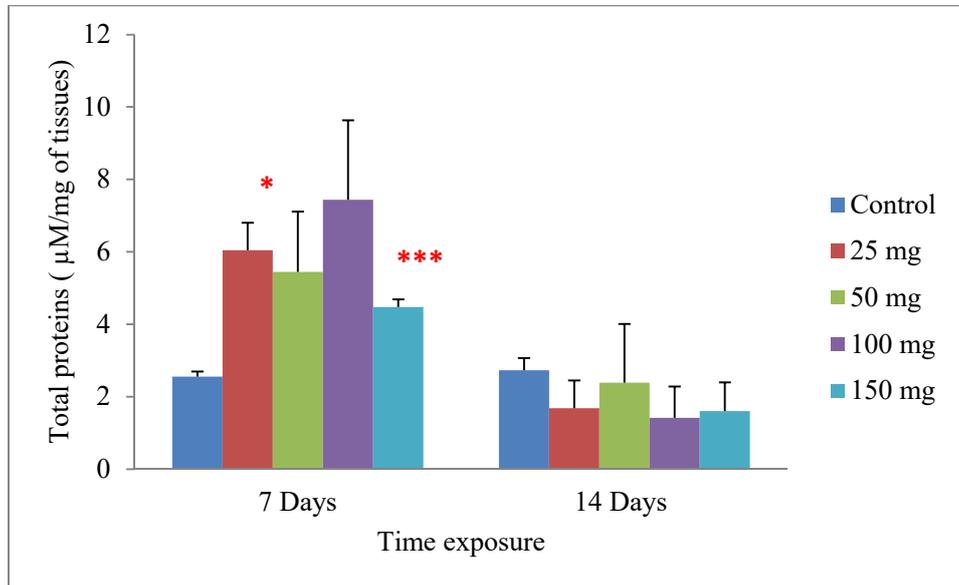
The obtained results were expressed as the Mean  $\pm$ Standard Deviation (SD) with three independent replicates. Data were analyzed by one-way analysis of variance (ANOVA). To look for differences by pairs between treated samples and the control, we applied the Student's t-test. Statistical analyses were performed using the Minitab software (14.0).

## **3. Results**

### **3.1. Total proteins rate**

Data are the mean  $\pm$  S.E. of three replicates. In the period of 7 days, a significant increase in the total proteins rate compared with the control was observed following exposure to the lowest concentration of cadmium 25 mg/500g d.w ( $p=0.016$ ), and for worms treated with the highest concentration of cadmium 150 mg/500g d.w this rate was very highly significantly increased to be  $4.47\pm 1.59$  when compared to the control  $2.55\pm 0.13 \mu\text{M mg}^{-1}$  of tissues ( $p = 0.001$ ). However, the effects of cadmium were less intense in the concentrations of 50 and 100 mg/500g d.w, where there were no significant differences in the total proteins rate compared with the control. While in a period of 14 days, the exposure of worms to cadmium resulted in no significant decrease of the total proteins rate compared with the control

$p = 0.156$ . Significant differences from the control are indicated as:  $p \leq 0.05$ (\*) and  $p \leq 0,001$  (\*\*\*) very highly significant (Fig.1).



**Fig.1:** Effect of cadmium stress on the total proteins rate in *Allolobophora caliginosa*

### 3.2. GSH rate

Data are the mean  $\pm$  S.E. of three replicates. A non-significant increase in the GSH rate compared with the control was observed following exposure to all concentrations of cadmium in a period of 7 days. However in a period of 14 days, it was found that cadmium causes a dose dependent decrease in the GSH level. Compared to the control ( $3.60 \pm 0.16$ ), GSH level was significantly determinate to be  $2.04 \pm 0.46 \mu\text{mol mg}^{-1}$  protein ( $p = 0.031$ ) in earthworms treated with the concentration of 50 mg/500g d.w. For worms treated with 100 and 150 mg/500g d.w this rate was very highly significantly reduced to be  $1.22 \pm 0.26$  and  $1.31 \pm 0.28 \mu\text{mol mg}^{-1}$  protein ( $p = 0.001$  and  $p = 0.001$ ), respectively. Significant differences from the control are indicated as:  $p \leq 0.05$ (\*) and  $p \leq 0,001$  (\*\*\*) very highly significant. (Fig.2).

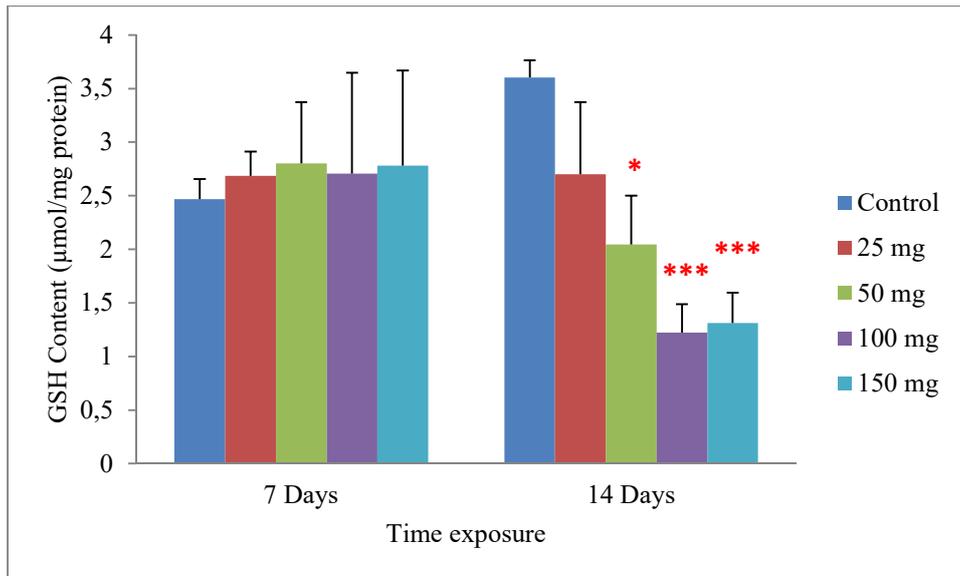


Fig. 2: Effect of cadmium stress on the GSH rate in *Allolobophora caliginosa*

### 3.3. GST rate

Data are the mean  $\pm$  S.E. of three replicates. In the period of 7 days, GST activity showed a tendency of decrease, but only became significant ( $P < 0.05$ ) with concentrations 50 and 150 mg/500g d.w compared to the control. While in a period of 14 days, the exposure of worms to cadmium resulted in an induction of the GST activity. The enzymatic activity appeared with concentrations 25, 100 and 150 mg/500g d.w, which had significant increase when compared to the control ( $p = 0.043$ ,  $p = 0.023$  and  $p = 0.043$ ), respectively. However, the effects of cadmium were less intense in the concentration 50 mg/500g d.w, where there were no significant differences in GST activity  $p = 0.123$ . Significant differences from the control are indicated as:  $p \leq 0.05$  (\*) (Fig.3).

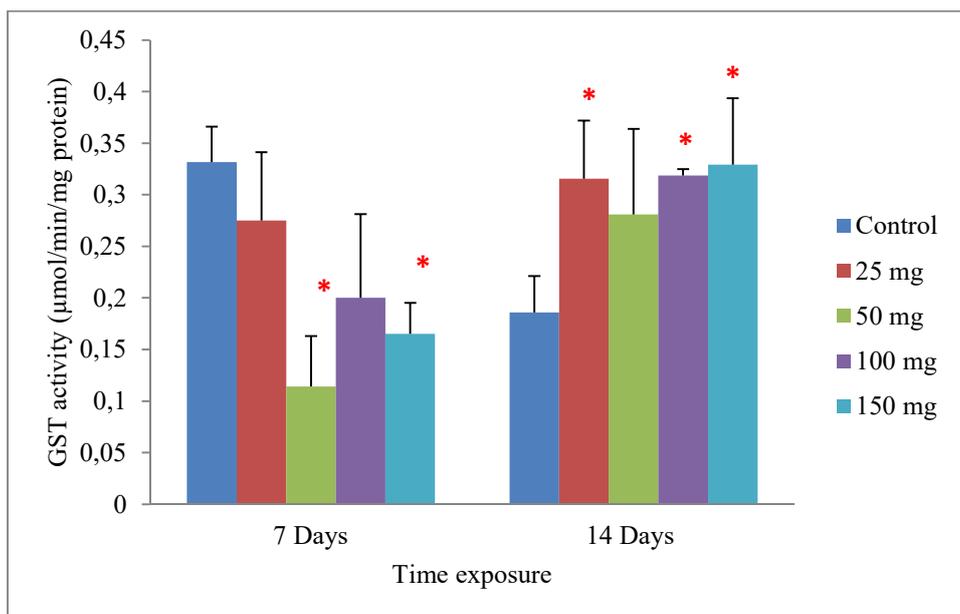


Fig.3: Effect of cadmium stress on the GST activity in *Allolobophora caliginosa*

### 3.4. CAT activity

Data are the mean  $\pm$  S.E. of three replicates. The results show a non-significant increase in CAT activity compared with the control, in presence of the xenobiotic (Fig.4).

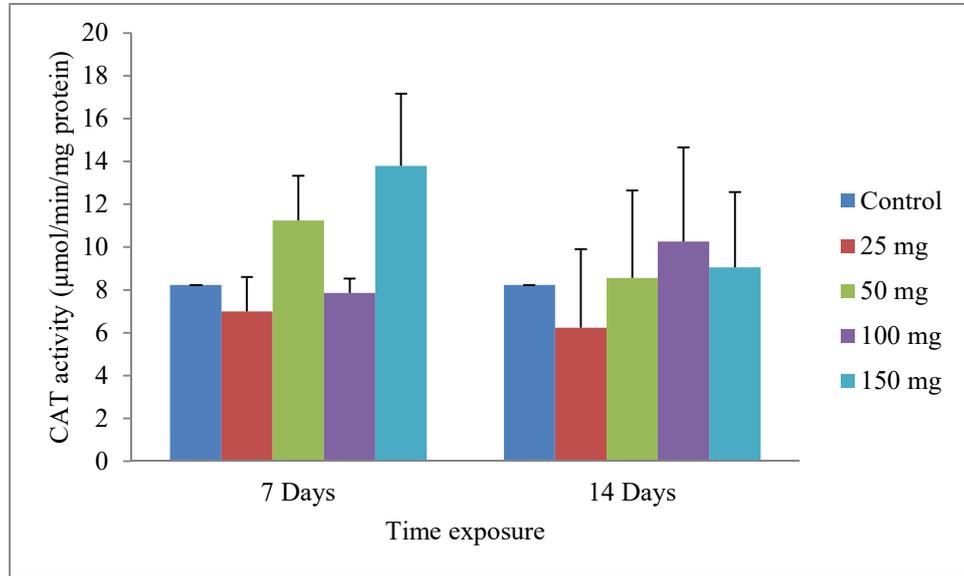


Fig.4: Effect of cadmium stress on the CAT activity in *Allolobophora caliginosa*

### 3.5. MDA level

Data are the mean  $\pm$  S.E. of three replicates. The results show a non-significant increase in MDA level compared with the control, in presence of the cadmium (Fig.5).

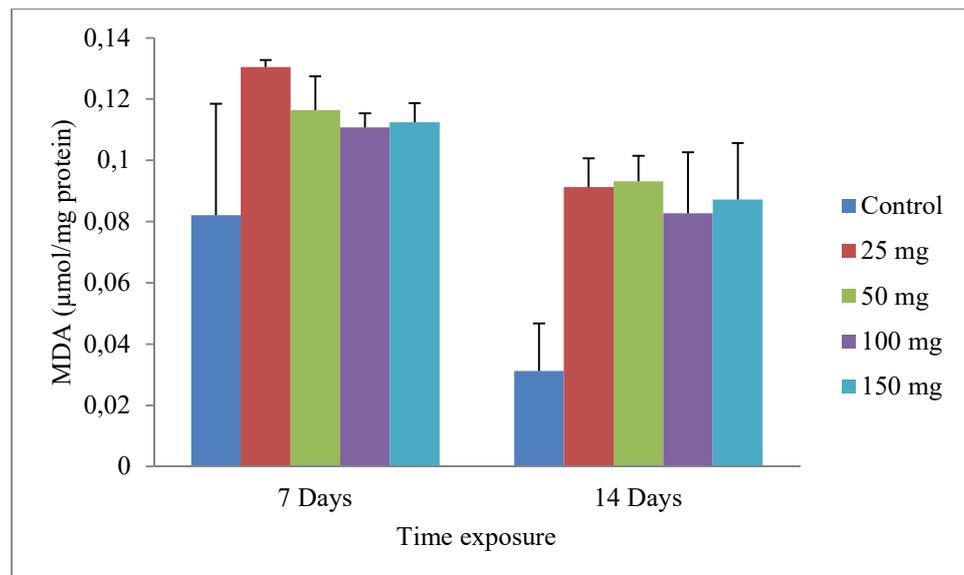


Fig.5: Effect of cadmium stress on MDA level in *Allolobophora caliginosa*

### 3. Discussion

In agricultural ecosystems, where animal farming and related agricultural practices are intensive, heavy metals can also reach the soil due to application of liquid and soil manure (or their derivatives, compost or sludge) or inorganic fertilizers. The presence of heavy metals in terrestrial ecosystems is of concern for their high toxicity and ability to be biomagnified through the food web, posing a threat to the Environmental health and survival of the resident organisms (El-Shenawy *et al.*, 2012). This contamination causes a stress in bioindicator organisms of pollution by distorting their physiological and biochemical capacities in different degrees (Zeriri *et al.*, 2012). Earthworms have great potential in risk assessment of contaminated land and act as an indicator for ecosystem health (Nahmani and Lavelle, 2002). The worms are capable to uptake and tolerate high levels of heavy metals, suggesting its suitability for environment monitoring (Usmani and Kumar, 2015). The Majority of studies have been conducted on toxicological effects of cadmium on earthworms *Eisenia fetida*. From this vision, the aim of the present study is to evaluate the toxicity of cadmium on earthworms rarely reported: *Allolobophora caliginosa*, by monitoring some physiological and antioxidant responses of worms exposed to a battery of high cadmium concentrations. Biomarkers can be used as early warning indicators of environmental contamination and potential adverse effects on populations (Peakall, 1994). In earthworms, two coexisting intracellular pathways are responsible for metal accumulation. The first involves retention of metals (particularly Pb) in insoluble phosphate granules called chloragosomes (Morgan and Morris, 1982). The second pathway is the intervention of sulfur-rich ligands like metallothionein-like proteins (MTLP) able to bind metals (particularly Cd) within cadmosomes (Morgan and Morgan, 1990; Morgan *et al.*, 1993). Since proteins “MTs” are physiologically involved in essential metal ions transport and regulation, and function as intracellular distributors of copper and zinc, they also play a role in non-essential metal regulation and detoxication (Klaassen, 1995; Klaassen *et al.*, 1999). They are particularly known to protect cells from Cd toxicity and oxidative stress (Andrews, 2000). In this work, we noted in the period of 7 days a significant increase in the total proteins rate of the lowest concentration of cadmium 25 mg/500g d.w, and for worms treated with the highest concentration of cadmium 150 mg/500g d.w this rate was very highly significantly increased. This finding is in agreement with those of Bernard *et al.* (2010) and Xiaohui *et al.* (2012), who showed an increase in the rate of total proteins of worms *Eisenia fetida*, *Lumbricus rubellus* and *Lumbricus terrestris* treated with increasing concentrations of cadmium. Similar induction of proteins after Cd exposure was already observed in aquatic organisms treated with increasing concentrations of cypermethrin which contain heavy metals (Amamra *et al.*, 2015). However, we noted a regulation of total proteins rate in a dose dependent manner in the period of 14 days, this finding is in agreement with those of Spurgeon *et al.* (1994) and Matthias (2010), who showed a significant up-regulation of MT in both species *Eisenia fetida* and *Lumbricus terrestris* exposed to cadmium, The high tolerance of earthworms to cadmium poisoning is due probably to detoxification by metallothionein proteins in the posterior alimentary canal (Morgan *et al.*, 1989). Ireland (1978) found that cadmium and lead are particularly concentrated in chloragogen cells in *L. terrestris* and *Dendrobaena rubidus*, where it bounds in the form of Cd-metallothioneins and Pb-metallothioneins. The chloragogen cells in earthworms appear to accumulate heavy metals

absorbed by the gut and immobilize the metals in small spheroidal chloragosomes and vesicles found in these cells (Sinha *et al.*, 2008; Usmani and Kumar, 2015). All the studies including our results suggest that MT could be developed as a more effective biomarker for determining soil cadmium contamination levels. The Glutathione considered as the vital second-line defenses against oxidative damage which is the major non enzymatic radical scavenger in the animal cells (Moumeni *et al.*, 2016) and Glutathione-s-transferase (GST) as one of phase II Enzymes can catalyze the conjugation reaction of GSH and electrophilic xenobiotics, and thus contribute to the removal of reactive electrophiles. This enzyme also plays a role in cellular protection against Oxidative stress (Xiaoxia Yang *et al.*, 2012; Sulata *et al.*, 2008). In the cell, there is equilibrium between the reduced and oxidized forms of glutathione which have the efficiency to provide antioxidant defenses against the heavy metal Cd on Earthworms. In our experiment, we noted a dose dependent increase in the GSH level parallel to the significant decrease of the GST activity for 7 days of exposure may be due to an up regulation of the GSH synthetic pathway and may be a protective mechanism against the toxic effects of the metal. (Sulata *et al.*, 2008; Xiaoxia Yang *et al.*, 2012) indicating the earthworms failed to cope with the toxic stress from exposure to Cd through their detoxifying and antioxidant mechanisms during a short exposure period. After 14 days of exposure, the present investigation clearly demonstrates a strong dose-response correlation between the cadmium and GSH expression. GSH expression seems to be sensitive to cadmium: it was strongly declined even at low cadmium concentration that depletion might be due to its massive utilization through the oxidation of two molecules of GSH to a molecule of GSSG. GSH also scavenges reactive oxygen species (ROS) and the Cd can be sequestered by GSH to prevent its adverse interaction with biomolecules. GSH and other thiols play a crucial role in cleaning ROS (Broerse *et al.*, 2012; Sulata *et al.*, 2008; Xiaoxia Yang *et al.*, 2012). similar conclusions was reached by Saint-Denis *et al.* (1999), Sulata *et al.* (2008), and Zeriri *et al.* (2012), who showed a significant decrease of GSH rate on *Eisenia fetida andrei* exposed to benzo [a] pyryne and *Octodrilus complanatus* exposed to pesticide. This is in agreement with earlier studies (Moumeni *et al.*, 2016; Amamra *et al.*, 2015). That elucidated a significant depletion of the GSH content in paramecia exposed to insecticide, parallel to a significant increase in GST activity known that it exert the function of detoxification, Usually the depletion of the GSH leads to GST activation with a simultaneous re-synthesis of GSH for the replacement of the normal level, so it plays a major role in the metal metabolism by organisms and provide to the cell a state of equilibrium and protection against oxidized reactive species (Mofredj *et al.*, 1999). Studies conducted on earthworms exposed to Heavy metals (such as Cd) (Brown *et al.*, 2004, Ribera *et al.*, 2001, Saint-Denis *et al.*, 1999; Xiaoxia Yang *et al.*, 2012) and to pesticide (Zeriri *et al.*, 2012), and studies conducted on paramecia exposed to pesticide (Moumeni *et al.*, 2016) and cypermethrin (Amamra *et al.*, 2015) demonstrated the same results. Catalase represents an important link in the detoxification of ROS generated as a result of a toxicity, which is regarded as an enzyme presenting a clear and early response to contamination. In the present study, CAT activity was found to be increased after 7 and 14 days of exposure. This increase might be due to up-regulation of CAT activity, which could be an adaptive mechanism to prevent the accumulation of toxic Reactive Oxygen Species ROS (El-Shenawy *et al.*, 2012). This result was in agreement with the findings of Zhang *et al.* (2009) 48 h *Eisenia fetida*. According to Torres *et al.* (2002) these data suggest

that the increase in antioxidant defenses would be due to enhanced oxygen free radicals production, which could stimulate antioxidant activities to cope with this increased oxidative stress and protect the cells from damage. LPO is a complex process in which polyunsaturated fatty acids in the biological membrane system undergo changes by chain reactions and form lipid hydroperoxides, which decompose double bonds of unsaturated fatty acids and disrupt membrane lipid (Radwan *et al.*, 2010). After 7 and 14 days of exposure, a noticeable increase in lipid peroxidation biomarker MDA was observed in *Allolobophora caliginosa* earthworms treated with different concentrations of cadmium. These findings are in good agreement with those of (Radwan *et al.*, 2010) who recorded a significant elevation in MDA after 48 h of exposure in *T. pisana* snails contaminated by Cd- and Zn. Also (Zeriri *et al.*, 2012) indicated that the level of MDA increase in earthworms treated with different concentrations of methomyl. These results were in agreement with the present data where increasing the levels of Cd elevated the LPO level in earthworms (Li *et al.*, 2008; Xue *et al.*, 2009) suggested that one of the most damaging effects of ROS and their products in cells is the peroxidation of membranous lipids, which can be indicated by the detection of MDA. In conclusion, the results demonstrate that *Allolobophora caliginosa* earthworms are sensitive to metal toxicity. Since it showed an important oxidative stress translated by an outbreak of a system of detoxification (GSH, GST), a stimulation of the oxidative enzyme activity: the catalase. This effect is accompanied by an up-regulation of total protein and a peroxidation of the lipids (MDA). It emerges that the *Allolobophora caliginosa* earthworms are an excellent bio-indicator model of the pollution and the state environmental. However, more research is needed for better understanding and application of results from *Allolobophora caliginosa* earthworms, such as how heavy metals interact with different soil types. Also more works are needed such as MT gene expression, cocoon reproduction etc, and a suite of biomarkers is helpful in the evaluation of heavy metals toxicity on earthworms and could be instrumental in monitoring soils contaminated with multiple pollutants.

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