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Assessing cypermethrin-contaminated soil with three different earthworm test methods

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Abstract

A series of tests (lethal, sublethal, and behavioral) on earthworms were conducted as an eco-assessment of pesticides. In this study, the toxicity of cypermethrin-contaminating soil on adult and juvenile earthworms was assessed. Beside the acute and chronic tests, an avoidance response test was carried out. It was shown that the all-round toxicity from cypermethrin was weak on adult earthworms. Compared with adult earthworms, the toxicity of juvenile earthworms from cypermethrin especially chronic toxicity increased significantly. Growth and reproduction of earthworms appeared to be more severely affected by cypermethrin at juvenile stage than at adult stage. Applied at 10 mg/kg, cypermethrin had obvious adverse impact on the growth of juvenile earthworms, while 20 mg/kg, cypermethrin caused significant toxic effects in reproduction. The results also indicated that ecotoxicological risk assessment using only adult specimens may underestimate the effects of cypermethrin on soil invertebrate populations.

Key words: earthworms; pesticide; cypermethrin; survival; fecundity; avoidance response; environment disrupting chemicals

Introduction

Cypermethrin, a broad-spectrum pyrethroid insecticide, was considered an ideal substitute for organochlorine insecticides. Since some studies had indicated that cypermethrin could lead to adverse impacts on the endocrine, immune, and nervous systems of animals, it has been listed as an environment disrupting chemicals (EDCs) (Wen and Liu, 2005; Li and Li, 2007). Despite that, cypermethrin is still commonly used in agriculture in China, and a large portion of application ends up as residuals in the soil (Jin and Webster, 1998), posing great threats to soil-dwelling animals and microorganisms.

Earthworms are common in a wide range of soils and may represent up to 80% of the total soil biomass. The species adapts well to laboratory conditions and its high sensitivity to its surroundings makes them one of the most suitable bioindicator for soil chemicals (Spurgeon *et al*., 2003). Most toxicity tests for cypermethrin on earthworms had been conducted using adult earthworms (Alshawish, 2004). However, some studies indicated that juveniles are often more sensitive to EDCs than adults (Booth and O'Halloran, 2001). There are evidence that soil contaminants can affect growth and sexual development of juveniles at low contaminants' concentrations than that of adults. Therefore, using toxicity data obtained solely from adult specimens in evaluating eco-toxicological risk may underestimate the effects of cypermethrin on soil invertebrate populations.

A series of tests on different animal responses (mortality, changes of growth and reproduction rates, behavioral responses) were designed and carried out using adult and juvenile earthworms in an eco-assessment of cypermethrin. The major objectives of this study were: (1) to test the integrative toxic effects of cypermethrin on earthworms using ecotoxicity experiments with acute, chronic, and behavioral endpoints, (2) to compare the sensitivity of using adult and juvenile earthworms in assessing cypermethrin toxicity.

1 Materials and methods

1.1 Pesticides

The pesticide being tested in these experiments was pyrethrum insecticide cypermethrin. Cypermethrin was of analytical reagent grade purity. The other chemicals involved were obtained from Sigma-Aldrich Chimie S.A.R.L, France. Tap water was used as a control.

1.2 Earthworms

Earthworms of species *Eisenia fetida andrei* (*E. fetida andrei*) were purchased from Jialiming Earthworm Farm Co., Ltd., (China) and were cultured in the laboratory in

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artificial soil according to ISO 11268-1 (1993) (Annex A: example of breeding technique for *E. fetida*). The soils were mixed with decayed leaves and decomposed cow manure, and were kept at room temperature (20°C). Soil water content was measured and distilled water was added every week to reach 60% of maximum water-holding capacity. The soils were changed every four weeks. The earthworms used in these experiments were juveniles of fresh weights between 100 and 200 mg and adults with well-developed clitella that weighed between 350 and 400 mg.

1.3 Earthworm acute test

The soil used in this test was the OECD artificial soil (OECD, 1984), consisting of 70% quartz sand, 20% Kaolin clay and 10% sphagnum peat, and a small amount of calcium carbonate was added to adjust the pH to 6.0 ± 0.5. In the experiment, different concentrations of cypermethrin were added to the soil, which were 62.5, 65, 72, 85, 105, and 125 mg/kg dry weight (dw).

For each cypermethrin concentration, desired amount of pesticide was thoroughly mixed into the soil as an aqueous solution, and was put into four 1-L glass jars. For the control treatment, tap water was used instead of cypermethrin and the substrate was placed into another four jars. Ten worms were then placed into each container and were covered with a polythene sheet with integrated gauze (+1 mm) to ensure sufficient ventilation. By the end of the 14-d test period, the worms were sorted by hand and the test endpoint was mortality rates.

1.4 Earthworm growth test

This test was modified from the method which introduced by Khalil *et al.* (1996). The soil was artificially contaminated by different concentrations of cypermethrin, which were 5, 10, 20, 40, and 60 mg/kg dw based on the results of pilot experiments (data not shown). Each concentration was represented by four replicates, and an additional four replicates were set up for the control treatment. Ten worms were added to each container, additional food (5 g ground cattle dung) was given to the worms in each jar at the beginning of the experiment.

During the test period of 8 weeks, the worms were weighed weekly to evaluate their growth response to cypermethrin. Before weighing, the worms were sorted, washed with tap water, and blotted with filter paper. Then worms were weighed using an electro-balance before they were returned to the soil. During the experiment, moisture content was checked weekly and maintained at 50% by adjusting the weight of the container against the weight known from the previous week prior to sampling. The substrate was replaced after 4 weeks with fresh contaminated soil.

1.5 Reproductive response test

This test was similar to the growth test described above, but the test endpoints were cocoon production after 4 weeks, the number of hatchlings, and their development after 8 weeks. The worms were hand-sorted after 4 weeks, the numbers of cocoons produced were counted and returned for further incubation. The juveniles were weighed after 8 weeks and their maturity were determined from the presence of a fully developed clitellum.

1.6 Avoidance response test

This test was based on the work by Schaefer (2004) and Zhou *et al.* (2007). The experiment was carried out in round opaque plastic containers (28 cm in diameter, 20 cm in height) with six chambers forming a circle that connected to a central chamber (Fig.1). Each chamber was separated from one another with plastic slides (with or without perforations), and was covered with plastic lids to prevent worms from escaping. Twenty earthworms were placed into the empty central chamber of each container at the start of the experiment. After they had migrated into the neighboring soil-filled chambers, the central chamber was closed, and free migration was allowed between the chambers. After an incubation of 48 h, slides (without perforations) were placed between the chambers to prevent further migrations and the number of worms from each chamber was counted.

The concentrations of cypermethrin in the exterior chambers were arranged in an ascending gradient as presented in Table 1. Four replicates and four controls were used in the experiment.

1.7 Statistical analysis

The results were tested for their normal distribution (Kolmogorov-Smirnov test) and homogeneity of variances (Levene test). Probability analysis was used for assessing the acute toxicity of contaminants to the earthworms. One-way ANOVA ($P < 0.05$) and correlation analysis were used for assessing the effects of contaminants on growth and reproduction. Avoidance response was analyzed using General Linear Models (Univariate) with treatment

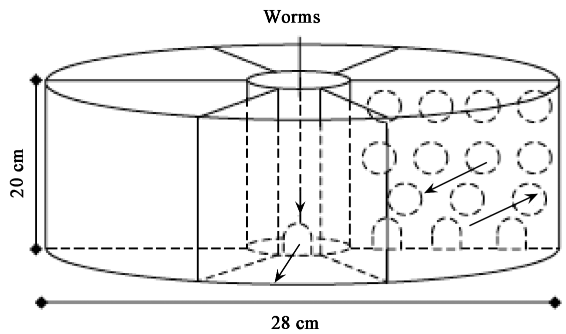

Fig. 1 Test container of the avoidance response test (Schaefer, 2004).

Table 1 Concentrations of cypermethrin applied in the avoidance response test

Substrate	CH0	CH1	CH2	CH3	CH4	CH5
Control (mg/kg)	0	0	0	0	0	0
Treatment (mg/kg)	0	5	10	20	40	60

CH0–CH5: serial number of chamber in the test container of the avoidance response test.

pesticides and earthworms as factors. For *post hoc*, in comparison of means (growth, reproduction), Scheffe test was applied. The results (mean value) of the earthworm avoidance response test were compared by the Mann-Whitney U-test, as data were not normally distributed.

All these statistical procedures were performed using software SPSS 12.0 for Windows (SPSS Inc., USA).

2 Results and discussion

2.1 Assessment of cypermethrin for acute toxicity on *E. fetida andrei*

The results of the acute test are summarized in Table 2. LC_{50} was estimated from the data using probability analysis in the SPSS software package. Only in the control treatment did all the worms survive at the end of the experement. In all other treatments with cypermethrin, a clear positive dose-response relationship was observed between earthworm mortality and pesticide concentration.

The most common variable for evaluating acute toxicity of chemicals to animals is LC_{50} (lethal concentration to 50% of individuals). LC_{50} is used to indicate the average lethal dose of such chemicals to the species. According to the regulation of environmental risk assessment for agricultural pesticides, the suggested standard of toxicity are LC_{50} < 1 mg/kg for highly-toxic pesticides, 1–10 mg/kg for medium-toxic pesticides, and > 10 mg/kg for low-toxic pesticides (MEPPRC, 1990). With this standard, the acute toxicity of cypermethrin on adult and juvenile earthworms was found to be low.

Table 2 Summary of results for the acute response test

Earthworm	LC_{50} (mg/kg)	Confection limits 95%
Adults	84.14	61.18–93.81
Juveniles	69.78	51.49–98.66

LC_{50} (lethal concentration to 50% of individuals) was calculated from regression model.

2.2 Impacts of cypermethrin on growth and reproduction of *E. fetida andrei*

The results of the growth and reproductive response tests are summarized in Tables 3 and 4. The results of these chronic toxicity tests demonstrated that cypermethrin could lead adverse impacts on both the growth and reproduction of adult and juvenile earthworms, while juveniles are more sensitive during the development stage. After 8 weeks of exposure, the cocoon production rates decreased significantly for the treatment group than the control group except at concentrations 5 and 10 mg/kg. On the other hand, even at low concentrations, toxic effects could be observed in the growth and cocoon production of juveniles, meaning that they are more susceptible to cypermethrin contamination.

2.3 Avoidance response of *E. fetida andrei* to cypermethrin

The avoidance response tests were conducted by using a six-chamber test system. The results are presented in Fig.2. Fig.2a shows that distribution of earthworms in each chamber of test system was similar to control treatment. However, as Fig.2b illustrates, cypermethrin exposure had a significant effect on the distribution of adult and juvenile earthworms in the six-chamber test system.

Earthworms can sense chemicals via a large number of chemoreceptors, and avoid the contaminations (Slimak, 1997; Capowiez *et al*., 2004). In the present study, we also observed earthworms that were introduced into glass containers staying on the top layer of the contaminated soil instead of moving into it, followed by worms dying

Table 3 Summary of results from the growth response test

Cypermethrin treatment (mg/kg)	Mean weight per earthworm (mg)			
	Adult		Juvenile	
	Week 0	Week 8	Week 0	Week 8
0 (control)	364.0±8.5	370.2±78.5	175.3±12.5	270.2±28.5
5	399.2±14.1	405.0±120.2	168.5±13.0	264.8±12.2
10	373.5±12.5	389.5±68.2	188.6±12.6	209.5±28.2*
20	354.6±6.5	361.5±58.5	178.5±13.2	214.0±37.0*
40	375.0±9.2	351.5±73.3*	160.3±12.8	181.5±28.5*
60	386.0±8.2	330.0±87.0*	182.5±12.2	141.5±13.3*

* Significant differences ($P < 0.05$) were found between treatment and control group.

Table 4 Summary of results from the reproductive response test on adult and juvenile earthworm

Cypermethrin treatment (mg/kg)	Adult earthworm		Juvenile earthworm	
	Cocoon number	Hatchling number	Cocoon number	Maturity percentage (%)
0 (control)	22.5±4.55	32.38±4.25	4.5±0.95	100±0
5	20.62±5.90	26.25±9.25	3.62±0.90	90±4
10	20.00±6.12	23.12±8.25	3.10±0.72	86±5
20	17.5±2.88	19.50±2.88	1.27±0.88*	55±8*
40	19.62±2.40	20.00±6.45*	0.62±0.40*	56±6*
60	14.38±2.38*	13.75±3.22*	0.38±0.08*	37±7*

* Significant differences ($P < 0.05$) were found between treatment and control group.

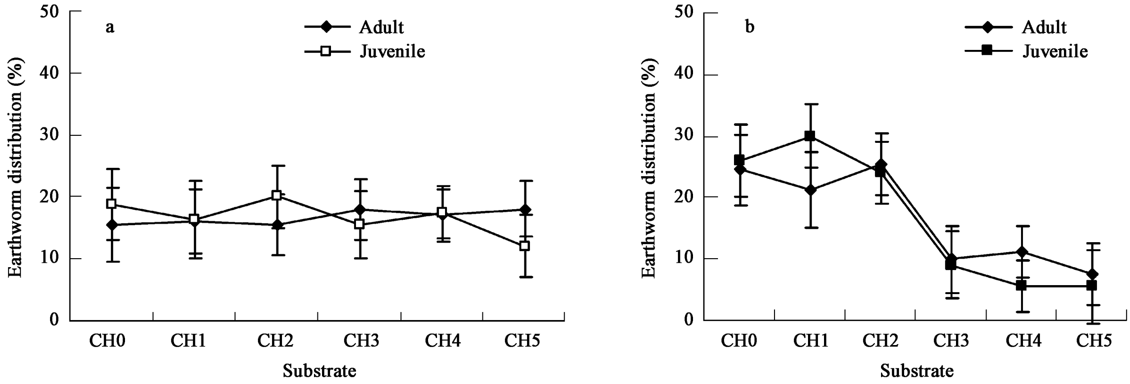

Fig. 2 Distribution of earthworms in control (a) and exposed to cypermethrin (b) after 48 h incubation in test system. Arithmetic mean of total distribution (n = 4). Each replicate contained 20 earthworms.

a few days later. The responses observed were similar between adults and juveniles. This can be interpreted as avoidance behavior, which supported the justification of behavioral tests in addition to acute and reproduction testing in earthworms. For cypermethrin, concentration affecting avoidance response in juveniles and adults were similar.

2.4 Comparison of different test methods using adult and juvenile earthworms

The sensitivity of different tests on cypermethrin toxicity on earthworms is summarized in Table 5. The results showed that cypermethrin has a low toxicity to adult earthworms, it is in agreement with the results from Alshawish *et al*. (2004). Meanwhile, the results from different toxicity tests showed that cypermethrin posed greater impacts on juveniles than on adults, especially of chronic responses. The results from this study demonstrated that cypermethrin can hamper the growth of juvenile earthworms at a low concentration than that of adults, and can delay their development and reproduction. In addition, the concentration of cypermethrin could cause more significant avoidance response of juveniles than that of growth response, despite that *E. fetida*, an epigenic earthworm living and feeding in the litter and topsoil layer. *E. fetida* were not able to live in the deep subsoil layer to avoid pollutant, but they could move away in the same level to avoid pollutant. According to the test, it seemed likely that juvenile earthworms were not able to escape from pesticide-contaminated soil into the underlying clean soil in field and hence were exposed continuously to pesticides.

Table 5 Lowest response concentrations of cypermethrin leading to test endpoints in different experiments

Lowest response concentration	Juvenile	Adult
Acute response		
LC_{10}(14 d) (mg/kg)	56.5	72.0
Chronic response		
Growth (mg/kg)	10	40
Cocoon production (mg/kg)	20	60
Maturity percentage (%)	20	
Behavioral response		
Avoid response (mg/kg)	20	20

3 Conclusions

It can be concluded that earthworms have different sensitivity to cypermethrin at different aspects and stages of life. Thus, eco-toxicological risk assessments of cypermethrin and maybe of other contaminants should involve multiple test methods with both juvenile and adult test animals. However, most eco-toxicologically risk assessments of cypermethrin were based on adult earthworms, neglecting the effects of cypermethrin to juvenile earthworms. Consequently, the use of toxicity data, obtained exclusively from adult earthworm evaluation of eco-toxicological risk may underestimate the effects of cypermethrin on soil invertebrate populations.

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