Cadmium toxicity evaluation in the earthworm *Eisenia foetida*: behavior and histopathological effects

Martin G. Rodriguez[1],*, **Birmania H. Rivera[2], Javier Ventura-Juárez[3] and Martin H. Muñoz-Ortega[4]**

[1]Physiology and Pharmacology Department, Basic Sciences Center, Autonomous University of Aguascalientes, C. P. 20900, Mexico, [2]Posgrado en Ciencias Biológicas, Facultad de Ciencias, Universidad Nacional Autónoma de México, Mexico City, Mexico, [3]Morphology Department, Basic Sciences Center, [4]Chemistry Department, Basic Sciences Center, Autonomous University of Aguascalientes, Aguascalientes, México

ABSTRACT

Most studies of cadmium toxicity have focused on sublethal effects and there has been little research into the mechanism of its toxicity. The aims of this work were to assess the toxicity of cadmium to earthworm *Eisenia foetida* using contact and avoidance tests and to examine their effects on behavior, morphology and histopathology to explain the cadmium toxicity. The first contact of the toxic substance with the earthworm is with the cuticle. The LC50 for cadmium was 19 µg/cm^2. Earthworms avoided cadmium in artificial soil but this was not dependent on cadmium concentration. Cadmium toxicity initially caused abnormal behavioral/physiological responses, including curling, coiling, excessive mucus secretion, sluggish movements and, finally, loss of locomotion. Histological changes included destruction of cuticle, severe lesions and destruction of body wall, bleeding, and autotomy. Cadmium was found in the chloragogenous tissue. Together, these observations suggest a toxicity mechanism that involves disruption of the sensory system, anomalous behavior, loss of locomotion, and severe lesions, leading to death.

KEYWORDS: cadmium, toxicity mechanism, histopathology, avoidance, earthworm

*Corresponding author: romag18@hotmail.com

INTRODUCTION

Earthworms are ubiquitous, abundant and important for soil processes. They may get exposed to toxics from natural or anthropogenic sources, including toxic metals and chemical pollutants from industrial and agricultural activities [1]. Hence, earthworms have been used as bioindicators and biosensors of sublethal concentrations of pollutants, thus providing a warning of the early effects of soil contamination [2]. Among earthworm species, *Eisenia foetida* is most commonly used as a bioindicator of soil contamination because it can be easily bred on a variety of organic wastes with short generation times, under laboratory conditions [3]. Earthworms may be exposed to contaminants in two ways. First, living in the soil they are in direct contact with soil pore water which may contain contaminants. Rapid exchange of water takes place across the body wall. Second, during their burrowing, pollutants adsorbed onto solid particles may be ingested along with leaf litter and other organic soil constituents [4]. A wide variety of exposure methods have been utilized to examine the effects of environmental contaminants on earthworms. Of particular note are the artificial soil test and the filter paper contact test to assess acute toxicity [1, 5], and the avoidance test to evaluate the ecotoxicological risks [6, 7, 8, 9].

Cadmium has no known physiological function in organisms and, as a heavy metal element it is

non-biodegradable. It has been used extensively in industrial processes and is delivered as waste into soils and rivers [10]. Cadmium is very harmful to soil organisms, including earthworms and there have been numerous surveys of cadmium toxicity. Early studies indicated that earthworms collected from contaminated soil had cadmium stored within their tissues [11, 12, 13]. LC50 values for *E. foetida* obtained from contact tests and soil tests ranged from 253 to 1843 mg/kg [14, 15, 16]. At sub-lethal levels, cadmium did not affect the growth of earthworms but did inhibit the production of cocoons [17, 18].

As noted, earthworms may take up metals by ingestion of contaminated soil particles and by absorption through the skin [3]. Although the filter paper test is not directly comparable with soil exposure, it can provide information about the sublethal effects and mechanism of cadmium toxicity. Absorption through the skin is believed to be the dominant exposure route. Thus, it is necessary to understand the functional and structural properties of epidermis. Morphologically the earthworm epidermis consists of supporting and basal cells, at least two types of mucus-secreting goblet cells, and the overlying cuticle [19]. The cuticle is the outermost layer in contact with the pore water. It is composed mainly of collagen fibers and polysaccharides [20, 21]. The epidermal cells are involved in the secretion of cuticle, active ion transport and mucus secretion, and they provide the first line of protection against soil particles and bacterial infection [21].

Avoidance is one of the behavioral endpoints commonly used to study the effects of chemicals on animals, which has subsequently been integrated into earthworm toxicology [6]. Avoidance behaviors are phenotypic adaptations that allow animals to survive in changing environments. Behaviors provide a unique perspective on toxicology because they represent the link between the physiology and ecology of an organism and its environment [22]. Behaviors are sequences of quantifiable actions operating through the central and peripheral nervous systems. They are the cumulative manifestations of genetic, biochemical and physiological processes essential to life, including feeding, reproduction and predator avoidance. Behaviors allow organisms to adjust to external and internal stimuli and to meet the challenges of survival in a changing environment. Conversely, behaviors also result from adaptation to environmental variables. Thus, behaviors are selective responses that are constantly adapting through direct interaction with physical, chemical, and social aspects of the environment. Consequently, evolutionary selection conserves stable behavioral patterns in conjunction with morphological and physiological adaptations. This stability optimizes opportunities for survival and reproductive success by enabling the organism to exploit resources efficiently and by defining suitable habitats [22]. Rand [23] stated that behavioral responses most useful in toxicology should be: (1) well-defined endpoints that are practical to measure, (2) well understood relative to environmental factors that cause variation in the response, (3) sensitive to a range of contaminants and adaptable to different species, and (4) ecologically relevant. Kane *et al.* [24] added a further criterion that the response should help to elucidate the mode of action of specific chemical classes. To satisfy these requirements, the selection of a particular behavioral endpoint in any toxicity study must take into account the mechanism of action, the route of uptake, and the chemical and physiological properties of the toxin of interest [25]. The aim of this paper was to evaluate the cadmium toxicity in earthworm *Eisenia foetida* and study their effects on behavioral, morphological and histological changes to gain more insight on the mechanisms of cadmium toxicity.

MATERIAL AND METHODS

Animals

Earthworms, *Eisenia foetida* were purchased from a local dealer. Worms were maintained in a plastic case that contained a mixture of humid soil, banana peel and oat husk fine milled. They were incubated in a chamber with constant temperature (20 ± 2 °C). This medium favored their growth and many cocoons emerged on the soil surface. Experiments were performed at room temperature on sexuality mature earthworms weighing more than 300 mg.

Filter paper contact test

A modified contact filter paper test was performed [26]. A piece of filter paper of area 71 cm^2 was

placed in a Petri dish (diameter 9.5 cm) and 1 mL of the test substance dissolved in water was pipetted into the filter paper. An earthworm was placed on the wet surface and the Petri dish was covered with plastic film that had been punched with small holes using needles. Test was performed in the dark room at 20 °C for 48 h under 80 to 85% relative humidity and mortalities were recorded. An earthworm was considered dead if it failed to respond to gentle mechanical touch at the anterior end. Earthworms were held on wet filter paper for 24 h at 25 °C in the dark to purge gut contents before the dose-response test. A preliminary test was conducted to determine a concentration range for cadmium chloride in which mortalities of 0-100% were obtained. The final concentrations used corresponded to 5, 10, 20, 30 and 60 μg/cm^2 of filter surface. Control tests were performed using 1 mL of distilled water. Ten replicates were used, each consisting of one earthworm per dish. LC50 of cadmium chloride was calculated after transforming percentage response to probit values, and concentrations into logarithm [27].

Avoidance test

The avoidance test was performed as described in ISO guideline 1712-1 [28] on earthworms in standardized artificial soil [26] at four concentrations of Cd (8, 16, 48 and 150 μg/g soil). These concentrations were selected according to the Concentration lethal (LC50) found in the contact test. Each concentration and a control were tested with 12 replicates. Square plastic 500 mL chambers (12 × 11 × 8 cm) were filled with soil to a height of about 6 cm (about 250 g soil, dry weight). A plastic transverse partition was inserted to fill one half of the vessel with Cd-contaminated soil and the other half with uncontaminated control soil. The plastic separator was then removed and 10 adults of *E. foetida* were placed on the separation line of each test vessel. The soil was moistened and maintained at 50% maximum water-holding capacity and the vessels were closed with clear perforated lids. The tests were carried out in the dark in incubators at 20 °C. The animals were not fed during the test. At the end of the test period of 48 h, the control and the contaminated soil sections were carefully separated and the number of earthworms in each

half of the vessel was determined. Individuals on the line separating the sections were assigned to the section which contained the anterior part of the body (considered to indicate the direction in which they were moving). A dual-control test was conducted with 10 replicates using control soil in both sides of the test chambers.

An avoidance index for each concentration of Cd was calculated from the mean number of earthworms in the contaminated halves of the chambers and the mean number of worms in the untreated soil in the control halves. This was expressed as a percentage avoidance using the following equation: R (%) = [(C − T)/N] × 100, where R is avoidance; C is the mean number of worms in the control halves; T is the mean number of worms in the contaminated halves; and N is the total number of worms used in each trial. The Fisher's test was used to analyze the significance of the response. For the test with the soil contaminated with cadmium, the one-tailed test was chosen; the null hypothesis assumed that when there is no effect, half of the organisms stay in the contaminated soil [29].

Histology

Earthworms were washed with distilled water. After a depuration period of 24 h to purge gut contents, earthworms were narcotized in 7% ethanol, and fixed in paraformaldehyde 2.5% in PBS solution for 24 h. After dehydration in a graded series of ethanol concentrations they were embedded in paraffin wax. Slices with longitudinal or transverse sections of earthworms (7 μm thick) were stained with hematoxylin and eosin for observation and photography using an optical microscope equipped with digital color camera (Axioskop 40 FL, Carl Zeiss, Jena, Germany; CoolSNAP-cf, Photometrics GmbH, Munich) and analyzed using ImagePro Plus 5.0 software (Media Cybernetics Inc., Bethesda, MD). To detect Goblet cells a set of slices were stained with PAS-Alcia Blue (pH 2.5) to distinguish neutral and acidic mucopolysaccharides [30], so that the blue-stained goblet cells containing acid mucins could be located.

Scanning electron microscopy (SEM)

Earthworms were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer overnight at 4 °C, rinsed

three times with phosphate buffer, and dehydrated in an acetone series (50, 80, 90 and 100%). The worms were then subjected to critical point drying, coated with gold metal, and examined in a scanning electron microscope (model JSM 25S, JEOL Ltd, Tokyo, Japan), fitted with Electron Dispersive X Ray.

RESULTS

Mortality and signs of cadmium toxicity

The toxicity of Cd to *E. foetida* was determined using the contact filter paper test at five different concentrations of cadmium chloride. The mortality rate progressively increased with increasing Cd concentration. The 48 h LC50 for Cd was 19 $\mu g/cm^2$. The LC50 range at 95% confidence limit was found to be 15-26 $\mu g/cm^2$. With longer exposure times, mortality and behavioral signs of toxicity were observed at lower concentrations. The earthworms showed increasing signs of intoxication during the first minutes of exposure to Cd at all tested concentrations, including rapid movements, coiling, curling, and mucus secretion. Mucus secretion, observed as the expulsion of yellow pigmented fluid, was concentration-dependent (Fig. 1). Morphological abnormalities such as sluggish movements, swelling of the pre-clitellar region and bloody lesions were observed within 12 h at concentrations >10 $\mu g/cm^2$. Constrictions were observed around the mouth and in the first seven segments. Swellings appeared in segments seven to twelve and in the posterior regions (Figs. 1, 2b). Constrictions were followed by autotomy in the posterior region after 24 h of exposure of the worms to higher concentrations. Some earthworms showed one or more zones of intense swelling in the post-clitellar region, around segment thirty. The swelling was associated with a thinning of the anterior region of the body, which sometimes ruptured, leading to death.

Histopathology

Histopathological examination of body sections from the control group illustrated the normal body architecture with intact circular and longitudinal muscles (Fig 2a). Earthworms exposed to concentrations higher than 10 $\mu g/cm^2$ revealed structural changes in the ventral region (Fig. 2c).

In the contact test, Cd noticeably damaged the ventral body wall of the earthworms. Cd-exposed earthworms that showed swelling in the pre-clitellar region were submitted to histological examination. This revealed rupture of the alimentary canal with large lesions in the vessels and internal bleeding (Fig. 2a, b). The cuticular membrane and ectodermal layer were severely damaged and were detached from the muscle layer. Voids were present in the internal circular muscles (Fig. 3b, c) and the external surfaces of the damaged tissues had begun to dissociate (Fig. 3d).

Damage caused by Cd was analyzed using the scanning electron microscopy. Control earthworms showed normal integrity of the prostomial region and corporal surface (Fig. 4 a, b). In Cd-exposed earthworms, sloughing and loss of cuticle was observed in the prostomium (Fig. 4c), in addition to longitudinal shrinkage of the segments, folding of the epidermis, and its detachment from the muscle layer (Fig. 4d). The localization of Cd within the body was explored by X-ray microanalysis (EDX) in samples prepared for SEM. Cd was not detected on the external surface of the earthworm but was present in the mucus secretion, cell debris, and the chloragogenous tissue (Fig. 5 a, b). Additionally, in a transversal section, we observed the intestinal tissue with the chloragogenous epithelium at light microscope level (Fig. 6 a, b). In other histolgical preparations from Cd-exposed earthworms, we observed the chloragogenous epithelium detached from the basal membrane analyzed under scanning electron microscopy (Fig. 6 c, d).

Avoidance

The avoidance test was carried out to examine interactions with Cd present in soil. The overall mortality in our tests was 1.6%. In the dual-control tests, the earthworms were randomly distributed between the two sides of the test chamber with a range 60%-40%. Fig. 7 shows the avoidance values calculated from the distribution of the earthworms exposed to Cd; they exhibited avoidance behavior by choosing the control soil. However, some earthworms choose the cadmium contaminated soil. The magnitude of the avoidance response was similar at all concentrations tested (p > 0.05, ns). The earthworms that remained in

Fig. 1. Effects of cadmium on the morphology of the earthworms. (a) Contact exposure to cadmium produced an increase of mucus secretion. (b, c) Abnormal swellings in the clitellar region. (d) Autotomy. (e) Protrusions over the whole body surface. (f, g) Bloody lesions and coiling.
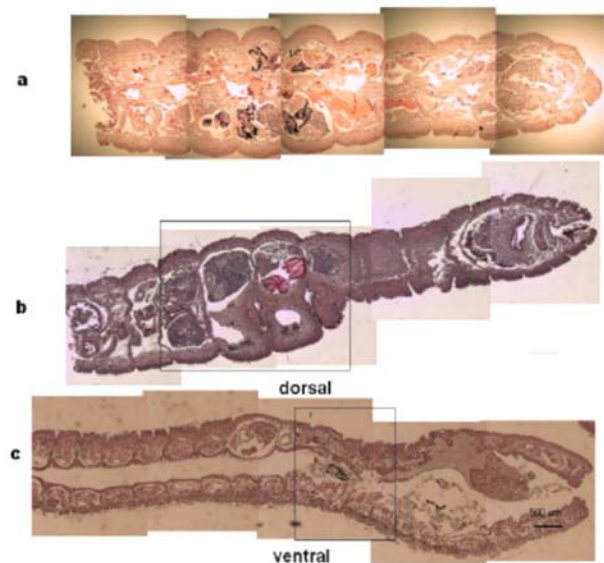

Fig. 2. Histopathological changes in cadmium-intoxicated earthworms. (a) Longitudinal section of a control earthworm. (b) A region of pre-clitellar swelling in segments 9-12 with internal tissue erosion and damaged intestinal duct. (c) Magnification of the ventral portion shows loss of external body wall and abnormal changes with internal destruction.
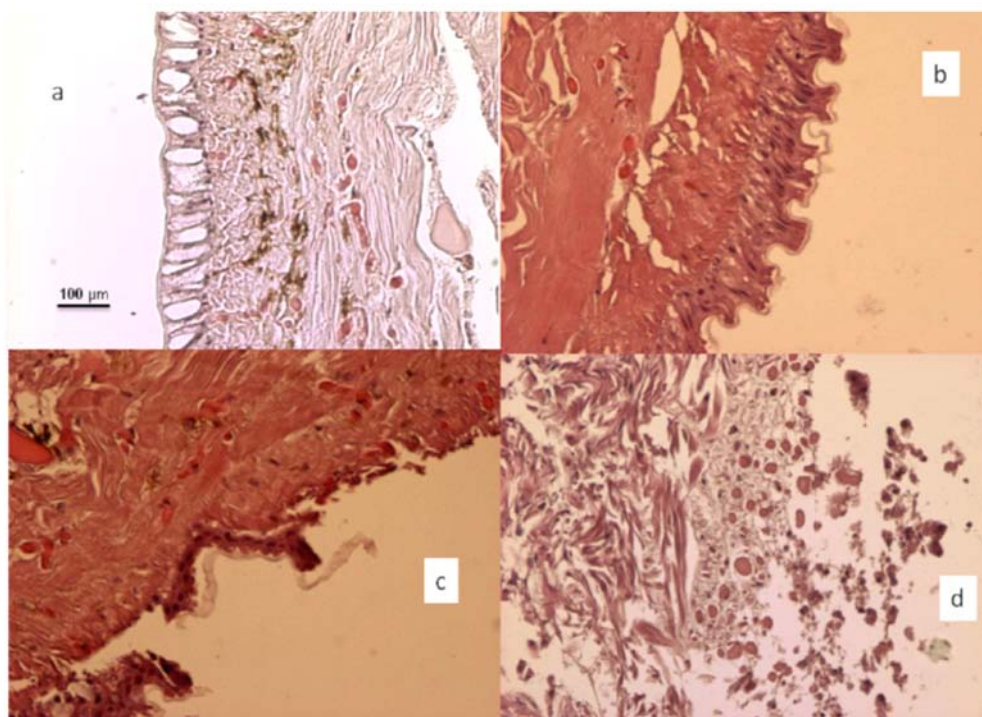
Fig. 3. Microphotography of the earthworm's clitellar region. (a) The cuticle and muscle layers are adherent in control earthworms. (b) Detached cuticle. (c, d) Rupture of the circular and longitudinal muscle layers.
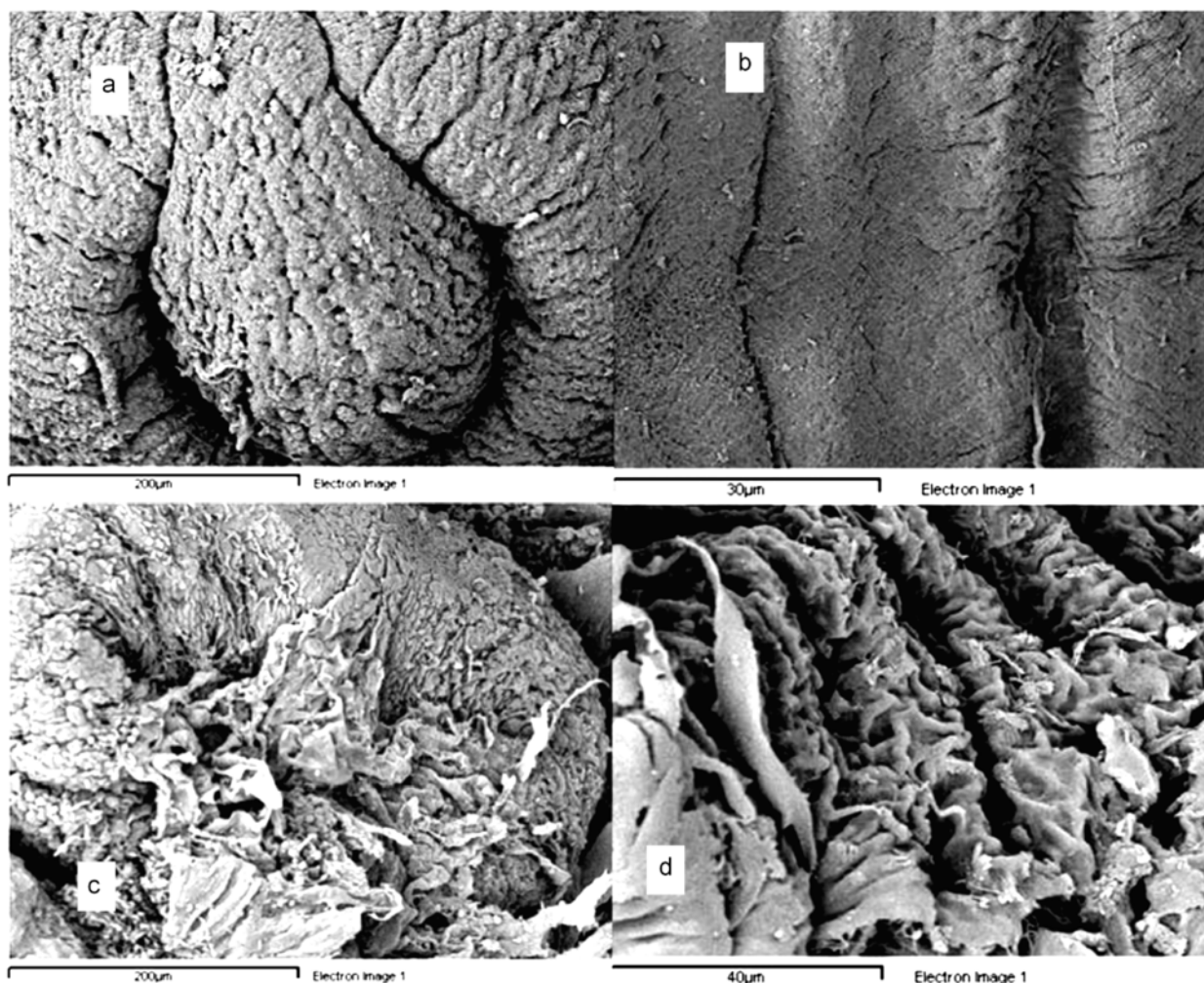
Fig. 4. Effects of cadmium on the earthworm surface. (a, b) The prostomium and the external surface of control earthworm appear intact. (c, d) Earthworms exposed to cadmium showed destruction of the external surface of the prostomium and segmental constriction with loosening of the cuticle.

the contaminated soil exhibited signs of toxicity compared with those in the control soil; e.g., adherent soil particles and decreased locomotion (Fig. 8 a, b).

The histopathological changes induced by Cd in the avoidance test were different from those observed in the contact test. Compared with the control treatment (Fig. 8 c), earthworms exposed to cadmium exhibited increased number of glandular epithelial cells in the epidermis, but not loss of the cuticular membrane (Fig. 8 d). The epidermis was severely degraded and necrotic, almost exposing the circular muscle. Some regions showed an almost complete disintegration

of the epithelial lining of the epidermis (Fig. 8 e). The circular muscle also showed changes in cell shape and disorganization of the muscle fibers (Fig. 8 f).

DISCUSSION

Cadmium toxicity: behavior and histopathological changes

Cadmium, a non-essential and non-biodegradable heavy metal has been included in a 'black list' of toxic chemicals [31]. Cd toxicity relates to oxidative stress, substitution on Ca-binding sites and binding to protein thiol groups [32]. Numerous studies have been performed to evaluate the
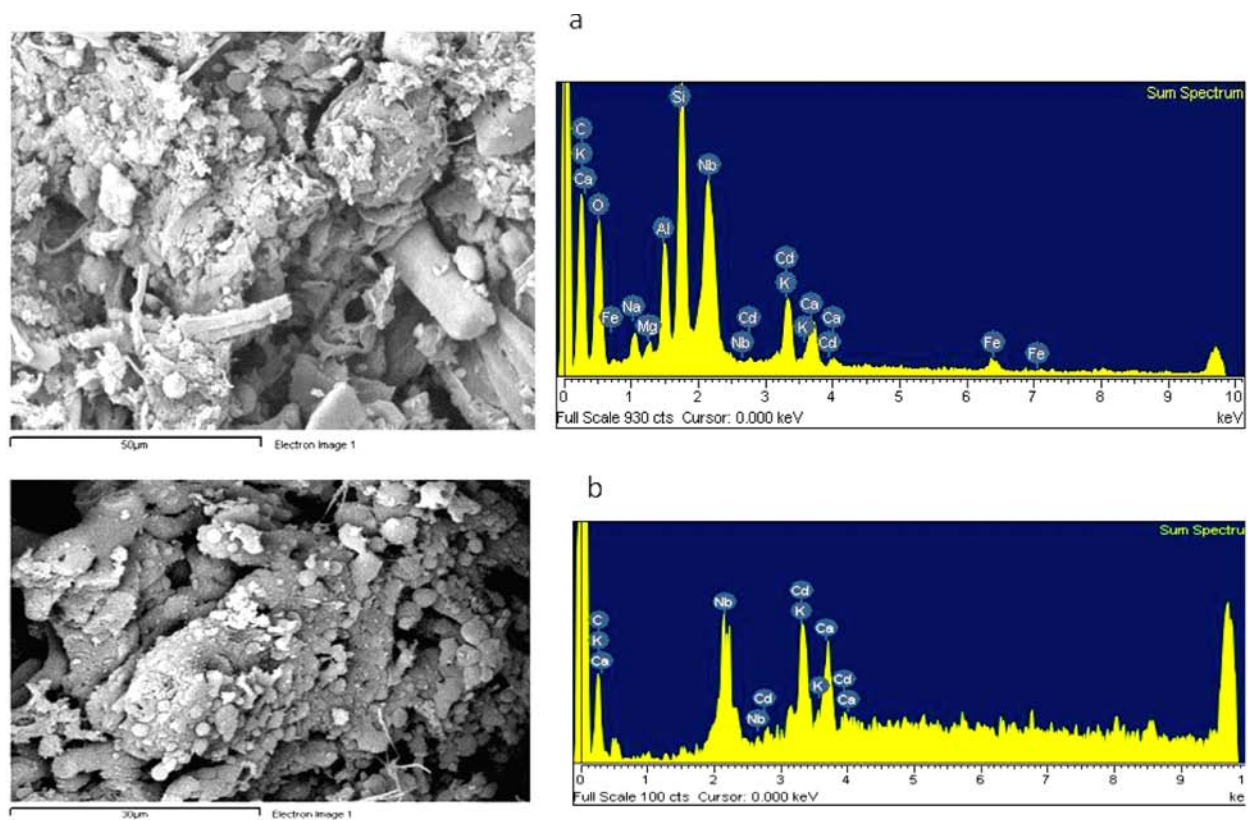
Fig. 5. Scanning electron microscopy with energy dispersive X-rays analysis. (a) Cadmium was detected in cellular debris containing coelomocytes and (b) in the distal region of the earthworm.
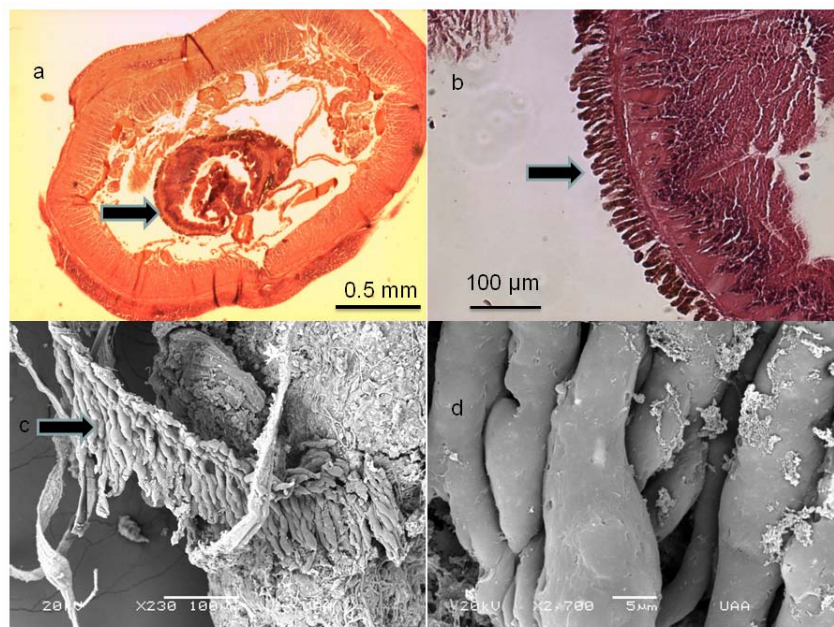

Fig. 6. (a) Transverse section of earthworm exposed to cadmium. (b) Enlargement showing the chloragogenous tissue. (c, d) SEM photos of this region showing the epithelial tissue detached from the intestinal layer with columnar cells.
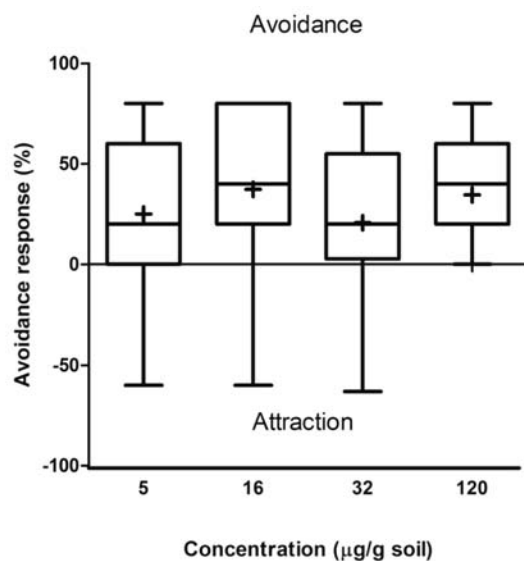
Fig. 7. Avoidance response of *Eisenia foetida* to Cd concentrations in OECD artificial soil. Groups of 10 earthworms were allowed to distribute between control and test soils. Box plots represent the median as the middle line and 25% and 75% as min and max values, respectively. + symbols are the mean values.

sublethal toxicity of Cd to earthworms in artificial soils and mining soils and have used a variety of criteria related to growth, and reproductive physiology [15, 33]. However, there are a few studies that address the cadmium toxicity.

We evaluated Cd toxicity using the filter paper test because it offers a quick and easy method of exposing earthworms to the metal. The method ensures that the element is in solution and makes intimate contact with the epidermis and it allows observation of the effects of cadmium on the animal [1]. The LC50 of cadmium in the filter paper test was 16.9 $\mu g/cm^2$. This is in good agreement with values published by others [1, 16]. Several studies have compared filter paper-derived LC50 values with artificial soil-derived values and have indicated that filter paper exposure is not a good predicator of soil acute toxicity. Filter paper exposure is generally considered to be more toxic to earthworms than soil exposure [14, 34]. Our study revealed that cadmium chloride had deleterious effects on various physiological functions of earthworms. Sudden changes in locomotion behavior when the animals made contact with Cd can be explained as

a sensory phenomenon. Cd was first detected by chemoreceptors mainly located in the prostomium [35], which then initiated the fast escape reflex described by Drewes and Vining [36]. This was accompanied by whipping movements and mucus secretion and by rapid locomotion around the Petri dish with the head raised, apparently in an attempt to avoid contact with the toxin. Finally the earthworms adopted a coiled configuration. These responses are not specific for cadmium. Similar responses have been reported for other metals [37, 38, 39], chemical pollutants [40], and insecticides [41]. Mucus secretion was concentration-dependent, as also noted by other researchers [10], and also occurs in slugs and fish [42]. Mucus secretion may provide a barrier that protects the epidermis from damage. The mucus is a heterogeneous mixture of coelomocytes, proteins, salts and coelomic fluid that together serve to chelate cadmium [43, 44, 45]. Bouche *et al.* [10] considered that mucus secretion is an adaptive response for longer term detoxification of cadmium. For example, coelomocytes exposed to cadmium may have the ability to express metallothioneins [46, 47, 48, 49]. Their expulsion with the coelomic fluid would eliminate Cd from the body.

The bleeding observed in the pre-clitellar region may be related to the fact that the heart is located in this region. Cadmium is known to cause injuries to small blood vessels in vertebrates and contractures of vascular smooth muscles leading to hemorrhages [50]. Damage induced by Cd in this region was also reported by Siekierska [51, 52], who noted that Cd induced injuries in the gonads and histological changes in the supra- and sub-pharyngeal ganglia of the earthworm *Dendrobaena benata*.

Locomotion is of central importance to earthworms in relation to feeding and burrowing. The effects of Cd, which included sluggish movements and contractures, could result from interference with cholinergic transmission and the contractile machinery [53, 54]. Cadmium induced autotomy by the earthworms, a phenomenon also observed with other metals, including Cu, Pb, Hg and Cr [37], and in freshwater oligochaeta [10], and with other classes of pollutants [55]. Generally, autotomy is considered to be a defensive
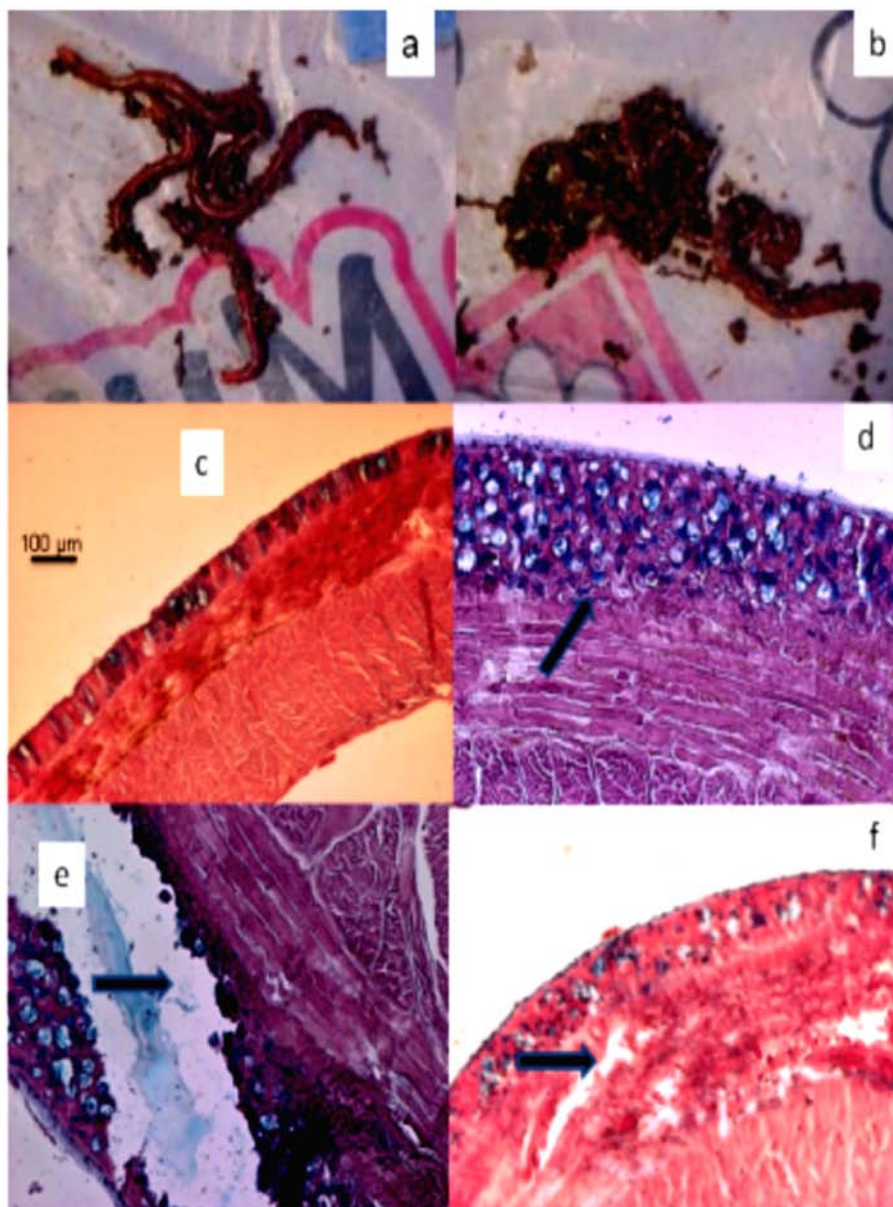
Fig. 8. Earthworms exposed to cadmium by avoidance test. (a) The control earthworms exhibited free locomotion. (b) Exposure to cadmium caused soil particles to adhere to the body. (c) Histology of control earthworms characterized by an intact external layer. (d) Worms exposed to cadmium showed larger numbers of goblet cells, (e) loss of the epithelial layer and (f) destruction of the circular muscle layer.

mechanism against predators. It is an immediate and rapid response by earthworms to tactile stimuli, such as touching and grasping [56]. However, in our experiments, the phenomenon appeared after a relatively long period of Cd exposure. Our observations are more in agreement with the hypothesis of Vidal and Horne [57] that earthworms can accumulate the metal in their caudal segments and that their subsequent elimination via the process of autotomy provides a mechanism of detoxification.

Histological changes observed in earthworm's body wall were evidently caused by direct contact with Cd in solution because there was differential damage in the ventral region which appeared only in the contact test. Thus Cd entered by dermal

contact and crossed the epidermis [58]. This caused severe wounds, detachment of the cuticle, and changes in the structural organization of muscle layers, before arriving in the coelom. Chloragogenous tissue is located in the gastrointestinal canal and separates the absorptive epithelium from the coelom. Our demonstration of Cd in the chloragogenous tissue by EDX is consistent with other observations in the literature that it is the main site of metal accumulation [59, 60, 61]. However, we were unable to detect Cd in muscle layers by EDX. Other authors have reported similar localizations of cadmium and similar histological changes in earthworms exposed to heavy metals [57, 62, 63, 64].

Avoidance response

We chose the avoidance test because behavioral endpoints have been used to study the effects of chemicals and drugs in animals, and have also been used in earthworm toxicology. Behavioral adaptations are phenotypic traits that facilitate survival of animals in changing environments. However, the relationships between experimental behavioral responses and ecologically-relevant issues, such as avoidance, stress resistance, growth, reproduction and longevity [23] are poorly understood. We did not find concentration-dependence in the avoidance tests over the concentration range tested, which is consistent with the results of Hund-Rinke *et al.* [7]. Other researchers found that earthworms did not avoid contaminated soils from mines [65] and were able to survive in soils with high cadmium concentrations. This phenomenon was explained as an adaptive response; animals without a cadmium history were more susceptible to cadmium toxicity [66]. The increased secretion of mucus in the avoidance test may function as a non-specific defense against toxicants by reducing the toxicant contact; i.e., mucus forms a barrier between the body and the toxic medium and minimizes its irritant effect [41]. Soil particles that adhered to the body surface would increase friction and impede the free movement and locomotion of the earthworms. Histologically, increased mucus secretion was associated with hypertrophy of the goblet cells in the glandular epithelium and damage to the external layers of the epidermis. Similar changes were noted in the

earthworm *Amynthas gracilis* inhabiting volcanic soil [67]. Although, cadmium was not found in muscle tissue, it induced loss of structural organization of the circular muscle, which is necessary for optimal contractile function.

A number of the earthworms remained in the contaminated soil in the avoidance test. Earthworms are believed to detect pollutants by sensory mechanisms involving chemoreceptors with external terminals located in the prostomium and epidermis [25, 68]. We observed that the contact with contaminated soil in the avoidance test was associated with disintegration of the epidermal layers and muscular tissue. These changes may have interrupted chemosensation and the ability of the earthworm to detect and move away from a noxious environment. Interference with the contractile machinery would exacerbate the difficulty of escaping from contaminated soil [5]. An alternate explanation for persistence of the earthworms in contaminated soil may be the well-known phenomenon of aggregation. Aggregation behavior maintains the community in a nutrient-rich medium [69], but is disrupted by anomalous behavior in response to the emission of cues caused by environmental stress [70].

CONCLUSION

From the results, it is suggested that Cd toxicity in earthworms involves serious damage to the body wall, break-up of the sensory signal due to rupture of the cuticle, internal lesions with locomotion decreased, and death. Cadmium can interfere with vascular and muscle function, weakening feed fitness, and the avoidance function. The earthworm's defensive mechanisms like mucus secretion and escape behavior are switched to counteract the cadmium toxicity. Therefore, careful analysis of the mode of action of toxic agents is a priority when performing toxicity tests, and morphological and histopathological tests help us to understand better the results of the test.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST STATEMENT

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

REFERENCES

1. Honeycutt, M., Roberts, B. and Roane, D. 1995, Ecotoxicol. Environ. Saf., 30, 143.
2. Yasmin, S. and D'Souza, D. 2010, App. Environ. Soil Sci., doi:10.1155/2010/678360, 9.
3. Sousa, A. P. A. and Andréa, M. M. 2011, Sensor, 11, 11056.
4. Vijver, M. G., Vink, J. P. M., Miermans, C. J. H. and Van Gestel, C. A. M. 2003, Soil Biol. Biochem., 35, 125.
5. Zhou, S. P., Duan, C. Q., Fu, H., Chen, Y. H., Wang, X. H. and Yu, Z. F. 2007, J. Environ. Sci., 19, 1019.
6. Yeardly, R. B., Lazorchak, J. M. and Gast, L. C. 1996, Environ. Toxicol. Chem., 15, 1532.
7. Hund-Rinke, K., Lindemann, M. and Simon, M. 2005, J. Soil Sediments, 5, 233.
8. Sousa, A., Pereira, R., Cachada, A., Pereira, E., Duarte, A. C. and Goncalves, F. 2008, Ecotoxicol. Environ. Saf., 71, 661.
9. Li, D., Hockaday, W. C., Masiello, C. A. and Alvarez, P. J. J. 2011, Soil Biol. Biochem., 43, 1732.
10. Bouche, M. L., Habets, F., Biagianti-Risbourg, S. and Vernet, G. 2000, Ecotoxicol. Environ. Saf., 46, 246.
11. Morgan, A. J. and Morris, B. 1982, Histochemistry, 75, 269.
12. Ma, W., Edelman, T. H., Beersum, V. I. and Jans, T. H. 1983, Bull. Environ. Contam. Toxicol., 30, 424.
13. Spurgeon, D. J. and Hopkin. S. P. 1999, App. Soil. Ecol., 11, 227.
14. Neuhauser, E. F., Loehr, R. C. and Malecki, M. R. 1986, Contact and artificial soil tests using earthworms to evaluate the impact of wastes in soils. pp 192-203. In: Hazardeus and industrial solid waste testing: Fouth symposium. ASTM STP 886. J. K. Jr. Petros, W. J. Lacy and R. A. Conway (Eds) American Society for testing and materials. Philadephia.
15. Van Gestel, C. A. M., Dis, W. A., Breemen, E. M. D-V., Sparenburg, P. M. and Baerselman R. 1991, Biol. Fertil. Soil, 12, 117.
16. Fitzpatrick, L. C., Muratti-Ortiz, J. F., Venables, B. J. and Goven, A. J. 1996, Bull Environ. Toxicol., 57, 67.
17. Van Gestel, C. A. M., Breemen, E. M. D-V., Baerselman, R., Emans, H. J. B., Janssen, J. A. M., Postuma, R. and Van Vliet, P. J. M. 1992, Ecotoxicol. Environ. Saf., 23, 206.
18. Spurgeon, D. J., Hopkin, S. P. and Jones, D. T. 1994, Environ. Poll., 84, 123.
19. Jamieson, B. 1981, Ultrastructure of the Oligochaeta, Academic Press London.
20. Golstein, A. and Adams, E. 1968, J. Biol. Chem., 243, 3550.
21. Fischer, E. and Horbath, I. 1977, Histochemistry, 54, 259.
22. Little, E. E. and Brewer, S. K. 2001, Neurobehavioral toxicity in fish. D. Schlenk, and W. H. Benson, (Ed.), Target organ toxicity in marine and freshwater teleost new perspectives: Toxicology and the environment, Tylor and Francis, London and New York, 2, 139.
23. Rand, G. M. 1985, Behavior. In Fundamentals of Aquatic Toxicology: Methods and Applications. G. M. Rand and S. R. Petrocelli (Eds.), pp 221-256. Hemisphere Publishing, New York.
24. Kane, A. S., Salierno, J. D. and Brewer, S. K. 2005, Fish models in behavioral toxicology: Automated techniques, updates and perspectives. Pages 559-590 In: G. K. Ostrander, Editor. Methods in Aquatic Toxicology (Chapter 32), Volume 2, Lewis Publishers, Boca Raton, FL.
25. Pereira, J. L., Antunes, S. C., Ferreira, A. C., Alves, F. G. and Pereira, R. 2010, J. Environ. Sc. and Health, 45, 229.
26. OECD, 1984, Earthworm, Acute toxicity tests. OECD Guidelines Test. Chem., 1, 1-9.

27. Litchfield, J. T. and Wilcoxon, F. A. 1949, J. Pharmacol. Exp. Ther., 96, 99.
28. ISO Guideline 17512-1 (ISO 2008).
29. Natal-da-Luz, T., Römbke, J. and Sousa, J. P. 2008, Environ. Toxicol. Chem., 27, 1112-1117.
30. Martoja, R. and Martoja-Pierson, M. 1970, Toray-Masson, Barcelona, 350.
31. Taylor, D. 1983, Ecotoxicol. Environ. Saf., 7, 33.
32. Moulis J. M. and Thévenod F. 2010, Biometals., 23, 763.
33. Spurgeon, D. J., Svendsen, C., Kille. P., Morgan, A. J. and Weeks, J. M. 2004, Ecotoxicol. Environ. Saf., 57, 54.
34. Callahan, C. A., Russel, L. K. and Peterson, S. A. 1985, Biol. Fert. Soils, 1, 195.
35. Knapp, M. F. and Mill, P. J. 1971, Tissue and Cell, 3, 623.
36. Drewes, C. D. and Vining, E. P. 1984, Pestic. Biochem. Physiol., 22, 93.
37. Labrot, F., Narbonne, J. F., Ville, P., Saint-Denis, M. and Ribera, D. 1999, Arch. Environ. Contam. Toxicol., 36, 167.
38. Venkateswara, J. R., Kaviha, P. and Padmanabha, A. R. 2003, Environ. Res., 92, 271.
39. Venkateswara, J. R., Surya, P. and Madhavendra, S. S. 2003, Ecotoxicol. Environ. Saf., 54, 296
40. Ebere, A. G. and Akintonwa, A. 1995, Bull Environ. Contam. Toxicol., 55, 766.
41. Sternesen, J. 1979, Pesticide Sci., 10, 66.
42. Dube, P. N. and Hosetti, B. B, 2010, Biotechnol. Anim. Husb., 26, 91.
43. Heredia, R. B., Dueñas, S., Castillo, L., Ventura, J. J., Briano, M. S., Posadas del Rio, F. P. and Rodríguez, M. G. 2008, Comp. Biochem. Physiol. (A), 151, 407.
44. Diogene, J., Dufour, M., Poirier, G. G. and Nadeau, D. 1997, Lab. Anim., 31, 326.
45. Muangphra, P. and Gooneratne. R. 2011, Appl. Environ. Soil Sc., 7p doi:10.1155/2011/218929.
46. Fugere, N., Brousseau, P., Krystyniak. K., Coderre, D. and Fourtinier, M. 1996, Toxicology, 109, 157.
47. Sturzenbaum, S. R., Kille, P. and Morgan, A. J. 1998, FEBS Lett., 431, 437.
48. Morgan, A. J., Stürsenbaum, S. R., Winter, C., Grime, G. W., Aziz, N. S. and Kille, P. 2004, Ecotoxicol. Environ. Saf., 57, 9.
49. Homa, J., Olchawa, E., Sturzenbaum, S. R., Morgan, A. J. and Plytycz, B. 2005, Environ. Poll., 135, 275.
50. Evans, D. H. and Weingarten, K. 1990, Toxicology, 61, 275.
51. Siekierska, E. and Urbanska-Jasik, D. 2002, Environ. Pollut., 120, 289.
52. Siekierska, E. 2003, Environ. Pollut., 126, 21.
53. Asai, F., Nishimura, M., Satoh, E. and Urekawa, N. 1983, Br. J. Pharmac., 75, 561.
54. Dulhunty, A. F. and Gage, P. W. 1989, Biophys. J., 56, 1.
55. Reddy, N. C. and Venkateswara, R. J. 2008, Ecotoxicol. Environ. Saf., 71, 574.
56. Tsai, C-F. and Tsai, S-H. 2001, Zological Studies, 40, 21.
57. Vidal, D. E. and Horne, A. J. 2003, Arch. Environ. Contam. Toxicol., 45, 462.
58. Vijver, M. G., Wolterbeek, H. T., Vink, J. P. M. and Van Gestel, C. A. M. 2005, Sci. Tech. Environ., 340, 271-280.
59. Ireland, M. P. and Richards, K. S. 1977, Histochemistry, 51, 153.
60. Richards, K. S. and Ireland, M. P. 1978, Histochemistry, 56, 55.
61. Morgan, J. E. and Morgan, A. J. 1989, Histochemistry, 90, 405.
62. Lourenco, J., Silva, A., Carvalho, F., Oliveira, J., Malta, M., Mendo, S., Goncalves, F. and Pereira, R. 2011, Chemosphere, 85,1630.
63. Sharma, V. J. and Satyanarayan, S. 2011, Environ. Monit. Assess., 180, 257.
64. Kilic, G. A. 2011, Chemosphere, 83, 1175.
65. Langdon, C. J., Hodson, M. E., Arnold, R. E. and Black, S. 2005, Environ. Pollut., 138, 368.
66. Reinecke, S. A., Prinsloo, M. W. and Reinecke, A. J. 1999, Ecotoxicol. Environ. Saf., 42, 75.
67. Cunha, N., Campos, I., Montiel, R., Rodrigues, A. and Morgan, A. 2011, Ecotoxicol. Environ. Saf., 74, 25.
68. Laverack, M. S. 1960, Comp. Biochem. Physiol., 1, 155.
69. Zirbes, L., Brostaux, Y., Mescher, M., Jason, M., Haubruge, E. and Deneubourg, J-L. 2011, PLoS One, 7, 1.
70. Zirbes, L., Mescher, M., Vrancken, V., Wathelet, J-P. and Verheggen, F. J. 2011, PLoS ONE, 6, 1-7.