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ECOTOXICOLOGICAL BIOASSAYS OF THE EARTHWORMS *ALLOLOBOPHORA CALIGINOSA* SAVIGNY AND *PHERETIMA HAWAYANA* ROSA TREATED WITH ARSENATE

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ABSTRACT

Little research has been carried out on the effect of arsenate on earthworms. Ecotoxicological laboratory tests are fundamental tools for assessing the toxicity of arsenate to soil organisms. In this study, the impact of arsenate on the survival, reproduction and behaviour of the endogenic earthworms *Allolobophora caliginosa* and the anecic earthworms *Pheretima hawayana* has been quantified. The 96-h LC 50 of arsenate was estimated as 233.43 mg arsenate Kg^{-1} soil. d.w. for *P. hawayana* which is significantly higher than that of *A. caliginosa* 147.24±27.16 mg arsenate kg^{-1} soil d.w. The number of juveniles of *P. hawayana* was significantly higher than that of *A. caliginosa* at the arsenate concentrations 180, 240 and 400 mg kg^{-1} soil dry weight. With the exception of the control (6.5 mg arsenate kg^{-1} soil.d.w.), *P. hawayana* showed an avoidance behaviour for soils treated with all tested concentrations. *A. caliginosa* preferred soils treated with 6.5, 60, 110, 180 mg arsenate kg^{-1} soil d.w., while the avoidance behaviour has been recorded only at 240 and 400 mg arsenate kg^{-1} soil. d.w. This means that *A. caliginosa* individuals feed less when exposed to arsenate. On the contrary, the *P. hawayana* worms could be escaped into their deep vertical burrows when exposed to arsenate.

Keywords: Acute Toxicity, Earthworm, Chronic Toxicity, Avoidance, Arsenate

1. INTRODUCTION

Large quantities of contaminants have continuously been leaked into ecosystems as a consequence of urbanization and industrial processes (Begum *et al*., 2009). Heavy metal pollution is the case where the quantities of these elements in soils are higher than the natural environmental concentrations and is potentially harmful to biological life. Arsenic is a metalloid pollutant widelyintroduced into nature and the environment through industrial processes and agricultural practices (Button *et al*., 2009; 2010; 2011; 2012). Samad *et al*. (2010) ranked arsenic in abundance as 20th in the earth's crust, 14th in seawater and 12th in the human body. There has been growing concern about the environmental effects resulting from arsenic compounds and, therefore, different countries have established various arsenic concentration limits for the protection of wild life in all ecosystems. Arsenic exhibits both metallic and non-metallic properties. In general, for metallic forms, arsenite (As^3) is considered more toxic than arsenate (As^5) (Molly *et al*., 2013). Since arsenic is a normal constituent of the environment, there is a need for effective monitoring and measurement of arsenic at arsenic-containing soil and waste sites. Arsenic occurs naturally in soils as a result of theweathering of the parent rock. Over 200 arsenic-containing minerals have been identified, with approximately 60% being arsenates, 20% sulphides and sulphosalts and the remaining 20% including arsenides, arsenites and oxides (Murciego *et al*., 2011). Ecotoxicological laboratory tests are considered a preliminary step in bioassays of environmental risk. Because of their relatively quick results, these tests can quantify the risks to animals posed by the use of certain substances in the soils of a given terrestrial ecosystem. Toxicity studies on soil fauna have not yet been carried out for several heavy metals. Such tests are also required to implement rules that can effectively regulate agricultural protection products. Since earthworms are one of the most important biotic components in the soil, they have been utilized extensively in studies of the effects of heavy metals (Ander *et al*., 2009; 2010a, 2010b; Button *et al*., 2010; 2011; 2012 ; Carbonell *et al*., 2009; Contreras-Ramos *et al*., 2009; Huang *et al*., 2010; Jones *et al*., 2009; Langdon *et al*., 2009; Natal-da-Luz *et al*., 2009; Oni and Hassan, 2013). Earthworms are known to play a major role in the mixing of soil constituents and are more susceptible to metal pollution than many other groups of terrestrial invertebrates. An avoidance response test has been used in many studies to assess the toxicity of contaminated soils to earthworms (Artuso *et al*., 2011; Kobeticova *et al*., 2010; Owojori and Reinecke, 2009). Most studies have evaluated arsenic toxicity in mammal cell lines and aquatic animal species. However, the studies of arsenic toxicity in terrestrial invertebrates, including earthworms are relatively limited.

The aim of this study was to characterize the effects of varying concentrations of arsenate on survival, growth, reproduction and behaviour of two earthworm species belonging to two different ecological categories: endogeic (*A.caliginosa*) and anecic (*P.hawayana*) under laboratory conditions.

2. MATERIALS AND METHODS

2.1. Soil

Samples of a sandy loam soil have been collected from an orange orchard that has not been treated with inorganic as for 40 years. Background arsenic concentration in soil (control) was determined according to the methods of Anderson *et al*. (2013).

2.2. Chemicals

All reagents used were analytical grade and all aqueous solutions were prepared using distilled water. Inorganic sodium arsenate ($Na_2HASo_4.7H_2O$) was purchased from Sigma-Aldrich, Egypt.

2.3. Experimental Animals

Clitellated specimens of two earthworm species *A. caliginosa* and *P. hawayana* were sampled in same duration in selected sites (non-polluted soil) by digging soil and were separated by hand sorting. Samples of each species were taken at the time of each sampling and placed in ventilated plastic boxes with their native soils and some leaf letter. The boxes containing earthworms and soil were returned to the laboratory in Faculty of Science, Zagazig University. The concentrations of arsenate used in the laboratory tests were determined through range finding tests. Experiments were started with an acute toxicity using increasing concentrations up to the limit of 400 mg kg^{-1} soil d. w. The chronic toxicity bioassays used sublethal concentrations based on the present definitive lethality tests. The avoidance tests used a different set of sublethal concentrations, lower than that used for chronic toxicity tests.

2.4. Acute Toxicity

Approximately 400 g of uncontaminated orchard soil were partially air dried, sieved (3 mm) and placed in circular plastic containers with a diameter 12.5 cm and a height of 9.5 cm such that each container contained soil to a depth 5-7 cm. The soil was then rewetted to a moisture content (55%) using distilled water (control) or a solution of sodium arsenate to give soil concentrations of 60, 110, 180, 240 and 400 mg arsenate kg^{-1} soil d. w. Three replicates of each concentration in addition to the control (untreated soil) were examined. The concentrations used were based on the results of pilot experiments (not reported here). Earthworms were washed and 20 individuals of each species were placed in each test container which was closed with plastic mesh. Lethal concentrations (96-h LC 50) of *A. caliginosa* and *P. hawayana* were assessed by probit analysis (Finney, 2009). After linearization of response curves by logarithmic transformation of concentrations, 95% confidence limits and slope function were calculated to provide a consistent presentation of toxicity data. The worms were fed cow manure each day during the 4 days of the test. In the last day of bioassays, worms were removed from the containers. Individuals that did not respond to the mechanical stimulation of the anterior portion of the body were recorded as dead.

2.5. Chronic Toxicity

Chronic toxicity effects of arsenate on earthworm reproduction and growth were assessed according to Alves *et al*. (2013). The bioassays were installed as in the acute toxicity tests which differed only in duration, concentrations and assessment methods. The soil was treated with sublethal concentrations 20, 30, 50, 100 and 150 mg arsenate kg^{-1} soil d. w., for *A. caliginosa*and *P. hawayana*. Three replicates of each concentration in addition to the control were used for 50 days. These concentrations were chosen on the basis of the data of previous acute toxicity test. Earthworms were washed and weighed individually and 20 individuals of each species with 600 g native soil were placed in each test container which was closed with plastic mesh. In the 25 day of bioassay, worms were removed from the containers. Individuals that did not respond to the mechanical stimulation of the anterior portion of the body were recorded as dead. Live worms were washed and weighed and the difference between starting and ending body weight was calculated. For the next 25 days only the soils, juvenile worms and cocoons remained in the containers. On the 50th day, the containers were immersed in warm water (40±5°C) for 2 h and once the juveniles had emerged on the soil surface, the number of individuals generated was calculated. All worms were fed with cow manure once a week during time of experiment. In the chronic toxicity, the mean body weight difference and number of juveniles were calculated.

2.6. Earthworm's Behaviour

Avoidance tests were carried out following the recommendations of Alves *et al*. (2013). For both *A. caliginosa* and *P. hawayana,* the arsenate was tested at concentrations 10, 20, 30, 40 and 50 mg kg^{-1} soil d. w. with 3 replicates for each concentration. Plastic boxes (20×15×10 cm) were divided into two equal compartments with a plastic divider. To one compartment, 400 g of treated soil were added while the other compartment remained as a control (400 g of untreated soil). Immediately thereafter, the plastic divider was removed and 10 clitellated earthworms of each species were placed on the line separating the two compartments of the containers. Containers were covered with plastic mesh. No food was added during this experiment. After 48 h, the plastic dividers were inserted again and the soil in both compartments removed. The number of earthworms present in each container (treated and control) were counted. Worms that were along the dividing line between the two compartments were counted as 0.5 for each compartment. Percentage avoidance was calculated using the following equation: % avoidance = $[(C-T)/N] \times 100$, where C is the number of worms in control soil, T is the number of worms in treated soil and N is the total number of worms at the beginning of experiment. Positive percentages indicate avoidance of the treated soil, a zero indicates no avoidance and the negative percentages indicate an attraction for the arsenic-treated soil.

2.7. Statistical Analysis

Analysis of data was carried out by student's t-test for comparing the means of experimental and control groups.

3. RESULTS

3.1. Acute Toxicity

In the control (background as concentrations in uncontaminated soil were 6.5 ± 0.3 mg kg^{-1} soil d. w.) of the acute toxicity tests, mortality of adult *A. caliginosa* and *P. hawayana*was below 10%, achieving the requirements of bioassay validation. There were no significant differences between replicates for each treatment, therefore the data from the three replicates were pooled for statistical analyses. The 96-h continuous exposure LC50 for *P. hawayana* was significantly higher (233.43±42.45 mg arsenate Kg^{-1} soil. d. w.) than that of *A. caliginosa* (147.24±27.16 mg arsenate kg^{-1} soil d.w) ($p<0.05$, t-test).Surviving worms also had reduced body weight and in some cases, morphological abnormalities like, coiling and curling and lifting the body of *A. caliginosa*have been recorded.

3.2. Chronic Toxicity

In the chronic toxicity tests, the control showed means of 75 and 92 juveniles of *A. caliginosa* and *P. hawayana*, respectively. The number of produced *A. calignosa* juveniles were significantly ($p<0.05$, t-test) fewer than that of *P. hawayana* at the concentrations 180, 240 and 400 mg arsenate kg^{-1} soil d.w. Number of juveniles of *P. hawayana* were four times larger (40 individuals) than the number of juveniles of *A. caliginosa* (10individuals) at the concentration 400 mg arsenate kg^{-1} soil d.w. (**Fig. 1**). Examined *P. hawayana*showed significant ($p<0.05$, t-test) reduction in body weight after 25 days exposure to concentration 60 mg arsenate kg^{-1} soil d.w. compared to the control while *A. caliginosa* individuals showed significant ($p<0.05$, t-test) reduction in body weight after 25 days exposure to all examined concentrations (**Fig. 2a and b**).

3.3. Avoidance Responses

In avoidance tests, the number of dead and escaped worms was <10%. This criterion was fulfilled in all tests. *P. hawayana* worms significantly ($p<0.05$, t-test) avoided soils treated with all tested arsenate concentrations compared to the control (untreated). Avoidance percentages were 27, 37, 30, 43 and 40 at 60, 110, 180, 240 and 400 mg arsenate kg^{-1} soil d. w., respectively (**Fig. 3a**). Soil treated with arsenate attracted *A. caliginosa* worms at the concentrations 60, 110, 180 mg kg^{-1} soil d. w. However, soil treated with higher concentrations of the arsenate (240 and 400 mg kg^{-1} soil d. w.) was significantly ($p<0.05$, t-test) avoided by the worms (**Fig. 3b**).

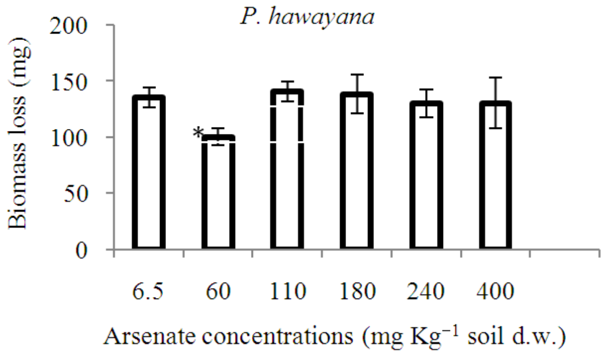


Fig. 1. Meannumber of juveniles of ♣: *P.hawayana* and ◆: *A. caliginosa* treated with varying concentrations of arsenate. *Means differ significantly from *A. caliginosa* (Student's t-test, p<0.05)

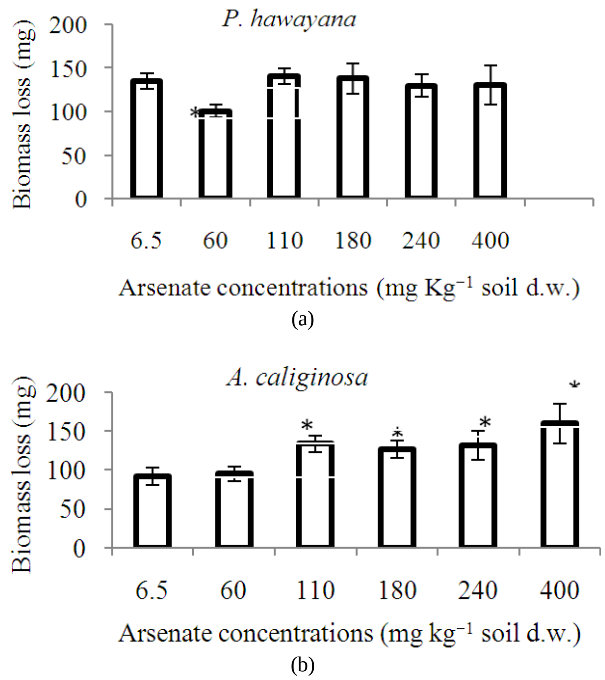


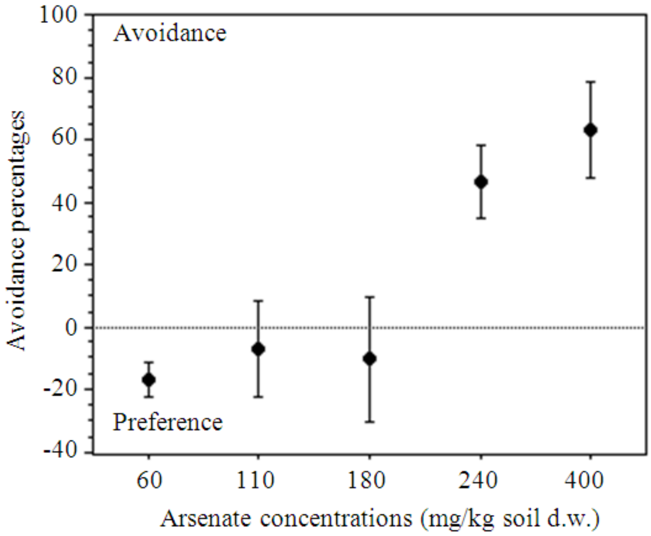
Fig. 2. Reduction in biomass (mg) of (a): *P. hawayana* and (b): *A. caliginosa* treated with varying concentrations of arsenate (25 days of exposure). *Means differ significantly from control (Student's t-test, p<0.05)

4. DISCUSSION

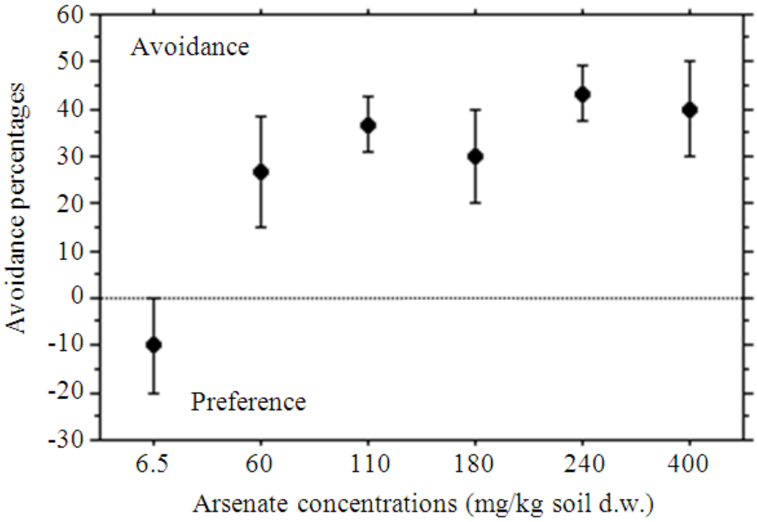
Earethworms are key organisms in soil ecology. As ecosystem engineers their activity affects not only many important soil processes such as soil aeration, decomposition or nutrient availability, but they also promote soil biodiversity, soil fertility and soil health (Palm *et al*., 2013). In this study both acute and chronic toxicity effects of arsenate have been demonstrated. The 96-h LC50 (233.43±42.45 mg kg^{-1} soil d. w.) of *P. hawayana*was significantly (p<0.05, t-test) higher than that of *A. caliginosa* (147.24±27.16 mg kg^{-1} soil d. w.). The difference between the acute toxicity of the two worms could be explained on the basis of the variety of their living and feeding habitats. *A. caliginosa* is an endogenic earthworm and the individuals of this species forage below the surface where they ingest large quantities of organic-rich soil and build burrows that are mainly horizontal in nature. Although they build permanent and vertical burrows that penetrate the soil deeply, the individuals of anceicspecies *P. hawayana* come to the surface to feed on partially decomposed litter, manure and other organic matter. Their burrows create microclimatic gradient and the worms can be found shallow or deep in their burrows depending on the prevailing conditions. The differences between the results reported in the literature and those of the present study are small and mostly attributable to different substrate composition and the earth worm species (Anderson *et al*., 2013).

Body weight loss may reflect reduced feeding by the worms as reported in other studies (Gomez-Eyles *et al*., 2009). In the present study, the body weight of *A. caliginosa* treated with arsenate concentrations (110-400 mg kg^{-1} soil d. w.) significantly (p<0.05, t-test) reduced in relation to the control while the body weight loss in *p.hawayana* was not affected by the arsenate concentrations except at 60 mg kg^{-1} soil d. w. This means that *A. caliginosa* individuals feed less when exposed to arsenate.

On the contrary, the anceic *P. hawayana* worms could be escaped into their deep vertical burrows when exposed to arsenate. However, Kreutzweiser *et al*. (2008) noted that worm feeding was similar in treated and control soils and attributed the reduction in biomass to physiological changes in worms. The lethal effectscould be attributed tothe blocking of nervous system receptors by pesticides. This blocking leads to an accumulation of acetylcholine which results in muscle and organ paralysis. In *P. hawayana*, the lowest concentration of arsenate (60 mg kg^{-1} soil d. w.) resulted in significant (p<0.05) less weight loss than the control (**Fig. 3b**) which could be the result of physiological stimuli caused by low concentrations of arsenate. Some authors have reported similar results and attributed it to a hormetic response which is defined as the ability of a substance to be toxic at high concentrations but stimulant at low concentrations (Zhang *et al*., 2009).



(a)



(b)

Fig. 3. Avoidance or attraction response of (a): *P. hawayana* and (b): *A. calignosa* to arsenate concentrations (mean net response and standard error bars). * Means differ significantly from control (student's t-test, $p<0.05$)

Few number of researches have been performed on the impact of heavy metals on earthworms avoidance under laboratory conditions. The earth worm avoidance test, originally developed in USA, was selected because it is quick and easy to perform and it is known to be sensitive towards a wide range of chemicals. Natal-da-luz *et al*. (2009) conducted avoidance tests with earthworms to demonstrate their feasibility as early screening tools for assessing the toxic potential of metal-polluted soils. Summarizing the results (**Fig. 3a and b**), it could be demonstrated that avoidance responses of *P. hawayana* is more sensitive to arsenate contamination than that of *A. caliginosa*. In this study, avoidance response of *A. caliginosa* occurred at a concentration 4 times (240 mg kg^{-1} soil d. w.) higher than that of *P. hawayana* (60 mg kg^{-1} soil d. w.). The ability of *A. caliginosa* to tolerate high levels of arsenate probably involves a combination of heritable and integrated physiological, morphological and behavioral modifications. Increased bio-immobilization capacity may be the most likely physiological strategy for arsenate resistance in earthworms (Ander *et al*., 2010a).

5. CONCLUSION

In this study ecotoxicological laboratory tests have been conducted on two eco-physiological different earthworms to determine the impact of arsenate on the survival, reproduction and behaviour of the endogenic, *A. caliginosa* and the anecic, *P. hawayana.* Based on the results of this work, it is concluded that body weight loss may reflect reduced feeding by the worms. The body weight of *A. caliginosa* treated with arsenate significantly reduced in relation to the control while the body weight loss in *P. hawayana* was not affected by arsenate concentration except at 60 mg kg^{-1} soil d. w. For assessing the toxicity of arsenate, *A. caliginosa* and *P. hawayana* are ecologically relevant species and they are sensiive to arsenate applications. However, when a rapid and efficient screening is required, *P. hawayana* is much more convenient.

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