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Effect of Methamidophos on the Morphology and Male Reproductive Parameters of the Earthworm *Eisenia foetida*

Efecto del Metamidofos sobre la Morfología y Parámetros Reproductivos Espermáticos de la Lombriz de Tierra *Eisenia foetida*

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SUMMARY: The earthworm *Eisenia foetida* (Annelida, Oligochaeta) is an excellent biosentinel model organism for assessing potential toxicological risks by xenobiotics in terrestrial ecosystems. Methamidophos is an organophosphorous insecticide widely used to control agricultural pests. Its main action is due to inhibition of the enzyme acetylcholinesterase. The aim of this study was to evaluate the toxicity of methamidophos on the morphology and male parameters in *Eisenia foetida*. After determining the LD50 for methamidophos (85.34 mg/kg soil), five groups were formed, including control subjects, and sprayed with tap water. Methamidophos treated groups were subjected to doses of 1/10, 1/6, 1/3 and 2/3 of the LD50. Each group was analyzed at day 1, 6, 14 and 30 post-treatment. The results show a significant decrease in the weight of all individuals treated with methamidophos, besides showing a coiled body in 100% of them. Sperm count showed a significant increase in the treated groups after 1, 6 and 14 days post exposure and a significant decrease by day 30. Using the technique of acridine orange, a significant increase in abnormal metachromatic sperm (red fluorescence) in treated animals was shown. Methamidophos alters the overall behavior in earthworms and the male reproductive parameters of *Eisenia foetida* possibly altering the structure and conformation of DNA.

KEY WORDS: Reproduction; *Eisenia foetida*; Methamidophos; Cytotoxicity.

INTRODUCTION

Ecotoxicology has included into its fields of study the impact that chemical substances may produce in the environment. Thus the estimation of potential damages versus the potential benefits allows taking convenient decisions about the risk/benefit of such substance in order to avoid the risk of future chemical disasters (Bustos-Obregon & Goicoechea, 2002; Sifakis *et al.*, 2011).

The earthworm *Eisenia foetida* is an excellent animal model as sentinel of environmental pollution because it is easy to handle and inexpensive to maintain. Likewise, its reproduction and growth are well documented. It is an important species that helps maintaining the soil structure and fertility, as well as being a food source for certain vertebrates. In addition, it is a recycler organism for organic matter and a natural decontaminant (Sorour & Larink, 2001). It can accumulate several toxic chemicals constituting an additional hazard to animals that consume it as food, and therefore it has a key position in the passage of toxic substances through the food chain; this is why they can be used as biomarkers for toxic risk assessments of xenobiotics in terrestrial ecosystems (Espinoza-Navarro & Bustos-Obregon, 2004; Härkönen, 2005; Espinoza-Navarro & Bustos-Obregon, 2005; Méndez *et al.*, 2008; Phugare *et al*., 2012).

Organophosphorous insecticides act by inhibition of the enzyme acetylcholinesterase which hydrolyzes acetylcholine, a nerve signal mediator in the end of postganglionic fibers of the parasympathetic system, neuromuscular junction and central nervous system. Inhibition of this enzyme results in accumulation of acetylcholine, responsible for the clinical manifestations of poisoning in animals and humans (Bello-Ramirez *et al*., 2000; Kwong, 2002; Méndez, *et al*., 2008; Joshi & Sharma, 2011).

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Methamidophos is a chemical organophosphorous compound with the common name O,S- dimethyl phosphoramidothioate. It is used as a systemic pesticide with action by contact and ingestion to combat chewing and sucking insects; it can be absorbed by ingestion, inhalation and contact. It is distributed under the trade name of Tamaron 600, Monitor 600, MTD 600SL and methamidophos 60 (García-Santos & Keller-Ferrer, 2011).

The aim of this research was to demonstrate the effect of methamidophos on morphology and male reproductive parameters of *Eisenia foetida*, a biosentinel species of environmental pollution.

MATERIAL AND METHOD

The earthworm *Eisenia foetida*, subclass Oligochaeta, phylum Annelida, was used as biological material. The selected individuals were sexually mature and with a well developed clitellum. Their general condition was assessed by visual inspection of their behavior and anatomy (Espinoza-Navarro & Bustos-Obregon, 2004). The initial weight of the selected individuals was approximately 0.35 grams.

To determine the mean lethal dose, or LD50, 40 individuals were selected for each treatment group, with two experimental groups (n=80) per dose. The worms were placed in 4-liter plastic containers containing 3 kg of dry soil and then humidified to 50% moisture. Four treatment groups were evaluated at concentrations of 50, 100, 150, and 200 mg methamidophos per kilogram of soil. A 60% solution was used for a single application at the beginning of the experiment; the exposure was performed at room temperature and the worms were fed during the whole process. After 14 days of exposure the percentage of dead individuals was determined and the results were expressed in percentage of dead worms per dose. From this data we determined a dose-response graph adjusted to a linear model (linear regression). We then calculated the concentration which corresponds to 50% of all deaths. With this procedure, the LD50 was established at 85.34 mg methamidophos per kilogram of soil.

Experimental design. To determine the effect of methamidophos on the morphology and male reproductive parameters of *Eisenia foetida*, five groups including control group and working groups at 1/10, 1/6, 1/3 and 2/3 of the LD50 (doses of 0, 8, 14, 28 and 56 mg per kg of soil methamidophos, respectively) were formed. Each group consisted of 40 individuals that were assessed at 1, 6, 14, and 30 days post treatment, respectively. Individuals were sprayed with methamidophos according to working doses. The control group was sprinkled with tap water. The applications were made only once on day 0 at 9:00 am to the respective groups. After treatment, changes in weight, external anatomy, sperm count and quality were assessed.

Changes in weight were detected as the difference in average weights of earthworms at the day 0 and after 1, 6, 14 and 30 days of exposure. The weight of earthworms was recorded on a MK-500C scale. The mean and standard deviation per time interval and treatment group were determined. Changes in external anatomy were observed by simply viewing the worms and focused on the curl of their bodies.

The sperm count was performed after weighing the individuals. The selected animals were anesthetized by placing them in an alcohol solution at 5% and then on a dissection plate. With fine surgical scissors and tweezers, the dorsal anterior body was opened and the male reproductive organs -located between the prostomium and clitellum- were extracted.

The seminal vesicles and testes were placed in Petri dishes containing 1 ml of PBS at pH 7.4 and macerated to permit release of the spermatozoa. Tissues were homogenized at 100 rpm for 30 seconds and a sperm count from the sample was performed in a Neubauer chamber. The count was expressed as number of sperm per individual in relation to the weight of the worm expressed in milligrams. Besides the sperm count, the mean and standard deviation per time interval and per treatment group were calculated.

To determine the quality of sperm suspensions from treated and control groups were evaluated in order to detect structural alterations in DNA by the acridine orange fluorescent test. This test is based on the properties of the fluorochrome acridine orange (Merck, CI No. 46005) which fluoresces metachromaticly red after binding to denatured DNA and fluoresces orthochromatic green upon binding to DNA in its native state.

Sperm suspensions were subjected to a 90° C heat shock for 10 minutes. Samples were fixed overnight in cold (4°C) Carnoy's solution (methanol-acetic acid, 3:1) in the dark. Then drops were taken from suspensions (50 microliters) to make smears. After air-drying, each smear was added 2-3 drops of acridine orange to a final concentration of 0.19 mg/mL, pH 2.5, for 5 minutes in cold and dark room. Washed three times with double distilled water and observed under an epifluorescence microscope with excitation filter of 436 nm (RS III Zeiss, Germany),

with 250 sperm evaluated per individual.

Statistical evaluation of the results, in all cases, was performed with the program INSTAT and nonparametric Kruskal-Wallis ANOVA and Dunn multiple comparison tests were applied. Values with $p < 0.05$ were considered statistically significant.

RESULTS

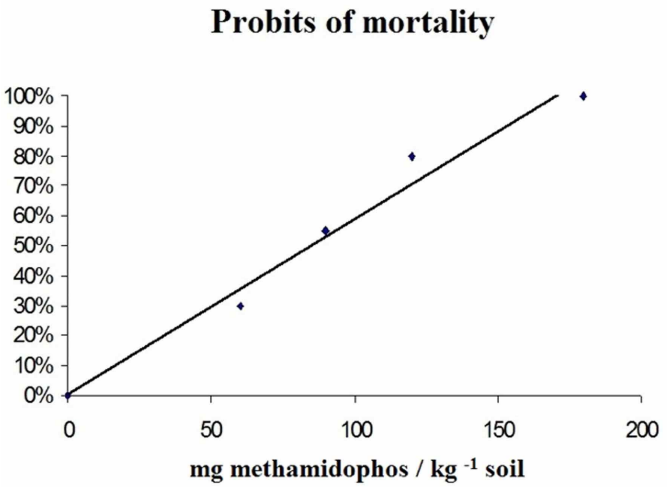

Fig. 1. Mean lethal dose (LD50) for *Eisenia foetida*. Doses of 50, 100, 150 and 200 mg of methamidophos per kilogram of soil.

The determination of methamidophos lethal dose 50 (LD50) for *Eisenia foetida*, expressed in milligrams of the pesticide, was 85.34 mg. per kilogram of soil. The dose-response curve was fitted to the linear regression equation $Y = 0.0058x + 0.005$ with $r2=0.9753$. Therefore, the doses equivalent to 1/10, 1/6, 1.3, and 2/3 of the LD50 were: 8, 14, 28, and 56 mg of methamidophos per kilogram of soil, respectively (Fig. 1).

Figure 2 shows a significant reduction in the weight of animals treated with concentrations of 8, 14, 28 and 56 mg of methamidophos after 1, 6, 14 and 30 days post treatment when compared with control group. This decrease was greater in the higher dose at 14 and 30 days after treatment, showing a dose-dependent response.

After calculating the sperm count and relate it to the weight of *E. foetida* in milligrams, it was observed that on day 1 and 6 after treatment, there was a significant increase in the number of sperm in all treated groups when compared to the control group in a dose dependent manner. At day 14, the increase was even more significant with respect to the control group. However,

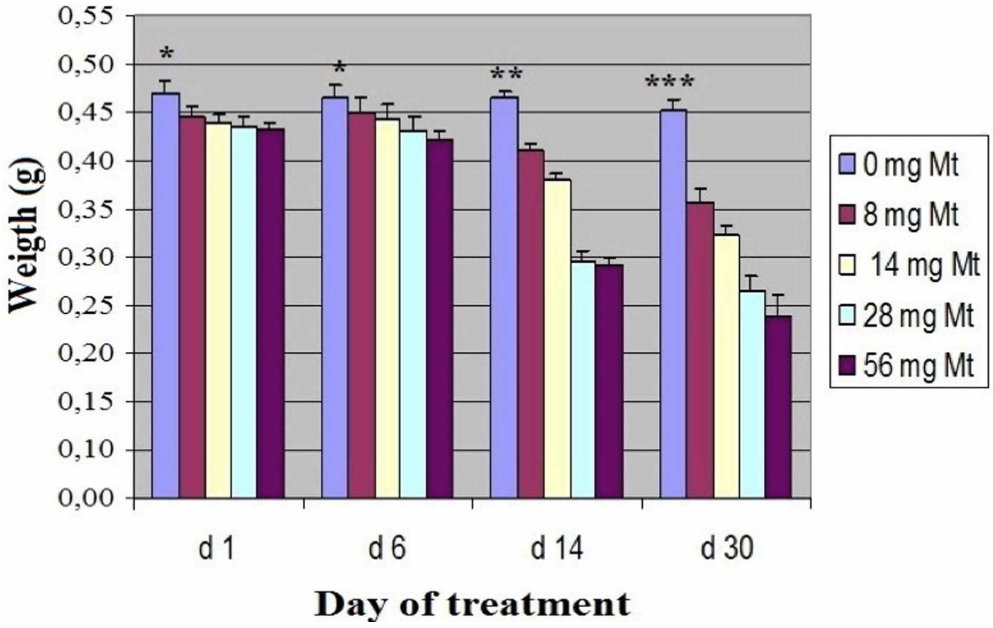

Fig. 2. Effect of methamidophos on body weight (grams) in *Eisenia foetida*. Doses of 0, 8, 14, 28 and 56 mg/kg of soil.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

day 30 samples showed a significant decrease in the number of sperm per milligram of gonadal tissue with respect to the control group at doses 14, 28 and 56 mg methamidophos/kg of soil, respectively (Fig. 3).

The results obtained with the technique of acridine orange fluorescence show a significant increase in the percentage of abnormal sperm with expression of red fluorescence (metachromatic) at all doses and treatment periods, with a dose dependent response. In the control groups, normal fluorescence patterns were detected, with only 10% of metachromatic sperm and 90% of orthochromatic sperm (i.e., green fluorescence; Fig. 4).

Figure 5 shows the changes in the external anatomy of *Eisenia foetida* in control groups and in groups treated with 1/10, 1/3 and 2/3 of methamidophos LD50 per kg of soil. By simple viewing, the coiling body behavior was observed in 100% of treated individuals. In controls earthworms this abnormal behavior was not observed.

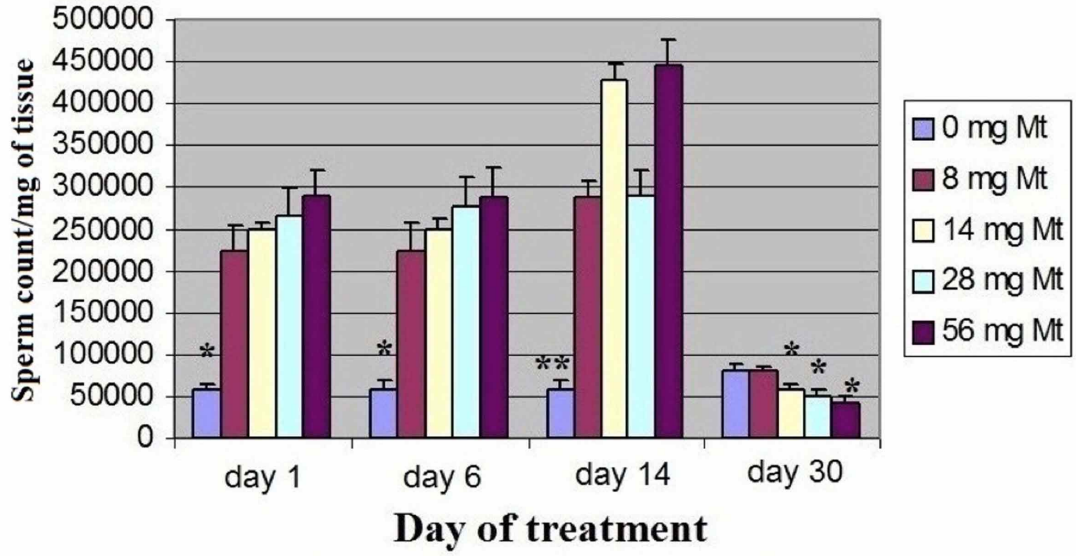

Fig. 3. Sperm count in *Eisenia foetida* per milligram of tissue after methamidophos exposure at doses of 0, 8, 14, 28 and 56 mg/kg of soil. * p <0.05; ** p <0.01.

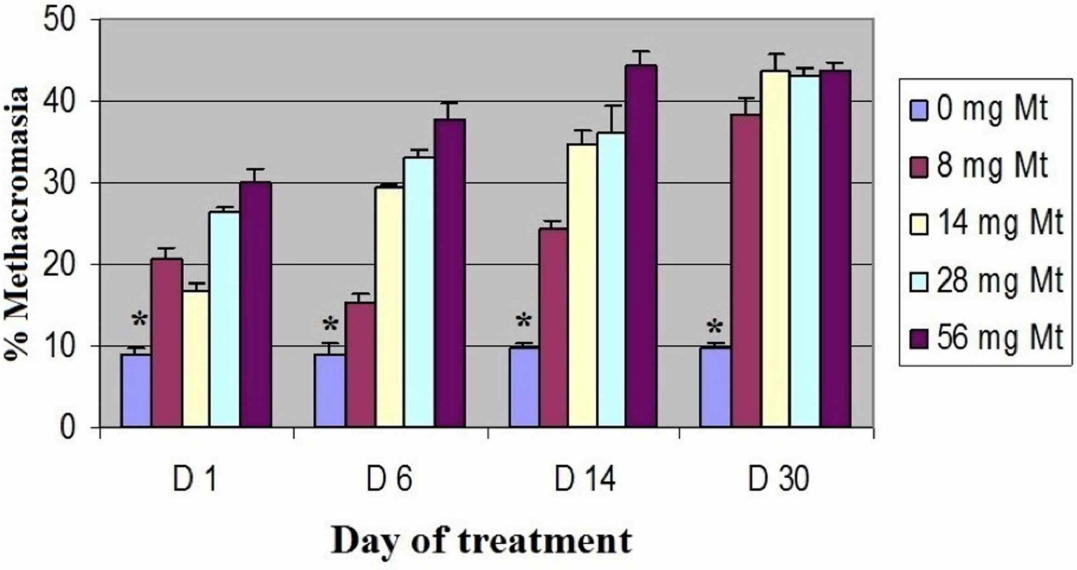

Fig. 4. Percentages of metachromasia in *Eisenia foetida* sperm using the method of acridine orange. Effect at doses of 0, 8, 14, 28 and 56 mg/kg of soil. * p <0.05.

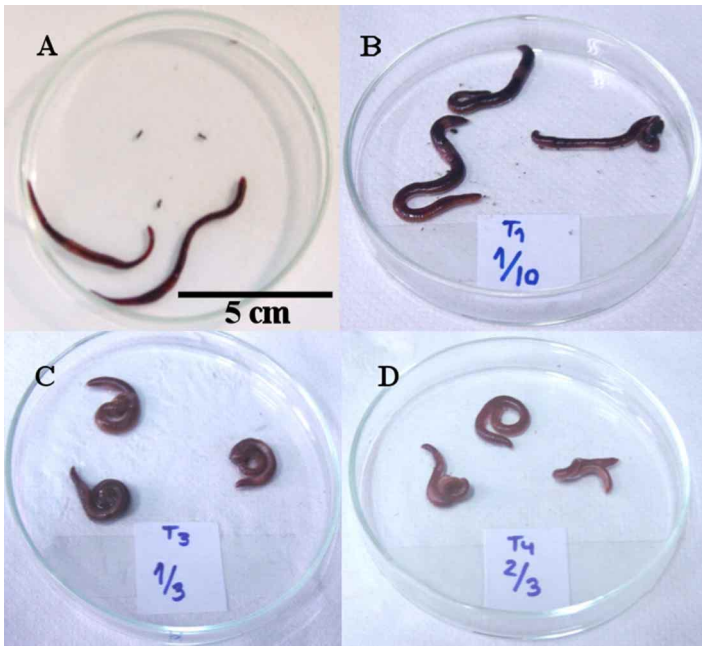

Fig. 5. Coiling body behavior in *Eisenia foetida* exposed to methamidophos. A: control group without coiling. B, C and D were exposed to 1/10, 1/3 and 2/3 of the LD50 (8, 28 and 56 mg/kg of soil, respectively) of methamidophos.

DISCUSSION

Earthworms are considered as sentinel organisms and permit good monitoring of genotoxic compounds that disrupt the normal functioning of the life cycle in terrestrial environments, where changes could be transmitted to offspring, altering reproductive patterns and threatening the viability of species. Earthworms like *Eisenia foetida* play a fundamental role on the structure and fertility of soils, making them one of the best model species in ecotoxicity studies (Espinoza-Navarro & Bustos-Obregon, 2005; Lin *et al*., 2010; Phugare *et al*.).

Methamidophos, insecticide and acaricide organophosphorus is widely used in agriculture, has a proven lethal effect on many living systems, but little is known about the chronic and sublethal effects on reproduction and genetic integrity of species (Chen *et al*., 2012).

Occupational exposure to organophosphates such as methamidophos may occur primarily through inhalation and dermal absorption by accident or as a result of improper handling. These pesticides are second in percentage of occupational poisoning with signs or symptoms that could be fatal (EPA, 2012).

Burrell *et al*. (2000) have shown adverse effects on sperm and offspring of mice exposed to methamidophos. In human males, alterations in sperm chromatin integrity have been reported (Salazar-Arredondo *et al*., 2008). Significant changes in the biology and morphology of *Eisenia foetida* have also been described (Yasmin & D'Souza, 2010; Chen *et al*.; Dureja & Tanwar, 2012).

In this work, methamidophos effects on *E. foetida* morphology and male reproductive patterns were assessed using sublethal doses of 2/3, 1/3, 1/6 and 1/10 of the LD50 -equivalent to 56, 28, 14 and 8 mg methamidophos per kilogram of soil, respectively- with evaluations after 1, 6, 14 and 30 days of exposure.

The results showed a significant weight loss in the worms treated with all doses which would indicate that this organophosphorus pesticide has a dose-dependent toxic effect. Espinoza-Navarro & Bustos-Obregon (2005) in a previous study associated the loss of body weight with an excessive secretion of body fluids in individuals treated with organophosphate pesticides. This loss of body mass has a multiplicative effect on *Eisenia foetida* since it also alters their ability to find food and therefore their reproductive capacity (Dureja & Tanwar).

In the evaluation of the effects of pesticides and other chemicals on reproduction, sperm count is one of the most sensitive parameters in determining reproductive impairment (Burrell *et al*.; Yasmin & D`Souza). The significant sperm count increase in earthworms treated with methamidophos for 1, 6 and 14 days could be explained by the cytotoxic cholinergic effect exerted by methamidophos on male reproductive structures of *Eisenia foetida*, which may allow the release of sperm stored and/or included in the ciliated epithelium of the spermatheca, thus altering the sperm count. Recent studies also report that the sperm count may be affected by the effect of pesticides on follicular clusters of spermatogonia (i.e., "morulae"), altering the constitution of cytophorus (an anuclear sincytial cytoplasmic mass of morulae), launching a great amount of immature sperm to the coelomic and seminal vesicles torrent thus affecting the normal development of spermatogenesis and spermiogenesis (Sorour & Larink; Chen *et al*.). This initial acute effect disappears by day 30 post treatment and then a significant reduction in the number of sperm per milligram of gonadal tissue was observed, suggesting that methamidophos -like other organophosphates- has been biodegraded and its metabolites may alter the cell cycle patterns and hence the reproductive patterns of *Eisenia foetida* (Zhou *et al*., 2008).

The effect of methamidophos on the sperm quality of *Eisenia foetida* shows a direct correlation between increased sperm chromatin decondensation and increased metachromatic (red fluorescence) sperm levels, indicating that this toxicant might be interfering with the DNA-histones complexes and thus allowing the fluorochrome acridine orange to bind to anionic groups in DNA which manifests as a red fluorescence. Normally, chromatin integrity is ensured by intact disulfide bonds, which is expressed as acridine orange orthochromasia (i.e., green fluorescence; Salazar-Arredondo *et al*, 2008).

Methamidophos, widely reported as an inhibitor of acetylcholinesterase (AChE), affects the neuromuscular functioning by altering the Ca++ and K+ balance which is reported to be responsible for changes in behavior and activity of earthworms. These alterations in the ionic balance further explain the phenomenon of winding of the tail observed in 100% of treated individuals (Dureja & Tanwar).

CONCLUSIONS

The mean lethal dose (LD50) of methamidophos for the earthworm *Eisenia foetida* after 14 days of exposure was determined in 85.34 mg methamidophos per kilogram of soil. Methamidophos provokes a significant decrease in the weight of treated individuals with respect to controls, thus indicating a cytotoxic effect. All treated individuals show body coiling, an important feature that evidences important nervous and neuromuscular damage. Methamidophos alters the sperm count, causing a significant initial increase, possibly by direct effects on seminal vesicles and the cytophorus of modular follicles. Subsequently, a significant decrease in sperm possibly indicates that methamidophos has been biodegraded, altering cell cycle patterns and hence fertility patterns in *Eisenia foetida*. Methamidophos destabilizes the sperm chromatin in *Eisenia foetida*, significantly increasing the levels of metachromasia or red fluorescence in sperm.

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ESPINOZA-NAVARRO, O.; FERREIRA, C. & BUSTOS-OBREGÓN, E. Efecto del metamidofos sobre la morfología y parámetros reproductivos espermáticos de la lombriz de tierra *Eisenia foetida*. ***Int. J. Morphol., 31(3)***:1097-1103, 2013.

RESUMEN: La Lombriz de tierra, *Eisenia foetida* (Annelida, Oligochaeta), constituye un excelente modelo de organismo biocentinela para evaluar los riesgos tóxicos de xenobióticos, en ecosistemas terrestres. Metamidofos es un insecticida organofosforado de amplio uso para controlar plagas agrícolas. Su acción principal se debe a la inhibición de la enzima acetilcolinesterasa. El objetivo de este estudio fue evaluar la toxicidad de metamidofos sobre la morfología y parámetros espermáticos de *Eisenia foetida*. Una vez determinada la LD50 para metamidofos (85,34 mg/kg de tierra), se tomaron 5 grupos, entre ellos los individuos del grupo control, los cuales fueron asperjados con agua potable. Los grupos tratados con metamidofos fueron sometidos a dosis de 1/10, 1/6, 1/3 y 2/3 de la LD50. Cada grupo fue analizado al día 1, 6, 14, y 30 post tratamiento. Los resultados muestran una baja significativa en el peso de todos los individuos tratados con metamidofos, además de presentar enrrollamiento corporal, en el 100% de ellos. El recuento espermático presenta un aumento significativo en los grupos tratados a 1,6 y 14 días, para luego decaer significativamente el día 30. El uso de la técnica del naranja de acridina, mostró un aumento significativo de espermatozoides metacromáticos anormales en los animales tratados (fluorescencia de color rojo). Se concluye que Metamidofos altera el comportamiento general de la lombriz de tierra y los parámetros reproductivos espermáticos de *Eisenia foetida* posiblemente alterando la conformación y estructura del ADN.

PALABRAS CLAVE: Reproducción; *Eisenia foetida*; Metamidofos; Citotoxicidad.

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