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Individual and combined toxic effects of cypermethrin and chlorpyrifos on earthworm

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Abstract

Toxicities were assessed for a pyrethroid (cypermethrin) and an organophosphate insecticide (chlorpyrifos) individually and in combination. A series of tests were conducted on different responses (acute, chronic, behavioral) of earthworms of species *Eisenia fetida andrei* in the ecological risk assessment of these pesticides. The results showed that the toxicity of the mixture of cypermethrin and chlorpyrifos was significantly higher than either of these pesticides individually, especially on the earthworm's chronic responses. At a concentration of 5 mg/kg, the mixture caused significant reductions on the growth and reproduction rates of earthworms, but did not cause any significant effect when the individual was tested. The increase in toxicity of the pesticide mixture means that the use of toxicity data obtained exclusively from single-pesticide experiments may underestimate the ecological risk of pesticides that actually present in the field.

Key words: earthworms; pesticide; combined effects; cypermethrin; chlorpyrifos

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Introduction

Pesticides are widely used all over the world, and have contributed greatly to the agricultural development. Nevertheless, extensive use of pesticides is known to have caused an increases of resistance of targeted pests, diminishing the effectiveness of these chemicals. Mixed pesticides are highly priced for their high efficiency, convenience and rapid actions, and are becoming increasingly popular in agricultural use. Due to structural and physiological similarities between pest and non-pest species, most pesticides are toxic not only to the target species, but to a range of non target organisms. Mixed pesticides, compared with single pesticides, could generally cause significant synergistic effects of toxicity on target species, while they are also effective to beneficial species in most cases. Anderson and Lydy (2002) reported that atrazine in combination with three organophosphate insecticides (chlorpyrifos, methyl parathion, and diazinon) could cause a significant increase in toxicity to *H. azteca* compared with individual pesticides. Clark et al. (2002) indicated that toxicity of chlorpyrifos was enhanced by atrazine and cyanazine to the fourth-instar larvae of the aquatic midge, *Chironomus tentans*. Thus the study of pesticide mixtures is even more important in evaluating ecological risk of pesticides on ecosystems.

A large portion of pesticides used in agriculture ends up as residuals in the soil, making soil-dwelling organisms more vulnerable to pesticide intoxication. Earthworm is one of the most common soil organisms in most environments, and plays an important role in the functioning of soil ecosystems (Spurgeon et al., 2003). Unlike many other soil organisms that are protected by thick cuticle on the exterior of their bodies, earthworms are particularly susceptible to soil chemicals. Most insecticides and heavy metals that they absorbed or ingested would undergo bioaccumulation, and passed on to animals at subsequent levels of the food chain. Bioaccumulation of substances in earthworm may not lead to significant effects to the animal itself, but may produce serious damages to the higher animals (Reinecke and Reinecke, 1996; Friis et al., 2004). Therefore, earthworms are suitable bioindicators of soil contamination, and can be used to provide safety thresholds for pesticide applications.

Although numerous eco-toxicity studies have been carried out in recent years using earthworms, these were mostly focused on single pesticides (Spurgeon et al., 2003). It is known that a mixture of pesticides could lead to an increase in toxicity, therefore, the results from

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single-species studies do not reflect field situations where multiple pesticides or pesticide mixtures are used, and should not be directly applied in ecological risk assessment. To date, only few studies have been published relating to the toxicity of mixed contaminants to earthworms, and these all have a focus on metal compounds (Khalil et al., 1996; Weltje, 1998). Little is known about the effects of multiple pesticides on earthworms.

In this article, the effects of the mixture of two pesticides, chlorpyrifos and cypermethrin are described in relation to the lethal and sub-lethal toxicity to earthworms, *Eisenia fetida andrei*. Chlorpyrifos and cypermethrin were selected because: (1) both pesticides are widely used in agriculture in China; (2) chlorpyrifos has a great potential for increased usage as a wide-spectrum organic insecticide with medium toxicity, and considered to be the ideal substitute for highly toxic pesticides; (3) chlorpyrifos and cypermethrin belong to pyrethroids and organophosphorus respectively, and such combination is common in pesticide applications; and (4) both pesticides are found to be toxic to earthworms (Alshawish et al., 2004; Booth and O'Halloran, 2001).

The objectives of this study are: (1) to test the effects of cypermethrin and chlorpyrifos individually and in mixture on earthworm's acute, chronic and behavioral responses, and (2) to compare the sensitivities as well as the advantages and disadvantages of different test methods in assessing the toxicity of soil contamination by mixed pesticides.

1 Materials and methods

1.1 Pesticides

The pesticides being tested in these experiments were the insecticides chlorpyrifos and cypermethrin, and their mixture at a ratio of 6.5:1 by weight, which is frequently-used in agriculture. Chlorpyrifos and cypermethrin were of analytical reagent grade purity. The pesticides and 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 the species *Eisenia fetida andrei* were purchased from Jialiming Earthworm Farm Co., Ltd., China and cultured in the laboratory in artificial soil according to International Standard 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 kept at room temperature (20°C). Soil water content was measured and distilled water was added every week to reach 50% of maximum water-holding capacity. The soils were changed every four weeks. The earthworms used in these experiments were adults with well-developed clitella and 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 pesticides (dry weight basis) were added to the soil, which were 75, 90, 100, 125, 150 and 200 mg/kg for chlorpyrifos, 62.5, 65, 72, 85, 105, and 125 mg/kg for cypermethrin, and 22.5, 25, 27.5, 30.0, 35.0 and 41.0 mg/kg for the mixture.

For each test concentration, desired amount of pesticide was thoroughly mixed into the soil as an aqueous solution, and was put into four 1-L glass containers. For the control treatment, tap water was used instead of pesticides and the substrates were placed into another four containers. 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-day test period, worms were sorted by hand and the test endpoint was mortality rates.

1.4 Earthworm growth test

The test method was similar to the acute test described above, and was modified from the work by Khalil et al. (1996). The test substrates were prepared by adding chlorpyrifos at 5, 20, 40, 60 and 80 mg/kg, cypermethrin at 5, 10, 20, 40 and 60 mg/kg, and their mixture at 5, 10, 20 and 30 mg/kg in the soil. The concentrations used were based on the results of pilot experiments (data not shown). Each pesticide concentration was tested by four replicates, and an additional four replicates 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 start of the experiment. During the test period of 8 weeks, the worms were weighed weekly to evaluate their growth response to test pesticides. Before weighing, all 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 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.

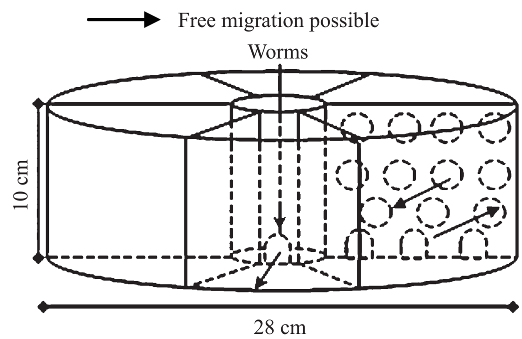

Fig. 1 Test container of the avoidance response test (Schaefer, 2004).

1.5 Reproductive response test

The test was similar to the growth test described above, but the test endpoints were cocoon production after 4 weeks and the amount of hatchlings after 8 weeks. The worms were hand-sorted and tallied after 4 weeks, and the number of cocoons produced were counted and returned to the soil for further incubation. By the end of the experiment, the juveniles were hand-sorted and their numbers were recorded.

1.6 Avoidance response test

The test was based on the work by Schaefer (2004) and Zhou et al. (2008). 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 of 5 mm in diameter), and were 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 hr, slides (without perforations) were placed between the chambers to prevent further migrations and the number of worms from each chamber was counted. The test substrates in consecutive chambers were arranged in sequence forming a concentration gradient (Table 1). Four replicates and four controls were used in the experiment.

Table 1 Concentrations of pesticides applied in the avoidance response test

Substrate	CH0	CH1	CH2	CH3	CH4	CH5
Control (mg/kg)	0	0	0	0	0	0
Chlorpyrifos (mg/kg)	0	5	10	20	40	60
Cypermethrin (mg/kg)	0	5	10	20	40	60
Mixture of two pesticides (mg/kg)	0	5	10	20	30	40

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

1.7 Statistical analyses

The 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 to the earthworms. One-way ANOVA ($P < 0.05$) and correlation analysis was used for assessing the effects of contaminants on growth and reproduction. Avoidance response was analyzed using General Linear Models (Univariate) with treatment pesticides and earthworms as factors. With *post hoc*, in 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.

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

2 Results

2.1 Assessment of test pesticides for acute toxicity on earthworms

The results of the acute response test are summarized in Table 2. Only in the control treatment did all the worms survive at the end of the test. In all other treatments, a clear positive dose-response relationship was observed between earthworm mortality and pesticide concentration. The results of the acute response test show that the mixture of cypermethrin with chlorpyrifos is more toxic to earthworms than pesticides applied separately.

2.2 Effects of test pesticides on growth and reproduction of earthworms

The effects of pesticides on growth and reproduction of earthworms are summarized in Tables 3 and 4. The results indicated that all pesticide treatments produced growth and reproductive responses, while the effects from the mixture were significantly greater than that of individual pesticides.

After 8 weeks of exposure, cypermethrin produced the least level of influence to the growth of earthworms, while chlorpyrifos and the mixture started to produce significant growth reductions at a concentration of 20 mg/kg.

The mixed pesticide of chlorpyrifos and cypermethrin showed a stronger influence on the reproduction of earthworm than that of their separate use. After 4 weeks of experiment, the reproductive rates from all concentrations of mixed pesticides were lower than the control group,

Table 2 Summary of results for the acute response test

Pesticides	LC_{50} (mg/kg)	Confection limits 95%
Cypermethrin	86.04	63.18–95.18
Chlorpyrifos	116.00	85. 62–137.42
Mixture of two pesticides	35.06	27.70–38.93

Table 3 Summary of results from the growth response test

Treatment	Mean weight per earthworm (mg)	
	0 week	8 weeks
Control	360.0 ± 8.2	380.2 ± 71.5
Chlorpyrifos		
5 mg/kg	404.2 ± 12.3	385.4±68.0
20 mg/kg	380.5 ± 11.0	335.5 ± 58.0 a
40 mg/kg	379.3 ± 7.5	285.2 ± 86.3 a
60 mg/kg	383.2 ± 15.5	306.0 ± 67.1 a
80 mg/kg	379.5 ± 10.5	265.0 ± 45.0 a
Cypermethrin		
5 mg/kg	399.2 ± 14.1	405.0 ± 120.2
10 mg/kg	375.5 ± 11.5	393.5 ± 68.2
20 mg/kg	366.0 ± 8.2	378.0 ± 87.0
40 mg/kg	374.6 ± 6.5	361.5 ± 58.5
60 mg/kg	385.0 ± 9.2	331.5 ± 73.3 a
Mixture of two pesticides		
5 mg/kg	365.4 ± 8.5	358.5 ± 83.5
10 mg/kg	344.9 ± 7.2	365.6 ± 114.5
20 mg/kg	387.3 ± 12.5	293.7 ± 76.0 a
30 mg/kg	352.0 ± 11.0	299.5 ± 44.0 a

a: Significant differences at $P < 0.05$ between treatment and control were compared each week.

Table 4 Summary of results from the reproductive response test

Treatment	Mean number of cocoons per earthworm	Mean number of juveniles per earthworm	Mean number of cocoons per earthworm
	4 weeks	8 weeks	8 weeks
Control	25.5 ± 5.45	34.83 ± 5.25	18.75 ± 3.25
Chlorpyrifos			
5 mg/kg	18.88 ± 6.25	25.62 ± 6.25	12.50 ± 4.55
20 mg/kg	6.88 ± 2.55 a	6.25 ± 2.38 a	4.38 ± 2.40 a
40 mg/kg	3.88 ± 0.96 a	4.88 ± 0.69 a	1.25 ± 0.58 a
60 mg/kg	4.50 ± 0.82 a	2.50 ± 0.82 a	0 ± 0 a
80 mg/kg	4.62 ± 3.75 a	0 ± 0 a	0 ± 0 a,b
Cypermethrin			
5 mg/kg	24.62 ± 5.90	30.25 ± 8.25	22.5 ± 3.22
10 mg/kg	22.00 ± 8.12	27.12 ± 8.25	22.5 ± 4.28
20 mg/kg	19.50 ± 8.28	22.50 ± 6.88	19.25 ± 3.15
40 mg/kg	18. 26 ± 5.40	21.00 ± 5.46 a	17.50 ± 3.22
60 mg/kg	13.68 ± 6.38 a	13.75 ± 8.22 a	2.50 ± 2.02 a, b
Mixture of two pesticides			
5 mg/kg	11.88 ± 3.75a	11.25 ± 2.50 a	5.00 ± 2.02 a, b
10 mg/kg	8.75 ± 3.22 a	7.50 ± 2.16 a	0 ± 0 a, b
20 mg/kg	2.50 ± 0.82 a	2.50 ± 0.89 a	0 ± 0 a, b
30 mg/kg	2.25 ± 1.89 a	1.25 ± 0.50 a	0 ± 0 a, b

a: significant differences ($P < 0.05$) between treatment and control are indicated for each week; b: significant differences ($P < 0.05$) between different exposure period are indicated for each concentration.

and the rates decreased with the increase of treatment concentrations. It was found that the influence of pesticides on the reproduction of earthworms was not only determined by the treatment concentration, but also related to the experiment duration. After 8 weeks of treatment with the mixed pesticide, no cocoons could be found except the one with a concentration of 5 mg/kg. Influence of pesticides was found with the number of hatchlings as well. After 4 weeks of exposure, viability of cocoons was found to be lower from the exposure of pesticides ($P < 0.05$). Cocoon viability of earthworm exposed to the mixed pesticide for 8 weeks was not evaluated due to extremely low cocoon production. The results from the reproductive response test demonstrated that the mixture of cypermethrin and chlorpyrifos produce greater adverse impacts on both cocoon production and cocoon viability compare to their individual use.

Table 5 Summary of concentrations of cypermethrin, chlorpyrifos and the mixture of both pesticide causing adverse impacts on endpoints in earthworms

Parameter assessed	The lowest-observed-response concentration (mg/kg)		
	Cypermethrin	Chlorpyrifos	Mixture of chlorpyrifos and cypermethrin
LC_{10} (day 14)	72.0	80.5	25.5
Growth (week 8)	60	20	20
Cocoon production (week 4)	60	20	5
Avoidance response	20	40	40

2.3 Avoidance response of earthworms to test pesticides

Figure 2a illustrates no avoidance response of earthworms, while Fig. 2b shows a significant response towards pesticides. During the study, significant avoidance response were observed in the treatment of mixed pesticides CH5 ($P = 0.014$) compared to the control CH0.

Earthworms have a large number of chemoreceptors on their body surface, and can produce avoidance response towards undesirable contaminants. During the study, the test animals were observed staying on the top layer of the contaminated soil only, followed by mortality a few days later. We interpreted such observation as avoidance behavior, which was consistent with the results from other response experiments. For the mixture of cypermethrin and chlorpyrifos, the concentration that led to avoidance response was found to be greater than that on growth and reproductive responses (Table 5).

3 Discussion

The sensitivity tests on pesticides toxicity on earthworm showed that both chlorpyrifos and cypermethrin have toxicity to earthworms, it is in agreement with other studies (Booth and O'Halloran, 2001; Alshawish et al., 2004; Zhou et al., 2007, 2008). Alshawish et al. (2004) tested the effect of cypermethrin, chlorpyrifos, dicofol, mancozeb and haloxyfopetotyl on their chronic toxicity on *Aporrectodea caliginosa* in laboratory cultures. They concluded that chlorpyrifos was the most toxic pesticide of all, which can produce significant impacts on earthworm fecundity at 50 mg/kg dry soil, while cypermethrin at the

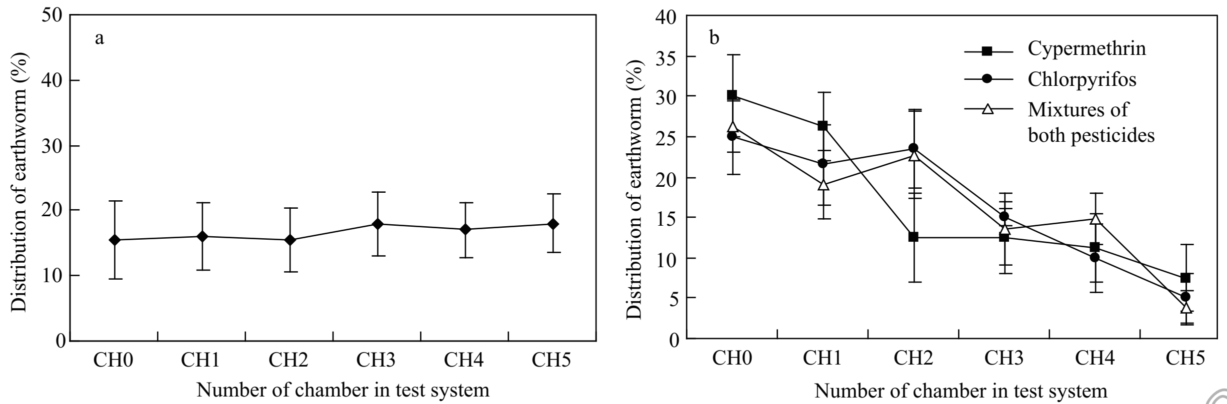

Fig. 2 Distribution of earthworm in the control treatment (a) and exposed to test pestcides (b) after 48 hr incubation time in the test system. Arithmetic mean of total distribution (replicates: $n = 4$). Each replicate contained 20 earthworms.

same dosage was the least toxic, which produced 20% reductions in cocoon viability and no effects on hatchlings development. Booth and O'Halloran (2001) studied the influence of diazinon and chlorpyrifos on the chronic toxicity of *A. caliginosa* at different pesticides dosages. They found that the lowest concentration of chlorpyrifos causing adverse impacts on growth and fecundity of adult earthworms was 28 mg/kg.

From this study, it indicates that the mixed use of cypermethrin and chlorpyrifos has obviously increased not only the acute toxicity, but also the chronic toxicity, i.e., growth and reproduction of earthworms. This increased toxicity can lead to adverse impacts on earthworm populations, threatening the normal functioning of soil ecosystems.

In this study, LC_{50} of earthworms from the mixed use of cypermethrin and chlorpyrifos were far lower than their separate use. The decrease of LC_{50} in the mixed use of cypermethrin and chlorpyrifos indicates that the mixture is more toxic compared to these pesticides applied individually, leading to higher mortality rate of earthworm populations.

The results also showed that the mixed pesticide can lead to the greater impacts on chronic response (i.e., growth and reproduction) than of acute response (i.e., mortality) in earthworms. In addition, the pesticides concentrations present as residuals in the soil is often lower than the lethal dose of earthworms. As in the findings of this study, the effective dose of the mixed pesticides of cypermethrin and chlorpyrifos that disrupted the normal processes of growth and reproduction is lower than that of their separate use, especially in reproduction. A dose of mixture at 5 mg/kg could lead to adverse effect on earthworm reproduction, while it was 60 mg/kg for cypermethrin and 20 mg/kg for chlorpyrifos. Toxicity of mixed used of pesticides led to significant reductions in cocoon production and viability of earthworms, thus greatly lowered their population fecundity. In addition, the impacts of mixed used of pesticides on earthworm reproduction means that the effects of contamination can last for more than one generation, leading to significant decline and reductions of genetic diversity, which may subsequently cause disruption of the functioning of the soil ecosystem. Therefore, the ecological risk of mixed pesticides on soil organisms should be studied in detail before applying to sensitive environments.

4 Conclusions

It can be concluded that the mixture of the two pesticides, chlorpyrifos and cypermethr causes greater effects on endpoints such as growth and reproduction on earthworms by a series of tests (lethal, sublethal, and behavioral) than the individual pesticides. Thus, the results from single-species studies do not reflect field situations where multiple pesticides or pesticide mixtures are used, and should not be directly applied in ecological risk assessment at such circumstances. The environmental risk of toxicants is, however, still judged on the effects of individual pesticides. Consequently, the increase in toxicity of the pesticide mixture means that the use of toxicity data obtained exclusively from single-pesticide experiments may underestimate the ecological risk of pesticides that actually present in the field.

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