Electron microscopy and the responses of terrestrial invertebrates against contaminants of soil

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Electron microscope allows the direct observation of ultrastructural aspects of the cells. This equipment is also a very efficient tool to analyze the toxic potential of certain compounds since it allows a more detailed understanding of the effects of contaminants on the organisms under study, revealing the real risks that they represent. Due to the high resolution that this equipment has it is possible to visualize what is the level of integrity and preservation of the organelles of the material analyzed. Thus, couple up studies of the ecotoxicology area with technological advances in transmission electron microscopy allows the assessment of the main biological responses of the living organisms to the action of pollutants in a more accurate form. In this sense, this text aims to present and discusses the ultrastructural alterations found in cells of the main organs of terrestrial invertebrates used in toxicity tests, as well as the intensity of the stressor factor, the type of target organ and the cellular alterations in their different levels.

.**Key-words:** contamination, organelles, edaphic organisms, toxicants, transmission electron microscopy.

1. Introduction

The creation of transmission electron microscope (TEM) resulted in a series of experiments concerning electrons and their behaviour. In 1926 the first studies aiming to focus beams of electrons arose and, based on these studies, in 1931 it was initiated the construction of the first TEM by Knoll and Ruska. Due to the enormous advance that transmission electron microscopy has brought to science, in 1939 it was already built the first commercial model of TEM. Since then, the improvements that the technical progresses promoted were so many that there are almost no barriers for the amplification of images promoted by this equipment [1].

Thus, the transmission electron microscopy has been a tool of fundamental importance for research in several areas of knowledge. Exceeding all the resolution limits of the human eye, which does not reach 0.2 mm, TEM with its high definition of images has contributed, in a representative way, for the characterization of the studied material, whether they are cells or tissues [1, 2]

Detailed knowledge of the ultrastructure of organisms allows not only the understanding but also, in many cases, even the prediction of their characteristics and behaviour [3]. Therefore, coupling electron microscopy to ecotoxicological studies becomes quite interesting, since it enables extreme precision to these studies, which helps in the localization of altered regions, identification of the level of such alterations and how invasive and harmful is the compound or pollutant studied, thus allowing to understand the main biological responses of the living organisms to the action of the toxicants.

Moreover, the ultrastructural analysis of target organs of several species of terrestrial invertebrates shows that the cell organelles have different susceptibilities to the pollutants and show different degrees of reaction in relation to them [4]. In these cases, depending on the stressor factor and the type of target organ, cellular alterations may vary at different levels [5], providing qualitative evidences of a functional adaptation to the external environment [6], as well as indicating early stages of toxicity [5, 7].

Thus, this paper aims to present and discuss the main ultrastructural alterations found in cells of organs of terrestrial invertebrates used in toxicity tests. A summary of the ultrastructural responses presented by these cells can be seen in Table 1.

2. Main ultrastructural alterations observed in terrestrial invertebrates

Edaphic saprofagous invertebrates such as Nematoda, Oligochaeta, Gastropoda, Isopoda, Diplopoda and Collembola, are among the most appropriate organisms to evaluate the effects of accumulation of toxic substances in the soil due to their direct contact with the contaminants therein [8-10].

Besides these organisms, Hymenoptera are also used to assess the environmental contamination. Bees are important pollinators and due to the destruction of their natural habitat, began to forage in agricultural areas. The contact with pesticides used in these areas has led to the decrease in the species number [11]. Ants have important characteristics that make them bioindicator organisms, such as enormous diversity, numerical dominance, wide geographic distribution, stationary nests and ease of sampling, which allows following alterations over time [12].

Ultrastructural morphological analysis of target organs such as midgut, fat body, hepatopancreas, tegument and salivary glands has become an efficient tool to identify damages caused by different harmful substances in studies with these invertebrates [7, 13].

2.1. Plasmatic membrane

Cell membrane is a structure of fundamental importance for the maintenance of the physiological conditions of any living organism and it is the first region to get in contact with any exogenous chemical substance. Membranes are not only passive barriers but, due to the group of specialized proteins, act in the promotion or catalysis of a variety of cell processes, such as transport of substances, including that potentially toxic.

For these characteristics, ultrastructural alterations can be observed in the plasmatic membrane of terrestrial invertebrates submitted to contaminants. Diplopods exposed to sewage sludge, for example, showed loss of plasmatic membrane integrity and also cellular spaces dilatation of the epithelium that cover the intestine wall [14]. Disruption of the plasmatic membrane was visualized in some cells of the ants midgut exposed to boric acid [15].

Changes in the plasmatic membrane characterize the process of necrosis related to the destabilization of junctional complexes [16]. In addition, necrotic cells suffer changes in the osmotic balance, which causes an increase in the cell volume and, consequently, rupture and release of their content to the extracellular environment, promoting inflammation in the adjacent tissues [16].

2.2. Microvilli

In Hymenoptera the microvilli of midgut cells and Malpighian tubules underwent dilatation after exposure to boric acid [17, 15]. In Isopoda, the microvilli diminished or were destroyed in hepatopancreas cells of the animals treated with heavy metals [18]. In diplopods exposed to sewage sludge, occurred a decrease in the number of microvilli in the midgut cells [14] (Figure 1A, B).

2.3. Cytoplasm

2.3.1. Formation of vacuoles, vesicles, granulations and cytoplasmic degradation

Boric acid was capable of causing cytoplasmic changes in the midgut cells, Malpighian tubules and postpharyngeal glands of ants. In the midgut were observed cytoplasmic vacuolization, presence of myelin figures and autophagic vacuoles; in the Malpighian tubules cells were observed mineralized granules with loss of concentric bodies and electron-density, as well as presence of myelin figures; in the postpharyngeal glands, cells presented intense cytoplasmic vacuolization [15].

In bees larvae, the compost fipronil was responsible for generating vacuolization and cytoplasmic disruption in intestine cells [19]. This substance was also responsible for the intense vacuolization of the cytoplasm of Malpighian tubule cells and presence of vacuoles in the trophocytes of worker bees. Mineralized granules present in the Malpighian tubules were also altered, since they were structurally distinct from those found in bees not exposed to the substance, in which it was evidenced only the initial stages of formation of granules concretions [20].

Also in bees, cells treated with boric acid presented intense cytoplasmic vacuolization in the Malpighian tubules, higher quantity of vacuoles in the trophocytes and presence of autophagic vacuoles in the oenocytes [20]. Hypopharyngeal gland cells exposed to the product Fenoxycarb presented cytoplasmic disorganization and presence of lysosomes.

In Diplopoda and Isopoda there was cytoplasmic condensation [18, 21] and in Collembola there was an increase in the formation of sphaerocrystals in the epithelial cells of the intestine [22]. For diplopods exposed to sewage sludge it was described the notable presence of autophagic vesicles in hepatic cells [21], increase in the number of cytoplasmic granules (sphaerocrystals) (Figure 1F), cytoplasmic vacuolization of cells midgut epithelium (Figure 1B) and intense release of secretory vesicles into the intestinal lumen [14].

Secretory vesicles of the apocrine type seem to help in the elimination of toxic substances initially absorbed by the organism and form a protective layer that would reduce the contact between the toxic agent and the analyzed tissue [14]. According to these authors, vacuolization of the cytoplasm indicates cell death by necrosis as well as impairment of other cell compartments that end up compromising the cell.

2.3.2. Mitochondria

Mitochondria are generally sensitive to several types of stressors and react very quickly with global pathological symptoms [23-25]. In Hymenoptera exposed to boric acid and fipronil, insecticides widely used in plantations, mitochondria of the organs such as postpharyngeal gland, intestine, Malpighian tubule and fat body presented as main alterations, swelling with loss of the typical structure, increase of the electron-density and dilatation of the internal membranes and increase in number [19-20].

However, in diplopods midgut cells exposed to different concentration of sewage sludge [14] and isopods hepatopancreas cells exposed to heavy metals [18] showed a reduction in mitochondria number. Besides these alterations, a decrease in the number of mitochondrial cristae was reported for collembolans midgut cells also exposed to metals [22].

In the epithelial cells of the earthworms intestine submitted to the albendazole drug was observed severe change in the morphology of this organelle indicated by the loss of the inner structure, such as reduction in the matrix content, decrease in the quantity of cristae, presence of a single membrane, agglomeration of small particles besides irregular organization of the cristae [26]. Nematodes larvae also submitted to albendazole presented a decrease in the mitochondria number of the non contractile muscle cells present in the body wall besides some mitochondria developed large vacuoles and became distorted and degenerated [27].

All the alterations pointed out constitute, frequently, the result of the interactions between the toxicants and the components of the membrane [28] and changes in the transport of ions along the mitochondrial membrane [29].

2.3.3. Endoplasmic reticulum

Endoplasmic reticulum also reacts very quickly to several types of intoxications, but comparing with mitochondria, they are more differentiated and allow to distinguish easily the progress of such changes [30]. In Hymenoptera treated with boric acid and fipronil, the rough endoplasmic reticulum of the intestine cells [19], Malpighian tubule, trophocytes and oenocytes [20] presented dilatation of their inner membranes and loss of ribosomes. Acini cells of the Hymenoptera hypopharyngeal gland exposed to Fenoxycarb did not present rough endoplasmic reticulum [31].

Endoplasmic reticulum cisternae of the isopods hepatopancreas cells showed a tendency for a parallel or circular arrangement [18]. In collembolans there was a reduction in the quantity of this organelle in the midgut cells, however, in the regenerative cells there was an increase in the number of cisternae both of the smooth and rough endoplasmic reticulum and accumulation of these organelles in the apical region of their microvilli [22]. In the observation of the smooth endoplasmic reticulum of the earthworms intestinal epithelium was verified a dilatation process, besides a progressive degeneration according to the exposure and growing concentrations of albendazole [26].

Alterations such as dilatation, loss of granulation of the endoplasmic reticulum have been related to functional modifications, such as induction of the oxidative biotransformation of enzymes, expression of recombinant membrane proteins, or the presence of mannitol oxidase [*apud* 30].

2.3.4. Golgi apparatus

Golgi apparatus seems to be less sensitive to intoxication and the reactions that occur are retarded in comparison with the endoplasmic reticulum [30]. However, it was observed, in terrestrial gastropods mucous cell, an alteration in the number of Golgi cisternae, which could appear dilated or compressed, besides alterations in the form or size of the secretory granules produced [30]. These alterations can be interpreted as an interference of toxic agents with an altered influx of material of the membrane from the endoplasmic reticulum [29].

2.4. Nucleus

Depending on the type of stressor, the nucleus of the cell can develop symptoms quickly or not [30]. Midgut nuclei cells of ants workers exposed to boric acid presented alterations that suggest programmed cell death, such as marginalization and chromatin condensation, formation of blebs, nuclear fragmentation, irregular morphology and pyknosis with central chromatin condensation. Malpighian tubules nuclei cells of Hymenoptera, in turn, suffered alteration in the morphology and increase in size of the heterochromatin regions, mainly near the nuclear envelope [15]. These same authors observed the intense chromatin condensation in the postpharyngeal gland nucleus, as the main alteration caused by the boric acid. Pyknosis of the cell nucleus was also observed in the larvae workers bees intestine exposed to fipronil [19]. Both the boric acid and fipronil were responsible for generating evaginations of the outer membrane of the nuclear envelope and increase in the size of regions with electron-dense chromatin in Malpighian tubules cells, oenocytes and trophocytes of bees [20].

Samples of sewage sludge also caused changes in the nuclear ultrastructure in diplopod, evidenced by the occurrence of nuclear envelope integrity loss of hepatic cells (Figure 1C, D) [14]. In collembolans submitted to heavy metals, the nuclei presented heterochromatin electron-dense spots located near the nuclear envelope. Moreover, it could be observed a displacement of the epithelial nucleus cells to the apical direction and lipid droplets and sphaerocrystals to the perinuclear region [22].

Body wall cells of nematode larvae presented degeneration of the nuclei. These alterations could be caused by the binding of toxic substances with the chromatin, but it can be also related with alterations in the membranes or any other metabolic alterations that lead to cell death [32].

2.5. Adverse reactions

In diplopod it was observed the formation of haemocytes clusters through of the fat body layer cells (Figure 1E), a reaction that is directly related to the mechanism of animals defence. The presence of haemocytes in a particular location represents an inflammatory reaction that helps in the removal of toxins and helps in the re-absorption of the damaged epithelium [33, 34, 35]. As the accumulation of haemocytes represents a common tissue response in invertebrates exposed to different conditions of environmental stress, the control of the haemocytes number can be used as a measure of stress caused by contamination of the environment [35].

A high increase in the tracheioles number between the hepatic cells that compose the diplopods midgut was observed in sub-chronically exposed animals to sewage sludge (Figure 1G) [14]. The authors suggest that, as the mechanisms of defence and detoxication imply in an excessive and continuous expenditure of energy, in special when the organism is exposed to a toxic agent for a long period, a higher oxygenation of the tissue is needed in order to allow the formation of adenosine triphosphate molecules (ATP).

3. Conclusions

The transmission electron microscopy is a tool that has successfully been used by many researchers in order to investigate how the cells respond to the contamination caused by man. As presented in this chapter, cell organelles can present different types of responses, depending on factors such as: (1) taxonomic group that the animal belongs to; (2) organ where the cell is located; (3) and substance that it is exposed.

Table 1 – Summary of the main ultrastructural alterations found in the cells of Hymenoptera, Diplopoda, Collembola, Isopoda, Oligochaeta and Gastropoda and the meaning of the alterations to these organisms.

Structure	Alteration	Animal	Meaning of the alteration
Plasmatic membrane	- disruption - dilatation of the intercellular spaces	Diplopoda	- Necrosis and cell death
	- disruption	Hymenoptera	
Microvilli	- dilatation	Hymenoptera	- Increase of the excretion surface
	- decrease and/or destruction	Isopoda	- Reduction of the absorption area
Mitochondria	- dilatation - increase in the electron-density - increase in the number	Hymenoptera	- Cell death - Oxidative stress - Increase in the energy production
	- reduction in number	Diplopoda	- Reduction in the energy production
		Isopoda	
	- decrease in the number of cristae	Collembola	
	- loss of the inner structure - reduction of the matrix content - decrease in the quantity of cristae - agglomeration of particles in the cristae - irregular organization of the cristae	Oligochaeta	- Mitochondrial inhibition, reduction in the energy production
	- decrease in the number - presence of large vacuoles - distortion/ degeneration	Nematoda	- Mitochondrial inhibition, reduction in the energy production
Endoplasmic reticulum	- dilatation of the inner membranes	Hymenoptera	- Cell death – apoptosis
	- deformation	Isopoda	- Detoxification
	- deformation - increase in the number of cisternae - accumulation in the apical region of microvilli	Collembola	- Detoxification
	- dilatation	Oligochaeta	- Cell death
Golgi apparatus	- alteration in the number of cisternae - dilatation or compactation of the cisternae - alteration in the shape or size of the secretory granules	Gastropoda	- Altered flow of membranous material produced by the endoplasmic reticulum
Cytoplasm	- vacuolization - formation of autophagic vesicles - myelin figures	Hymenoptera	- Cell death - necrosis - Cell death – apoptosis - Symptoms or early stage of cell death
	- vacuolization - formation of autophagic vesicles - increase in the number of sphaerocrystals	Collembola	- Cell death – necrosis - Cell death – apoptosis - Storage and elimination of toxic substances
	- vacuolization - formation of autophagic vesicles - increase in the number of sphaerocrystals - release of secretory vesicles	Diplopoda	- Cell death- necrosis - Cell death – apoptosis - Storage and elimination of toxic substances - Elimination of toxic substances
Nucleus	- chromatin condensation - formation of blebs - nuclear fragmentation - increase in the size of heterochromatin	Hymenoptera	- Decrease in the metabolic activity – cell death
	- pyknosis		- Advanced process of cell death
	- evaginations of the nuclear envelope		- Unknown
	- loss of integrity of the nuclear envelope	Diplopoda	- Cell death

Table 1 (cont.) – Summary of the main ultrastructural alterations found in the cells of Hymenoptera, Diplopoda, Collembola, Isopoda, Oligochaeta and Gastropoda and the meaning of the alterations to these organisms

Structure	Alteration	Animal	Meaning of the alteration
Nucleus (cont.)	- heterochromatin electron-dense spots near the nuclear envelope - presence of lipid droplets and sphaerocrystals in the perinuclear region	Collembola	- Cell death
Adverse reactions	- formation of haemocytes clusters	Diplopoda	- Inflammatory reaction
	- increase in the tracheioles number		- Increased energy requirement

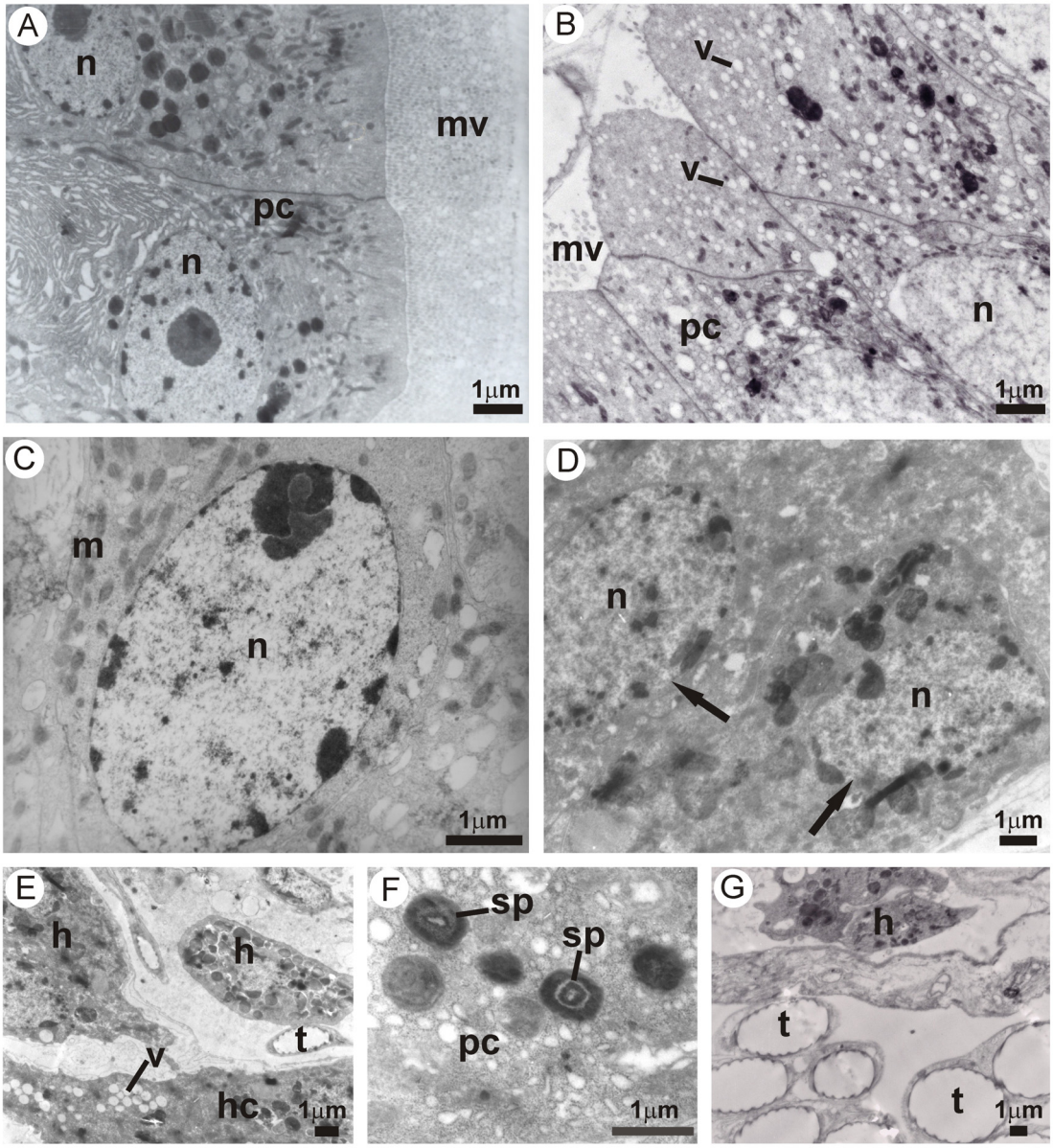

Figure 1 - Electron micrographs of the midg ut of diplopod. Control group (A, C). Group exposed to sewage sludge (B, D, E, F, G). Principal cell with microvilli (A), vacuolization of principal cell (B), intact nucleus cell (C), loss of the nuclear envelope integrity (D), concentration of haemocytes (E), sphaerocrystals (F) and tracheoles (G).

h: haemocytes; hc: hepatic cell; m: mitochondria; mv: microvilli; n: nucleus; pc: principal cell; sp: sphaerocrystals; v: vacuoles; t: tracheoles; arrows in D: damage of the nuclear envelope.

References

[1] Benchimol M, Attias M, Silva NLC, Carvalho TMU. Métodos de Estudo da Célula. In: *O Microscópio Eletrônico de Transmissão*. Rio de Janeiro, Brasil: Editoração Eletrônica-FENORTE/UENF; 1996: 142p.

[2] Junqueira LC, Carneiro J. *Histologia Básica* – Texto/Atlas. 11th ed. Rio de Janeiro: Guanabara Koogan SA; 2008.

[3] WILLIAMS DB, CARTER CB. Transmission Electron *Microscopy*. A Textbook for Materials Science. New York and London: Plenum Press; 1996.

[4] Köhler HR, Triebskorn R. Assessment of the cytotoxic impact of heavy metals on soil invertebrates using a protocol integrating qualitative and quantitative components. *Biomarkers.* 1998;3:109-127.

[5] Nogarol LR, Fontanetti CS. Acute and subchronic exposure of diplopods to substrate containing sewage mud: Tissular responses of the midgut. *Micron*. 2010;d41:239-246.

[6] Meyers TR, Hendricks JD. Histopathology. In: Rand GM, Petrocelli SR, eds. *Fundamental of Aquatic Toxicology: Methods and Applications*. Washington: Hemisphere Pub; 1985:283-331.
[7] Triebskorn R, Henderson IF, Martin AP. Detection of iron in tissues from slugs (*Deroceras reticulatum* Müller) after ingestion of iron chelates by means of energy-filtering transmission electron microscopy (EFTEM). *Pesticide Science*. 1999; 55:55–61.
[8] Spadotto CA, Gomes MAF, Luchini LC, Andréa MM. *Monitoramento do risco ambiental de agrotóxicos: princípios e recomendações.* Jaguariúna, SP: Embrapa Meio Ambiente; 2004.
[9] Gräff S, Berkus M, Alberti G, Köhler HR. (1997). Metal accumulation strategies in saprophagous and phytophagous soil invertebrates: a quantitative comparison. *BioMetals.* 1997; 10:45-53.
[10] Hopkin SP. *Ecophysiology of metals in terrestrial invertebrates*. London: Elsevier Applied Science; 1989.
[11] Malaspina O, Silva-Zacarin CM. Cell markers for ecotoxicological studies in target organs of bees. *Braz. J. Morphol. Sci.* 2006; 23:303-309.
[12] Matlock RB, Cruz R. Ants as indicators of pesticides impacts in banana. Environ. Entomol. 2003; 32:816-829.
[13] Fontanetti CS, Christofoletti CA, Pinheiro TG, Souza, TS, Pedro-Escher J. Microscopy as a tool in toxicological evaluations. In: Méndez-Vilas A, Diaz J, eds. *Microscopy: Science, Technology, Applications and Education.*, Badajoz: Formatex Research Center; 2010:1001-1007.
[14] Nogarol LR, Fontanetti CS. Ultrastructural Alterations in the Midgut of Diplopods after Subchronic Exposure to Substrate Containing Sewage Mud. *Water, Air & Soil Pollution*. 2011;128:539-547.
[15] Sumida S, Silva-Zacarin ECM, Decio P, Malaspina O, Bueno FC, Bueno OC. Toxicological and histopathological effects of boric acido n *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) workers. *J. Econ. Entomol.* 2010;103:676-690.
[16] Kumar V, Cotran RS, Robbins SL. *Patologia Básica*. 5 ed. Rio de Janeiro: Guanabara Koogan, 1994.
[17] Klotz JH, Amrhein C, McDaniel S, Rust MK, Reierson DA. Assimilation and toxicity of boron in the Argentine ant (Hymenoptera: Formicidae). *J. Entomol. Sci.* 2002;37:193-199.
[18] Köhler HR, Hüttenrauch K, Berkus M, Gräff, S, Alberti G. Cellular hepatopancreatic reactions in *Porcellio scaber* (Isopoda) as biomarkers for the evaluation of heavy metal toxicity in soils. *Applied Soil Ecology*. 1996;3:1-15.
[19] Cruz AS, Silva-Zacarin, ECM, Bueno OC, Malaspina O. Morphological alterations induced by boric acid and fipronil in the midgut of worker honeybee (*Apis mellifera* L.) larvae. *Cell Biol. Toxicol.* 2010;26:165-176.
[20] Ferreira, RAC. Análise morfológica e histoquímica do corpo gorduroso e dos túbulos de Malpighi de operárias adultas de Scaptotrigona postica (Latreille, 1807) (Hymenoptera, Apidae) tratadas com fipronil e ácido bórico. Rio Claro, SP: Universidade Estadual Paulista. 2010.
[21] Godoy JAP, Fontanetti CS. Diplopods as Bioindicators of Soils: Analysis of Midgut of Individuals Maintained in Substract Containing Sewage Sludge. *Water, Air and Soil Pollution*. 2010;210:389-398.
[22] Pawert M, Triebskorn R, Griffc S, Berkus M, Schulzd J, Köhler HR. Cellular alterations in collembolan midgut cells as a marker of heavy metal exposure: ultrastructure and intracellular metal distribution. The Science of the Total Environment, 181;187-200; 1996.
[23] Ghadially FN. *Ultrastructural Pathology of the Cell and Matrix'.* 3rd ed. London: Butter-worths; 1998.
[24] Triebskorn R. Ultrastructural changes in the digestive tract of *Deroceras reticulatum* (Müller) induced by a carbamate molluscicide and by metaldehyde. *Malacologia,*1989;31:141-156.
[25] Triebskorn R, Künast C. Ultrastructural changes in the digestive system of *Deroceras reticulatum* (Mollusca; Gastropoda) induced by lethal and sublethal concentrations of the carbamate molluscicide Cloethocarb. *Malacologia,* 1990;32:89-106.
[26] Gao Y, Sun Z, Sun X, Sun Y, Shi W. Toxic effects of albendazole on adenosine triphosphatase activity and ultrastructure in *Eisenia fetida*. *Ecotoxicology and Environmental Safety*, 2007;378-384.
[27] Arunyanart C, Kanla P, Chaichun A, Intapan PM, Maleewong W. Ultrastructural effects of albendazole on the body wall of *Gnathostoma spinigerum* third stage larvae. *Southeast Asian J Trop Med Public Health*, 2009;40:1199-1207.
[28] Reich T, Depew MC, Marks GS, Singer MA, Wan JKS. Effect of polychlorinated biphenyls on phospholipid membrane fluidity. *J. Environ. Sci. Health A. Environ. Sci. Eng*. 1981;16:65-72.
[29] Rez G. E1ectron microscopic approaches to environmental toxicity. *Acta Biol Hung*. 1986;37:31-45.
[30] Kammenga JE, Dallinger R, Donker MH, Köhler HR, Simonsen V, Triebskorn R, Weeks JM. Biomarkers in terrestrial in terrestrial invertebrates for ecotoxicological soil risks assessment. *Reviews of Environmental Contamination and Toxicology*. 2000;164:93-147.
[31] Heylen K, Gobin B, Arckens L, Huybrechts R, Billen J. The effects of four crop protection products on the morphology and ultrastructure of the hypopharyngeal gland of the European honeybee, *Apis mellifera*. *Apodologie.* 2011;42:103-116.
[32] Vogt G, Böhm R, Segner H. Mimosine-induced cell death and related chromatin changes. *J Submicrosc Cytol Pathol*. 1994;26:319-330.
[33] van Braak CBT. *Haemocytic defence in black tiger shrimp (Penaeus monodon).* Wageningen, The Netherlands: Wageningen University, 2002.
[34] Fontanetti CS, Nogarol LR, Souza RB, Perez DG, Maziviero GT. Bioindicators and biomarkers in the assessment of soil toxicity. In: Pascucci S, ed. *Soil Contamination.* Rijeka, Croatia: InTech; 2011:143-168.
[35] Perez DG, Fontanetti CS. Hemocitical responses to environmental stress in invertebrates: a review. *Environmental Monitoring and Assessment*. 2011;177:437-447.

