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Toxicity assessment for chlorpyrifos-contaminated soil with three different earthworm test methods

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Abstract

Earthworm toxicity tests are useful tools for terrestrial risk assessment but require a hierarchy of test designs that differ in effect levels (behavior, sublethal, lethal). In this study, the toxicity of chlorpyrifos contaminated soil on earthworms was assessed. In addition to the acute and chronic tests, an avoidance response test was applied. Earthworms were exposed to sublethal and lethal concentration of chlorpyrifos, and evaluated for acute toxicity, growth, fecundity and avoidance response after a certain exposure period. The test methods covered all important ecological relevant endpoints (acute, chronic, behavioral). Concentration of 78.91 mg/kg, chlorpyrifos caused significant toxic effects in all test methods, but at lower test concentrations, only significant chronic toxic effects could be observed. In present study, chlorpyrifos had adverse effect on growth and fecundity in earthworm exposed to 5 mg/kg chlorpyrifos after eight weeks. The avoidance response test, however, showed significant repellent effects at concentration of 40 mg/kg chlorpyrifos. For chlorpyrifos, concentration affecting avoidance response was far greater than growth and fecundity, it seemed likely that earthworms were not able to escape from pesticide-contaminated soil into the clean soil in field and hence were exposed continuously to elevated concentrations of pesticides.

Key words: chlorpyrifos; earthworms; avoidance response; survival; reproduction test

Introduction

Chlorpyrifos, a broad-spectrum organophosphorus insecticide, is considered an ideal substitute for virulent organophosphorus insecticides such as methamidophos. Recently, with the prohibition of virulent organophosphorus insecticides in the production of vegetables, the usage of chlorpyrifos is rapidly increasing. However, some studies indicated that residues and metabolites of chlorpyrifos caused soil, groundwater, and surface water contamination at many sites because of long-persist of chlorpyrifos in the soil. In the field situation, chlorpyrifos could persist in the soil for longer time periods (Kuhr and Tashiro, 1978), and chlorpyrifos had been found to persist for up to two years in soil (Cox, 1995). Newer products such as sustained-release pellets, which release low levels of pesticides into the soil over a much longer period, can persist for up to 18 months (Racke, 1993). The wide-use of chlorpyrifos may result in a hazard to the environment.

Earthworm, as an important organism in the soil ecosystem, is the important indicator for pesticide pollution. Acute and chronic toxicity tests using earthworm have been traditionally used to assess the toxicity of contaminants in soils to terrestrial invertebrates (ISO 11268-1, 1993; ISO 11268-2, 1998). Until recently, the measured common end point when evaluating toxicity of chlorpyrifos to earthworm were ecologically relevant toxicity criteria, such as mortality, body growth and cocoon production. Alshawish *et al*. (2004) had studied the chronic toxicity of chlorpyrifos on *Aporrectodea caliginosa*, and found that chlorpyrifos could greatly affect the fecundity of *A. caliginosa*. The research by Booth and O'Halloran (2001) has shown that not only the chlorpyrifos could greatly impact the growth and fecundity of *A. caliginosa*, but also the impact on the juvenile *A. caliginosa* is greater than the adult.

Although the test endpoints such as reproduction rates could reflect the toxicity of chlorpyrifos to some extent, but they could provide only a measure of toxicity in signal level such as fecundity and they could not reflect the all-round toxicity of chlorpyrifos on earthworm, therefore, the predicting of the ecological consequences of exposure to chlorpyrifos resulted from such end points may differ in the real situation. For example, the behavioural impact

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of pollution on earthworms may result in the avoidance response of earthworms on the pollution, which leads to the decrease of bioavailability of pollutants, and evaluate ecotoxicological risk may overestimate the real effects of pollutants on soil invertebrate populations.

The hierarchy of test designs differing in their levels of effect (lethal, sublethal, behavioral) should be applied to the eco-assessment of chlorpyrifos. The main aims of the present study were: (1) to test the integrative toxic effects of chlorpyrifos, using earthworm ecotoxicity experiments with acute, chronic and behavioral endpoints; (2) to compare the sensitivity as well as the advantages and disadvantages of different earthworm test methods in assessing chlorpyrifos contaminated soil.

1 Materials and methods

1.1 Pesticides

The pesticide tested in these experiments was organophosphorus insecticides chlorpyrifos. Chlorpyrifos was ordered from Sigma-Aldrich Chimie S.A.R.L. (Saint Quentin-Fallavier, France). Other chemicals of rigorously pure grade were also obtained from Sigma-Aldrich Chimie S.A.R.L. Tap water was used as a control.

1.2 Earthworm

Earthworms of the species *Eisenia fetida* andrei were purchased from Jia Liming Earthworm Farm Co., Ltd. (Tianjin, China) and maintained in the laboratory in a culture medium according to ISO 11268-1 (1993) (Annex A: Example of breeding technique for *E. fetida*). Earthworms were cultured in the laboratory at room temperature (20°C) on moist soil mixed with decayed leaves and well decomposed cow manure. Distilled water was given to reach 60% of maximum water-holding capacity. Water content was readjusted weekly. Soil was changed every four weeks and earthworms were maintained until required for experimentation. The earthworms used in this experiment were adults with well-developed clitella. The individual fresh weights of the adults earthworms used in the experiment varied between 350 and 400 mg.

1.3 Earthworm acute test

The test soil was the OECD artificial soil (OECD, 1984). The soil consisted of 70% quartz sand, 20% Kaolin clay, 10% sphagnum peat and calcium carbonate to adjust the pH to 6.0±0.5. The soil was prepared by adding different concentrations of pesticides (dry weight basis). Chlorpyrifos was added at 75, 90, 100, 125, 150 and 200 mg/kg soil.

The desired amount of pesticide was thoroughly mixed into soil as an aqueous solution to give the working concentration of pesticide, and soil was placed into glass jars. Ten adult worms were placed in 1-L glass containers filled with test substrate. To prevent the worms from escaping, test containers were covered with a polythene sheet with integrated gauze (+1 mm) to ensure optimal ventilation. After 14 d, 21 d of incubation, surviving worms were sorted by hand. The test endpoint was mortality.

Four replicates were applied for the acute tests. A control treatment was in parallel, also represented by four replicates.

1.4 Earthworm growth test

This test conformed to the acute test described above, but as the working concentration of pesticide was changed, and the incubation duration was also longer, additional food (5 g ground cattle dung/test container) was given to the earthworms at test start. A series of experiments were conducted to evaluate the responses of juveniles and adult earthworms to the test pesticides.

This test was based on work proposed by Khalil *et al.* (1996) but had been slightly modified. The soil was artificially contaminated by adding different concentrations of pesticides (dry weight basis). Chlorpyrifos was added at 5, 20, 40, 60, 80 mg/kg soil. The concentrations used were based on the results of pilot experiments (data not shown). Each pesticide concentration was represented by four replicates, and an additional four replicates for the control treatment.

Ten worms were added at each container. Soil was changed every four weeks and earthworms were placed into fresh contaminated soil. Earthworms were weighed weekly during experiment to determine the effect of each pesticide on growth. Before weighing, all worms were sorted out of the soil, washed with tap water and left on wet filter paper, then blotted off and weighed using an electrobalance. Worms were then returned to the soil. During all experiments, 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 experiment lasted for 4 weeks.

1.5 Earthworm reproduction test

This test conformed to the growth test described above, but the test endpoints were cocoon production after 28 d and the numbers of hatched juveniles after 56 d. Surviving worms were hand-sorted after 28 d, and the cocoons produced were returned and further incubated for another 28 d (total incubation time 56 d).

1.6 Earthworm avoidance response test

This test was based on work proposed by Stephenson *et al.* (1998) and Schaefer (2004) but had been slightly modified here. This test method consisted of round opaque plastic containers (28 cm in diameter, 20 cm in height) with six different chambers connected to a central chamber serving as the test arena (Fig.1). Plastic slides with holes (5 mm in diameter) separated each chamber from the neighboring unit. Plastic lids covered the test containers to prevent worms from escaping during each exposure period. Twenty earthworms were placed into the soil-free central chamber of each container. After they had migrated into the neighboring soil-filled chambers, the central chamber was closed with a plug. Free migration was now only possible between the chambers. After an incubation of 48 h, slides (without holes) were placed

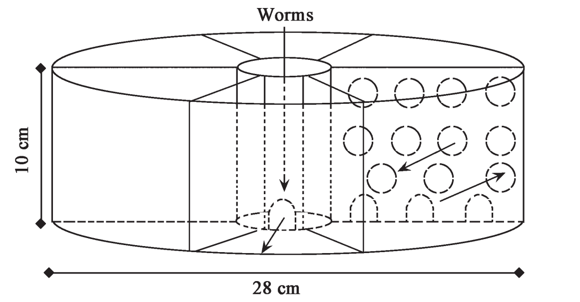

Fig. 1 Test container of the avoidance response test (Schaefer, 2004). Arrow presents free migration possibility.

between the different chambers to prevent further worm movement between the compartments and the worms were extracted by hand-sorting from each chamber. The number of worms in soil of each chamber was the measured test endpoint.

The grouping of test substrates was arranged in each replicate according sequence to form concentration grads. The concentration grads of test pesticides are presented in Table 1. Four replicates were applied for avoidance response test. A control treatment was in parallel, also represented by four replicates.

Table 1 Summary of parameter for the avoidance response of earthworm

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

1.7 Statistical analysis

Results were tested for their normal distribution (Kolmogorov-Smirnov test) and homogeneity of variances (Levene test). Probit analysis was used for assessing the acute toxicity of contaminants in soil to earthworm. One way ANOVA ($P<0.05$) and correlation analysis was used for assessing the effects of contaminants on growth and reproduction. Substrate avoidance was analyzed using General Linear Models (Univariate) with treatment pesticides and earthworms as factors. For post hoc comparison of means (growth, reproduction), Scheffe test was applied. Results (mean values) of the earthworm avoidance response test were compared by the Mann-Whitney U-test, as data were not normally distributed.

Statistical analysis was performed using the SPSS software (SPSS 12.0 for Windows; SPSS Inc., Chicago, IL, USA).

2 Results and discussion

2.1 Assessment of test chlorpyrifos on acute toxicity for *E. fetida*

The results of the acute test were presented in Table 1. Only in the control treatment did all the worms survive at the end of the test. In all exposure period, a clear concentration-response relationship was observed

Table 2 Summary of parameter estimates for the acute toxicity of chlorpyrifos

Exposure period	LC_{50} (mg/kg)	Confection limits 95%	X^2	P
14 d	118.5	87.26–138.65	0.870	0.833
21 d	91.78	73.49–98.76	0.937	0.816

The parameters X^2 and P were obtained after Pearson Goodness-of-Fit test; LC_{50} was calculated from regression model.

that earthworm mortality increased with increasing concentrations of each contaminant. Parameter estimates for the acute toxicity of contaminants are given in Table 2.

Acute toxicity test has been traditionally used to assess the toxicity of contaminants in soils to earthworm. The most common end point measured when evaluating acute toxicity of chemicals to earthworms was LC_{50} (the concentration that is lethal to 50% of individuals). The endpoint "LC_{50}" shows effects at relatively high pollutant concentrations and indicates the maximum damage likely to an organism. In this research, LC_{50} on earthworm by chlorpyrifos assessed is 91.78 mg/kg (14 d), which is similar to the report of Hu Xiuqing that LC_{50} is 83.63 mg/kg (14 d) (Hu *et al*., 2004). According to the regulation of environment safety assessment test for agricultural pesticide, the suggested standard of toxicity is LC_{50} < 1 mg/kg for high-toxic pesticide, 1–10 mg/kg for mid-toxic pesticide, >10 mg/kg for low-toxic pesticide, thus the acute toxicity of chlorpyrifos on earthworms is low.

2.2 Impacts of chlorpyrifos on growth and reproduction of *E. fetida*

As in the present study, results of chronic toxicity tests demonstrated that chlorpyrifos have adverse impact on growth and reproduction in earthworms, but this is largely dependent on pesticide concentration and exposure period.

Earthworm growth decreased with increased concentrations of pesticide (Table 3). After 6 and 8 weeks of exposure, growth differed from the control in all treatments except for 5 mg/kg chlorpyrifos. The average weight gain of earthworms exposed to chlorpyrifos was generally lower than the controls during the eight-week exposure period.

Results of the reproduction test were presented in Fig.2. Chlorpyrifos exposure had a significant effect on reproduction in earthworms. After four weeks of exposure, number of cocoons differed from the control in all treatment except for 5 mg/kg chlorpyrifos ($P<0.05$). However, after 8 weeks, cocoon production was significantly lower at 5 mg/kg chlorpyrifos than the controls ($P<0.05$). Cocoon production in earthworm exposed to chlorpyrifos for 8 weeks was lower than that for 4 weeks.

The results of the viability of cocoons are presented in Fig.3. After four weeks of exposure, viability of cocoons from earthworms exposed to pesticides was significantly lower than the controls except for 5 mg/kg chlorpyrifos ($P<0.05$). Cocoon viability in earthworm exposed to chlorpyrifos for 8 weeks was not evaluated due to very low cocoon production.

Considering cocoon production and cocoon viability, toxic effects could be observed in earthworms exposed

Table 3 Growth of adult *E. fetida* exposed to pesticides for eight weeks

Treatment with chlorpyrifos (mg/kg)	Mean weight per earthworm (mg)			
	Week 0	Week 4	Week 6	Week 8
Control	364±12	406±40	410±114	407±78
5	384±12	385±58	356±68	365±78
20	370±11	349±78	316±73 [a]	323±58 [a]
40	369±7	333±120	325±78 [a]	258±86 [a]
60	383±15	366±85	338±34 [a]	326±68 [a]
80	369±10	310±25 [a]	300±65 [a]	268±44 [a]

[a] Significant differences (P<0.05) between treatment and control are indicated for each week.

to chlorpyrifos, with significant decreases in reproduction at the lowest concentration. Results of the reproduction test demonstrated that chlorpyrifos have adverse impact on cocoon production and cocoon viability.

2.3 Toxicity of chlorpyrifos on the avoidance response of *E. fetida*

The avoidance response tests were conducted using a six-chamber test system. Results of the avoidance response test are shown in Fig.4. Fig.4a shows that distribution of earthworm in each chamber of test system was similar to control treatment. However, as Fig.4b illustrates, chlorpyrifos exposure had a significant effect on distribution of earthworm in six-chamber test system. For earthworm, significant avoidance response occurred in chlorpyrifos treatments CH4 (P = 0.024) and CH5 (P = 0.002) when compared to the control CH0.

Earthworms can sense chemicals via a large number of chemoreceptors, and avoid the contaminations. As in the present study, we also observed earthworms that were

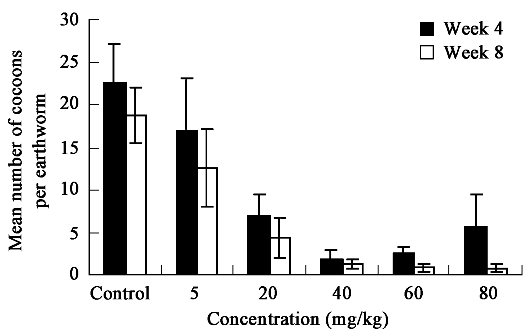

Fig. 2 Cocoon production in *Eisenia fetida* exposed to chlorpyrifos for different exposure period.

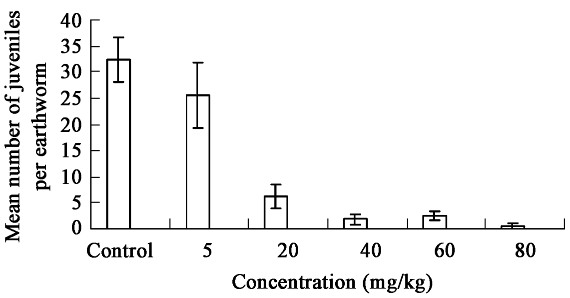

Fig. 3 Cocoon viability in *Eisenia fetida* exposed to chlorpyrifos for exposure week 4.

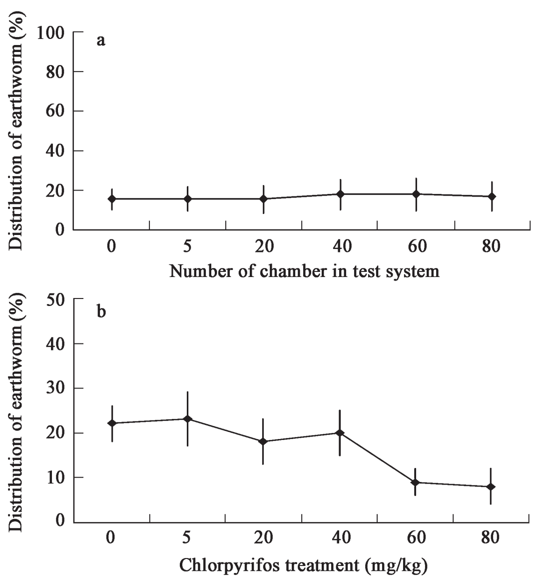

Fig. 4 Distribution of earthworm in control after 48 h (a) and exposed to chlorpyrifos after 48 h (b) incubation time in test system. Arithmetic mean of total distribution (n=4). Each replicate contained 20 earthworms.

introduced into glass containers staying on the top layer of the contaminated soil instead of moving into it, followed by worms dying a few days later. This can be interpreted as avoidance behavior, which supports the justification of behavioral tests in addition to acute and reproduction testing in earthworms. For chlorpyrifos, concentration affecting avoidance response was far more than that for growth and fecundity (Table 3), it seems likely that earthworm were not able to escape from pesticide-contaminated soil into the underlying clean soil in field and hence were exposed continuously to elevated concentrations of pesticides. However, most ecotoxicologically based risk assessment methods often operate at different ranges of sensitivity (lethal, sublethal) using endpoints such as mortality, change of biomass, reproduction rates, by neglecting behavioral response. Consequently, the use of toxicity data obtained exclusively from acute and chronic toxicity tests to evaluate ecotoxicological risk may underestimate the effects of pollutants on soil invertebrate populations.

2.4 Summary of sensitivity of test methods using different endpoints

Assessing the toxicity of a soil requires not only suitable tests that address different soil organisms representative of the different habitat functions and ways of bioavailabil-

Table 4 Summary of concentrations of chlorpyrifos causing adverse impacts on endpoints in *Eisenia fetda* Andrei

	Lowest-obsered-response concentration (mg/kg)	
Acute toxicity	LC_{10} (14 d)	78.91
Chronic toxicity	Growth (week 4)	80
	Growth (week 6)	20
	Cocoon production (week 4)	20
	Cocoon production (week 8)	5
	Cocoon viability (week 4)	20
Behavioral	Avoid response	40

ity but also tests with different sensitive population test endpoints (Schaefer, 2001). The summary of sensitivity of test methods using different endpoints was presented in Table 4.

As Table 4 illustrates, reproduction variables were clearly more sensitive test endpoints than other endpoints. Chlorpyrifos had adverse effect on fecundity in earthworm exposed to 5 mg/kg chlorpyrifos after 8 weeks. According to Booth's study, when chlorpyrifos is applied at the field reate of 2 L of Lorsban (40 EC) per hectare, the maximum concentration in the soil would be 6.15 g a.i./m^3, which equates to 4 mg/kg (Booth and O'Halloran, 2001). The calculation assumed that the pesticide would not penetrate the soil below 13 mm (Kuhr and Tashiro, 1978). This means that, in the field situation, fecundity in earthworm exposed to chlorpyrifos were more sensitively affected, even though population densities of earthworms may not be immediately affected by the chlorpyrifos exposure, adverse reproductive consequences may subsequently result in a reduction in population density.

3 Conclusions

The hierarchy of test designs differing in their levels of effect (lethal, sublethal, behavioral) should be applied to the eco-assessment of chlorpyrifos. However, most ecotoxicologically based risk assessment of chlorpyrifos often operate at different ranges of sensitivity (lethal, sublethal) using endpoints such as mortality, change of biomass, reproduction rates, by neglecting behavioral response. Consequently, the use of toxicity data obtained exclusively from acute and chronic toxicity tests to evaluate ecotoxicological risk may underestimate the effects of chlorpyrifos on soil invertebrate populations.

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