Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 21(2009) 691–695
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Effect of lead on survival, locomotion and sperm morphology of Asian earthworm, *Pheretima guillelmi*

ZHENG Rongquan*, LI Canyang

College of Chemistry and Life Science, Zhejiang Normal University, Jinhua 321004, China. E-mail: zhengrq@zjnu.cn

Received 10 May 2008; revised 23 June 2008; accepted 15 July 2008

Abstract

To provide basic toxicity data for formulating risk characterization benchmarks, the effects of lead on survival, locomotion, and sperm morphology were investigated in the Asian earthworm *Pheretima guillelmi*. The LC_{50} of *P. guillelmi* for 7 and 14 d were 4285±339 mg/kg and 3207±248 mg/kg, which shows *P. guillelmi* can tolerate a higher concentration of lead nitrate. The average weight of the surviving earthworms decreased at concentration of 2800 mg Pb/kg soil, and the locomotor ability of earthworms exposed to a range of soil Pb concentrations showed a general decrease with increasing Pb concentrations. We also presented data depicting the sperm morphology of earthworms, which shows potential as a sensitive biomarker for measuring the effects of heavy metal on reproduction.

Key words: lead; *Pheretima guillelmi*; sperm morphology; locomotion

DOI: 10.1016/S1001-0742(08)62325-6

Introduction

The metal Pb is a ubiquitous environmental contaminant in soils because of the extensive use of Pb in batteries, paints, alloys, and other commercial products. Although there is abundant evidence of lead pollution in the environment, it is difficult to determine its effects on the environment (Davies *et al*., 2003). Earthworm is ideal experimental animals for assessing soil quality and contamination (Heikens *et al*., 2001; Lanno *et al*., 2004). When assessing soil quality and contamination, many parameters on earthworm have been generally used before, such as survival, growth, reproduction, accumulation of substances and avoidance responses (Maboeta *et al*., 1999; Currie *et al*., 2005; Langdon *et al*., 2005; Jia *et al*., 2005; Zhou *et al*., 2007). Among these parameters, the measurement endpoints are easily measured and can predict the toxic effects of chemicals.

The reproductive system is sensitive to environment and the sperm morphology is one of most important parameters when accessing sperm quality. The abnormal sperms would result in deformity or infertile. Alterations in the reproductive process in individuals could both correspond to and portend the effects at the population level. As such, they have potential as measurement endpoints for assessing sublethal toxicity of terrestrial pollutants. Pb affects the fertility of earthworms, e.g., *Eisenia fetida* (Spurgeon *et al*., 1994; Reinecke and Reinecke, 1996; Reinecke *et al*., 2001), *Eudrilus eugeniae*, *Perionyx excavatus* (Reinecke *et al*., 2001), and *Eisenia andrei* (Savard *et al*., 2007). Consequently, a benchmark of 500 mg/kg Pb was established for this invertebrate (Efroymson *et al*., 1997). Recently, the United States Environmental Protection Agency (USEPA) has issued an Ecological Soil Screening Level (Eco-SSL) of 1700 mg Pb/kg for soil invertebrates, but this value is based on reproduction tests with the Collembola *Folsomia Candida* (USEPA, 2003).

However, Stenersen *et al*. (1992) maintained that if earthworms are to be exploited as test organisms in ecotoxicological studies, more should be known about their interspecific differences. Many studies have been conducted on the effects of heavy metals on earthworms (e.g., Guo *et al*., 1996; Langdon *et al*., 2005; Inouye *et al*., 2006), however, by now, no information has been available on the effects of Pb on *Pheretima guillelmi*. The Asian species *P. guillelmi* mainly distribute in the east of China. As a waste decomposer, *P. guillelmi* can also be utilized as a Chinese medicine.

The primary aim of the study was to determine the toxicity of Pb in solution forms to *P. guillelmi* by calculating the LC_{50}, and the effects of sublethal concentrations of lead nitrate on the locomotory abilities and sperm morphology of *P. guillelmi*.

1 Materials and methods

1.1 Earthworm

The soil used in these experiments was prepared according to Organization for Economic Cooperation and

* Corresponding author. E-mail: zhengrq@zjnu.cn

Development (OECD, 1984) and consisted of 70% sand, 20% kaolin clay, and 10% organic matter. After air-dried the soil was sieved to < 2 mm. Earthworms (fresh mass range 2.18–2.78 g, mean ± SD 2.49 ± 0.15 g) used in the test were clitellum *P. guillelmi* obtained from a local pristine land near the North Mountain located in Jinhua of Zhejiang Province in China. Based on clitellum and the weight of fresh body, the earthworms were about 3–4 months old.

1.2 Earthworm acute test

The toxicity experiments were conducted in plastic containers containing 5 kg dry soil and 1100 mL demineralized water, giving a 22% water content (50% of the water holding capacity). The water, in the relevant cases also containing the final lead content, was added one week prior to the start of the experiment. The earthworms (10 per replicate) were exposed to eight lead concentration levels, including controls, ranging between 0 and 7500 mg/kg (dry weight by dry weight), specifically 0, and 1000, 1400, 2000, 2800, 3800, 5400, 7500 mg/kg, with four replicates per concentration. Earthworms were acclimatized in uncontaminated test soil for 1 week before being introduced to the lead amended soil. For all soils the pH was adjusted to 5.5–6.0, by the addition of $CaCO_3$. The container lids were perforated and covered with gauze to allow aeration and to keep the worms from escaping. The experiment was continued for two weeks at constant temperature 20±1°C. The surviving earthworms were hand collected and the biomass and mortality were determined at 7 and 14 d after the addition of the lead.

Sublethal toxicity tests were conducted in a similar way as the toxicity experiments described above except the metal spiked with the soil were 1000, 1400, 1800, 2500 mg/kg. Each of the 4 concentrations and the control had four replicate containers with 10 worms per container.

1.3 Earthworm locomotor ability test

The locomotor ability was measured after 7 d. The worms were removed from each container and washed in distilled water. They were then put on a clean wooden experimental table where there was a red line drew by 2% coccinellin. A wet print paper (150 mm × 150 mm) was put on the table in 1 cm from the red line. After crossing the red line, the earthworms would crawl to the print paper. As they had dipped some coccinellin on their body, the trail they had crawled would be marked. The trail that a worm had crawled in one minute was overlaid by a thread. Then the thread was stretched and the length was measured by a ruler.

1.4 Earthworm sperm morphology test

The sperm deformity of the worms was assessed after exposed to each concentration of Pb for 14 d. After anesthetization in 12% ethanol, each earthworm was dissected from the dorsal side to collect the four spermathecaes. The dissected organs, blotted with filter paper to remove excess fluids, were then placed into 20 mL glass vials containing 2 mL of earthworm physiological saline (Lore *et al.*, 1999). After sperm had been released into the fluid by teasing apart the spermathecaes, 8 mL fluid were added to the vials, and the entire volume was filtered through a funnel lined with fine double gauze (375 μm) into fresh 20 mL vials. The sample was loaded into a haemocytometer, and sperm in a 0.1-mL volume were observed under an optical microscope (×400) to detect abnormalities in sperm morphology. The volume of the spermathecaes was a negligible addition to the 10 mL of saline, so sperm numbers were calculated by multiplying sperm counts by a dilution factor of 100. One thousand sperm were counted for every slide and percentage (%) of sperm deformity was calculated.

1.5 Statistical analysis

The data in this study were analysed using SPSS 13.0, and all the values are presented as mean ± SD. The LC_{50} (lethal concentration when 50% of the population were killed) was calculated by probit analysis. Statistical differences between controls and treated groups for locomotion and sperm deformity test were determined by ANOVA followed by Dunncan's multiple comparison tests on a 5% significant level.

2 Results and discussion

2.1 Assessment of Pb on acute toxicity for *P. guillelmi*

With Pb concentration increasing, the pH of soil decreased. The lowest pH recorded at the beginning of toxicity experiments was 5.5 ± 0.2 in the 7500 mg/kg Pb. The pH range across the range of concentration over 7 d was 5.7–7.1. Although the pH of Pb treated soil showed a significant decrease with increasing Pb concentration, previous work (Spurgeon and Hopkin, 1996) has shown that pH alone has little effect on worm mortality. Therefore, it is thought that the earthworm mortality could not be attributed to a change in pH.

The mortality of the earthworms increased with increasing soil Pb concentration. All worms exposed to 7500 mg/kg died. The LC_{50} of *P. guillelmi* for 7 and 14 d exposure were 4285 ± 339 mg/kg and 3207 ± 248 mg/kg, respectively. Compared to the LC_{50} of *E. fetida* for 7 d (2662 ± 193 mg/kg) and 14 d (2589 ± 381 mg/kg) (Currie *et al.*, 2005), *P. guillelmi* can tolerate a higher Pb concentration.

The weight of the earthworms after 7 and 14 d exposure is given in Table 1. The difference of mean weight between control group and the exposure groups at the start of the experiments was not significant ($P > 0.05$). The weight of the earthworms between control group and exposure groups of 1000, 1400, 2000, 2800 mg/kg Pb was not significantly different following 7 d exposure to Pb, while significant difference ($P < 0.05$) existed between control group (3800 mg/kg) and exposure groups (5400 mg/kg). The average weight of the surviving worms decreased at concentration of 2800 mg/kg. On day 14, the mean weights of the earthworms among concentrations were more variable. After 14 d, the reduction in weight of the

Table 1 Mortality and body weight of earthworms after treatment by Pb

Lead concentration (mg Pb/kg soil)	Day 0		Day 7		Day 14	
	Mortality (%)	Body weight (g)	Mortality (%)	Body weight (g)	Mortality (%)	Body weight (g)
0	0	2.51 ± 0.14	0	2.50 ± 0.15 a	0	2.49 ± 0.14 a
1000	0	2.45 ± 0.14	0	2.44 ± 0.16 a	0	2.40 ± 0.15 a
1400	0	2.56 ± 0.19	0	2.52 ± 0.20 a	0	2.47 ± 0.24 a
2000	0	2.60 ± 0.18	3 ± 5	2.51 ± 0.16 a	10 ± 8	2.45 ± 0.22 a
2800	0	2.41 ± 0.10	13 ± 5	2.11 ± 0.17 a	33 ± 10	1.99 ± 0.13 b
3800	0	2.53 ± 0.17	28 ± 5	2.07 ± 0.21 b	68 ± 15	1.77 ± 0.09 c
5400	0	2.40 ± 0.15	73 ± 10	1.88 ± 0.19 b	95 ± 6	1.35 ± 0.29 c
7500	0	2.49 ± 0.13	100 ± 0	–	100 ± 0	–

Values are mean ± SD of average earthworm in each replicate (n = 4). Where no value is given mortality was 100%. Identical letters indicate statistically identical values. Statistical differences between controls and treated groups for body weight of earthworms were determined on a 5% significant level. Identical letters and no letter in each column indicate statistically identical values.

earthworm was significantly greater in the 3800, 5400 mg/kg. Because of mortality at the higher Pb concentration and therefore insufficient data, an EC_{50} for weight change could not be determined.

2.2 Toxicity of Pb on locomotor ability of *P. guillelmi*

Locomotor ability was measured on day 7. Crawled distance in one minute is shown in Fig. 1. The locomotor ability was affected by Pb at the concentration of 1000 mg/kg. Although the locomotor ability of earthworms exposed to a range of soil Pb concentrations showed a general decrease with increasing Pb concentrations, there is no difference between the three higher nominal-exposure concentrations of 1400, 1800, 2500 mg/kg.

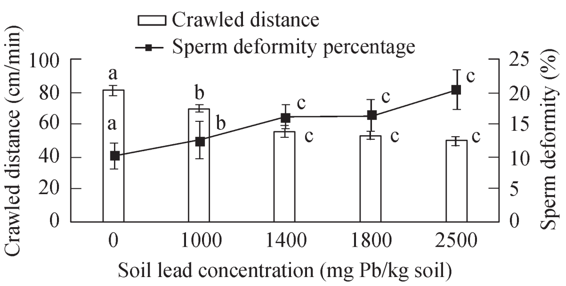

Fig. 1 Mean (±SD) crawled distance in one minute on day 7 and mean (±SD) of sperm deformity percentage (%) on day 14. Identical letters indicate statistically identical values.

Earthworms are capable of detecting and avoiding metal contaminated soils. They clearly avoided contaminated soil at lower metal concentrations than those inducing negative responses in standardized acute and reproduction tests. Their locomotor ability, couples with the sensitivity to chemicals, contributed much to their avoidance response. As the lead concentration increasing, the locomotor ability of earthworms decreased. It may be speculated that the locomotor ability could be influenced by lead.

In our study, the variation between individuals from the same exposure concentration was very small. Given this reason, it is thought that the decrease of locomotor ability at high Pb concentration was due to high concentration of Pb rather than individual variation. However, there may be several reasons for the observed variation in the decrease of locomotor ability between individuals. For example, this parameter could be affected by conditions other than the presence of toxic metals. We used print paper as a substrate to conduct the experiment, which may not be optimal for earthworms. In addition, as earthworms use scarfskin to breathe, when exposed to air, their locomotor ability decreases may result from breathing difficulty. What is more, the light and temperatures may also affect this ability. Therefore, in order to use it as a parameter in the field, factors other than the toxicological ones which may affect this parameter need to be identified.

2.3 Impacts of Pb on sperm morphology of *P. guillelmi*

The morphology of *P. guillelmi* sperm is shown in Fig. 2. The normal sperm of *P. guillelmi* is composed of a bacilliary head and a flagellum. The most usually abnormalities are swelling head (Fig. 2b) and head helix (Fig. 2c), while some of the sperms showed the morphology abnormalities of head bending (Fig. 2d). Mean ± SD percentage deformity is shown in Fig. 1.

Earthworms are ecologically important soil organisms, and toxic effects on their gametes should correspond directly to their populations and, indirectly, to other wildlife. Here we present data depicting the sperm morphology of earthworms, *P. guillelmi*, which shows potential as a sensitive biomarker for measuring effects of heavy metal on reproduction.

Long-term exposure to metals and organochlorine pesticides has been reported to affect cocoon production and hatching success (Spurgeon *et al*., 1994; Maboeta *et al*., 1999; Inouye *et al*., 2006), and Pb was found to influence on the spermatozoa of *E. fetida* (Reinecke and Reinecke, 1997) and cocoon viability (Reinecke *et al*., 2001; Savard *et al*., 2007). Although these reproductive parameters show promise for assessing the effects of chronic exposure to environmental pollutants, sperm morphology appears to offer a more rapid measurement endpoint biomarker for laboratory and *in situ* field studies. There is an increasing interest in the use of sperm deformity test to estimate the reproductive toxicity of different compounds in the environment (Zang *et al*., 2000; Navarro and Obregón, 2005). In agreement with these previous studies, the percentage sperm deformity augmented with the increasing of Pb concentrations in soil. The abnormal phenotype mainly occurred on the head of sperm. As the head contains most of the genetic materials, it is speculated that lead may act

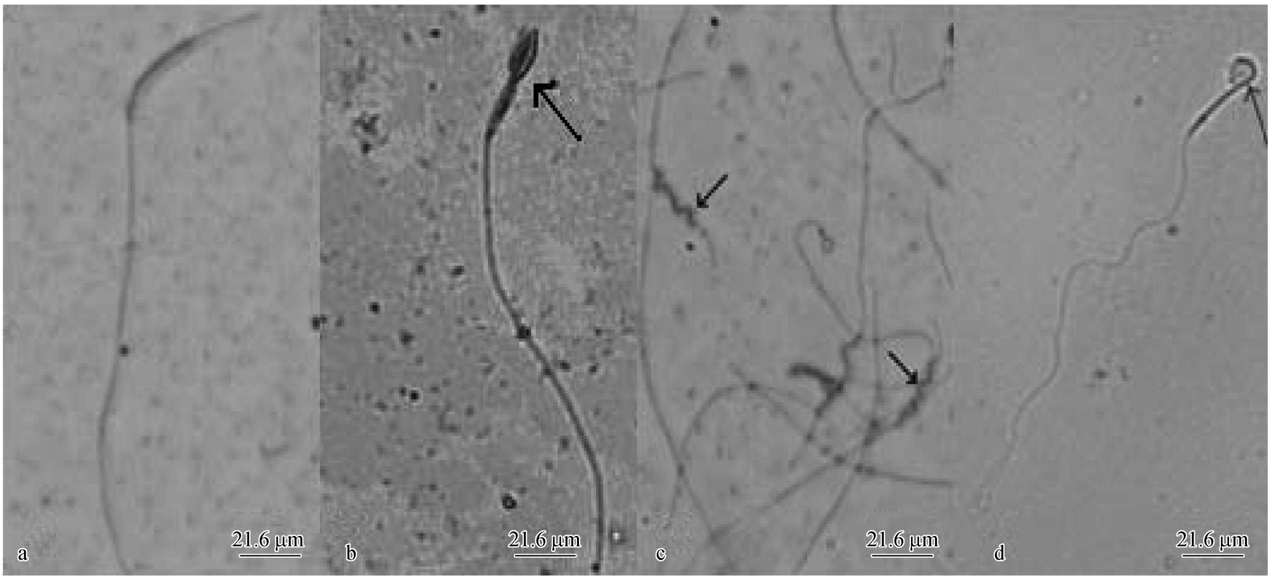

Fig. 2 Micrograph (×400) of the sperm morphology of earthworm, *P. guillelmi*. (a) normal; (b) swollen head; (c) head helix; (d) head bending.

on DNA and result in abnormal morphology.

3 Conclusions

Earthworms are to be exploited as test organisms in ecotoxicological studies, more should be known about their interspecific differences. This study shows *P. guillelmi* can tolerate a higher Pb concentration as compared with other earthworms. However, most ecotoxicologically based risk assessment of Pb can operate at different ranges of sensitivity (lethal, sublethal) using endpoints such as mortality, change of weight, locomotor ability and percentage sperm deformity of *P. guillelmi*.

Acknowledgments

This work was supported by the Natural Science Fund of Zhejiang Province (No. Y506255). Grateful thanks were given to Dr. DU Weiguo for his suggestions on the manuscript.

References

Currie M, Hodson M E, Arnold R E, Langdon C J, 2005. Single versus multiple occupancy – effects on toxicity parameters measured on *Eisenia fetida* in lead nitrate-treated soil. *Environmental Toxicology and Chemistry*, 24(1): 110–116.

Davies N A, Hodson M E, Black S, 2003. The influence of time on lead toxicity and bioaccumulation determined by the OECD earthworm toxicity test. *Environmental Pollution*, 121(1): 55–61.

Efroymson R A, Will M E, Suter II G W, 1997. Toxicological benchmarks for contaminants of potential concern for effects on soil and litter invertebrates and heterotrophic process: 1997 revision. United States Department of Energy, Washington DC.

Guo Y C, Wang Z Z, Zhang Y M, Lai Q, Yan H M, Xia W S, Deng J F, 1996. Study on toxicity and toxicology of heavy metals to earthworms in polluted soil. *Chinese Journal of Applied and Environmental Biology*, 2(1): 132–140.

Heikens A, Peijnenburg W, Hendriks A J, 2001. Bioaccumulation of heavy metal in terrestrial invertebrates. *Environmental Pollution*, 113(3): 385–393.

Inouye L S, Jones R P, Bednar A J, 2006. Tungsten effects on survival, growth, and reproduction in the earthworm, *Eisenia fetida*. *Environmental Toxicology and Chemistry*, 25(3): 763–768.

Jia X Y, Li X M, Yang Y Q, Hu L F, Zhu Y F, 2005. Acute toxicological effects of Cr(VI) and Cu single and combined pollution on earthworm *Eisenia foetida*. *Journal of Agro-Environment Science*, 24(1): 31–34.

Langdon C J, Hodson M E, Arnold R E, Stuart B, 2005. Survival, Pb-uptake and behaviour of three species of earthworm in Pb treated soils determined using an OECD-style toxicity test and a soil avoidance test. *Environmental Pollution*, 132: 368–375.

Lanno R P, Wells J, Conder J M, Bradham K, Basta N, 2004. The bioavailability of chemicals in soil for earthworms. *Ecotoxicology and Environmental Safety*, 57(1): 39–47.

Lore A B, Hubbell J A, Bob D S, Ballinger M L, Loftin K L, Smith J W *et al*., 1999. Rapid induction of functional and morphological continuity between severed ends of mammalian or earthworm myelinated axons. *Journal of Neuroscience*, 19(7): 2442–2454.

Maboeta M S, Reinecke A J, Reinecke S A, 1999. Effects of low levels of lead on growth and reproduction of the Asian earthworm *Perionyx excavatus* (Oligochaeta). *Ecotoxicology and Environmental Safety*, 44(3): 236–240.

Navarro O E, Obregón E B, 2005. Effect of malathion on the male reproductive organs of earthworms, *Eisenia foetida*. *Asian Journal of Andrology*, 7(1): 97–101.

OECD (Organization for Economic Co-operation and Development), 1984. Test 207: Earthworm, acute toxicity tests. Organization for Economic Co-operation and Development ed. OECD Guidelines for Testing of Chemicals.

Reinecke A, Reinecke S, 1996. The influence of heavy metals on the growth and reproduction of the compost worm *Eisenia fetida* (Oligochaeta). *Pedobiologia*, 40: 439–448.

Reinecke A, Reinecke S, Maboeta A, 2001. Cocoon production and viability as endpoints in toxicity testing of heavy metals with three earthworm species. *Pedobiologia*, 45(1): 61–68.

Reinecke S, Reinecke A, 1997. The influence of lead and manganese on the Spermatozoa of *Eisenia fetida* (Oligochaeta).

Soil Biology and Soil Biochemistry, 29: 737–742.

Savard K, Berthelot Y, Auroy A, Spear P A, Trottier B, Robidoux P Y, 2007. Effects of HMX-lead mixtures on reproduction of the earthworm *Eisenia Andrei*. *Archives of Environmental Contamination and Toxicology*, 53(3): 351–358.

Spurgeon D J, Hopkin S, 1996. Effects of variations of the organic matter content and pH of soils on the availability and toxicity of zinc to the earthworm *Eisenia fetida*. *Pedobiologia*, 40: 80–96.

Spurgeon D J, Hopkin S P, Jones S A, 1994. Effects of cadmium, copper, lead and zinc on growth, reproduction and survival of the earthworm *Eisenia fetida* contamination in terrestrial ecosystems. *Environmental Pollution*, 84(1): 123–130.

Stenersen J, Brekke E, Egelstad F, 1992. Earthworms for toxicity testing: Species differences in response towards cholinesterase inhabiting insecticides. *Soil Biology and Soil Biochemistry*, 24(12): 1761–1764.

USEPA (United States Environmental Protection Agency), 2003. Ecological soil screening levels for lead: Interim Final OSWER Directive 9285.7-70. Washington DC: Office of Solid Waste and Emergency Response. 244.

Zang Y, Zhong Y, Luo Y, Kong Z M, 2000, Genotoxicity of two novel pesticides for the earthworm, *Eisenia fetida*. *Environmental Pollution*, 108(2): 271–278.

Zhou S P, Duan C Q, Fu H, Chen Y H, Wang X H, Yu Z F, 2007. Toxicity assessment for chlorpyrifos-contaminated soil with three different earthworm test methods. *Journal of Environmental Sciences*, 19(7): 854–858.