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Individual and combined toxic effects of herbicide atrazine and three insecticides on the earthworm, *Eisenia fetida*

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Abstract In the present study, we evaluated the individual and combined toxic effects of herbicide atrazine and three insecticides (chlorpyrifos, *lambda*-cyhalothrin and imidacloprid) on the earthworm, *Eisenia fetida*. Results from 48-h filter paper test indicated that imidacloprid had the highest toxicity to *E. fetida* with an LC_{50} of 0.05 (0.041–0.058) μg a.i. cm^{-2}, followed by *lambda*-cyhalothrin and atrazine with LC_{50} values ranging from 4.89 (3.52–6.38) to 4.93 (3.76–6.35) μg a.i. cm^{-2}, while chlorpyrifos had the least toxicity to the worms with an LC_{50} of 31.18 (16.22–52.85) μg a.i. cm^{-2}. Results from 14-days soil toxicity test showed a different pattern of toxicity except that imidacloprid was the most toxic even under the soil toxicity bioassay system. The acute toxicity of atrazine was significantly higher than that of chlorpyrifos. In contrast, *lambda*-cyhalothrin was the least toxic to the animals under the soil toxicity bioassay system. The binary mixture of atrazine–*lambda*-cyhalothrin and ternary mixture of atrazine–chlorpyrifos–*lambda*-cyhalothrin displayed a significant synergistic effect on the earthworms under the soil toxicity bioassay. Our findings would help regulatory authorities understand the complexity of effects from pesticide mixtures on non-target organisms and provide useful information of the interaction of various pesticide classes detected in natural environment.

Keywords Soil invertebrate · Ecotoxicology · Pesticide · Combined toxicity · Acute toxicity

Introduction

Pesticides are widely used in agricultural applications all over the world. However, there are serious concerns about these substances introduced into the soil environment due to runoff and leaching (Dabrowski et al. 2014; Wahanthaswamy and Patil 2004). The effects of pesticides on soil fauna have become an important focus in studies of soil pollution (Reinecke and Reinecke 2007; Gupta et al. 2011). Earthworms play important roles in terrestrial food chains, energy and nutrient cycling (Ellis et al. 2010). The adverse effects on keystone species may exert a disproportionate impact on the stability of the community, leading to serious ecological damages to the entire terrestrial ecosystem (Edwards and Bohlen 1992; Landrum et al. 2006).

Many studies have evaluated the toxicity of single pesticide to soil organisms (Liang and Zhou 2003; Stepić et al. 2013). In the natural environment, pesticides often exist in mixtures of two or more compounds (Choung et al. 2011; Phyu et al. 2011; Saxena et al. 2014). However, little information about their combined effects on soil organisms is available (Daam et al. 2011). The joint toxicity of pesticide mixtures on a specific organism can be less than, equal to, or more than the summed effects of individual pesticides (Jin-Clark et al. 2008). Correspondingly, the toxicity of toxicants can be qualitatively described as antagonistic, additive, or synergistic, respectively. Moreover, ecological risk assessment and/or environment

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quality criteria based on a single pesticide exposure may not adequately protect terrestrial ecosystems (Zhou et al. 2011). Extensive ecotoxicological tests using earthworms have been conducted in recent years, but most of them have focused on effects of single pesticides (Gupta et al. 2011; Sanchez-Hernandez et al. 2014). However, the effects of pesticide mixtures on earthworms remain largely unclear (Wu et al. 2012).

Mixtures of herbicides and insecticides are very often used in agricultural applications, where herbicides are used to control weeds and insecticides are co-applied to control insect pests (Choung et al. 2011). Atrazine (a triazine herbicide), chlorpyrifos (an organophosphate insecticide), *lambda*-cyhalothrin (a pyrethroid insecticide) and imidacloprid (a neonicotinoid insecticide) are all commonly used agricultural chemicals on crops in China (Piner and Üner 2012; Xing et al. 2014). Although the individual and equitoxic ratio effects of these four pesticides on non-target organisms are quite well documented, the equivalent concentration combined effects of their mixtures have not been examined (Sanchez-Hernandez et al. 2014; Zhang et al. 2014). In the present study, we aimed to assess both individual and combined toxicity with equivalent concentrations of herbicide atrazine and three insecticides (chlorpyrifos, *lambda*-cyhalothrin and imidacloprid) to the earthworm, *Eisenia fetida*. Our findings could provide a scientific basis for accurate assessment the ecological risk of pesticide mixtures on soil invertebrates.

Materials and methods

Earthworms

The earthworm, *Eisenia fetida* (Oligochaeta, Lumbricidae) was obtained from the College of Animal Sciences, Zhejiang University, China, and selected as the test organism in our study. They were maintained at room temperature (20 ± 1 °C) in artificial soil according to OECD guidelines (OECD 1984). Soils were mixed with ground sphagnum peat and decomposed pig manure under laboratory conditions. Adult earthworms (weighing 350–500 mg) with well-developed clitella were randomly selected for the toxicity tests. Additional control tests were carried out using chloracetamide as a toxic reference standard.

Pesticides

Four pesticides were tested in this study, including one triazine herbicide atrazine, one organophosphate insecticide chlorpyrifos, one pyrethroid insecticide *lambda*-cyhalothrin and one neonicotinoid insecticide imidacloprid. The selected pesticides are widely used in agriculture worldwide. Atrazine [95 % technical product (TC)] and imidacloprid (95.3 % TC) were provided by Nanjing Red Sun Chemical Co., Ltd. (Nanjing, Jiangsu, China). Chlorpyrifos (96 % TC) was purchased from Jiangsu Yangnong Agrochemical Group (Yangzhou, Jiangsu, China). *Lambda*-cyhalothrin (97 % TC) was obtained from Jiangsu Changlong Chemical Industrial Group (Changzhou, Jiangsu, China). Since our study aimed to document the toxic effects of the chemical compounds and not of the adjuvants added to the commercial products, active ingredients were used instead of commercial formulations.

Toxicity tests

Filter paper contact test

Filter paper contact test was conducted as OECD TG 207 (OECD 1984) described with minor modifications (Wang et al. 2012a). Briefly, earthworms were held on wet filter paper at 20 ± 1 °C for 24 h in the dark to purge the gut contents. They were then washed and dried before the dose–response test. A piece of Whatman filter paper (Grade 1 quatative) was placed in a 9-cm Petri dish and treated with the test substance dissolved in 2 mL of acetone. After the solvent was evaporated, the piece of filter paper was remoistened with 2 mL of distilled water. Only one earthworm was placed on the filter paper in order to avoid the adverse effect caused by the death of worm in the same dish. Acetone was used as the control. Treated earthworms were maintained at 20 ± 1 °C under 80–85 % relative humidity in the dark. After exposure for 48 h, worm's mortality was recorded. An earthworm was considered dead if it failed to respond to a gentle mechanical touch on the front end.

In the contact filter paper test, the pesticides were classified as supertoxic (<1.0 μg a.i. cm^{-2}), extremely toxic (1–10 μg a.i. cm^{-2}), very toxic (10–100 μg a.i. cm^{-2}), moderately toxic (100–1000 μg a.i. cm^{-2}) or relatively nontoxic (>1000 μg a.i. cm^{-2}) based on the resulting LC_{50} values (Roberts and Dorough 1984).

Artificial soil test

Two exposure periods (7 and 14 days) were used in the artificial soil test according to OECD soil (OECD 1984). The soil consisted of (dry weight) 10 % ground sphagnum peat (<0.5 mm), 20 % kaolinite clay (>50 % kaolinite) and 70 % fine sand (OECD 1984, 2004). Deionised water is added to give an overall moisture content of about 35 % of the dry weight of artificial soil. For each tested concentration, the desired amount of pesticide was dissolved in 10 mL acetone and mixed with a small quantity of fine quartz sand. The sand was mixed for least 1 h to evaporate the acetone and then thoroughly mixed with the pre-moistened artificial soil in a household mixer. The final moisture contents of the artificial soil were adjusted to the above-mentioned level with distilled water. A total of 0.65 kg soil (equivalent to 0.5 kg dry artificial soil) was placed in a 500-mL glass jar (surface area, 63.6 cm^2), and 10 adult earthworms were added to each jar. Similarly, controls were prepared only with 10 mL acetone containing no insecticide. The jars were loosely covered with polypropylene lids to allow the air exchange and stored at 20 ± 1 °C with 80–85 % relative humidity under 400–800 lx of constant light. Mortality was assessed after treatment for 7 and 14 days.

Mixture toxicity test

Interaction of mixtures is classified as additive, synergistic and antagonistic as previously described (Jin-Clark et al. 2008). A fixed equivalent concentration ratio design was employed in mixture experiments of the herbicide atrazine and three insecticides to *E. fetida*. For the equivalent concentration mixture treatments, the initial concentration (1X) of each of the insecticides tested was 6.25 % LC_{50} of higher (or the highest) toxicity insecticide when exists individually. The concentrations of each individual pesticide in the mixture components were then sequentially doubled (1X, 2X, 4X, 8X, 16X, 32X) giving the six concentrations tested. The ratio of the binary, ternary and quaternary mixture components were kept constant (1:1, 1:1:1 or 1:1:1:1), while the total concentration of the mixture systematically varied.

Statistical analysis

A probit analysis was conducted to assess the acute toxicity of pesticides to *E. fetida* using a program developed by Chi (Chi 1997). The significant level of mean separation ($P < 0.05$) detected was based on the lack of overlap between the 95 % confidence limits of 2 LC_{50} values. The synergy of pesticide mixtures was determined on the basis of an additive index and 95 % confidence interval from LC_{50} data according to a previously described method (Marking 1985). This method defines an additive index for the combined effects of a mixture of chemicals. The biological activity (S) of test compounds A, B and C was determined by the equation as follows:

$$S = (Am/Ai) + (Bm/Bi) + (Cm/Ci),$$

where A, B and C are chemicals; i is the individual LC_{50} value for A, B or C; m is the LC_{50} value for the mixture of A, B or C; S is the sum of the biological activity. The calculated S values were then substituted into appropriate formulas to determine the additive index (AI). The AI was calculated using the following equations:

$$\mathrm{AI} = (1/S) - 1 \text{ for } S < 1.0;\ \mathrm{AI} = -S + 1.0 \text{ for } S \geq 1.0$$

The AI was used to indicate the property of observed toxicity (additive, synergistic, or antagonistic). An AI value =0 means that the toxicity of the mixture is simply additive, an AI value <0 means antagonistic or less than additive toxicity, and an AI value >0 means greater than additive toxicity or synergistic. The greater the additive index value, the greater the chemical synergy. Chemical synergy was considered as statistically significant if the 95 % confidence interval was >0.

Results

Contact toxicity

Individual pesticide toxicity

The results of filter paper contact test are presented in Table 1. The results demonstrated that the different pesticides widely varied in their contact toxicities to *E. fetida*. At 24-h interval, imidacloprid exhibited the highest toxicity to *E. fetida*, followed by atrazine and *lambda*-cyhalothrin, while chlorpyrifos was the least toxic to the worms. Based on the LC_{50} values, imidacloprid was 989.5 times more toxic than chlorpyrifos. The order of toxicity to the animals based on LC_{50} values was as follows: imidacloprid>atrazine, *lambda*-cyhalothrin>chlorpyrifos. At 48-h interval, imidacloprid still showed the highest intrinsic toxicity to *E. fetida*, *lambda*-cyhalothrin and atrazine exhibited relatively less toxicity, while chlorpyrifos displayed the lowest toxicity to the animals. The toxicity of imidacloprid was 640-fold higher compared with chlorpyrifos. The toxicity for the four tested pesticides could be ranked in a descending order as follows: imidacloprid>*lambda*-cyhalothrin, atrazine>chlorpyrifos.

According to the classification of Roberts and Dorough (1984), imidacloprid was categorized as supertoxic, atrazine and *lambda*-cyhalothrin were extremely toxic, whereas chlorpyrifos was moderately toxic to worms at 24-h interval base on the resulting LC_{50} values for *E. fetida* exposed to impregnated papers. However, imidacloprid was still classified as supertoxic, atrazine and *lambda*-cyhalothrin were classified as extremely toxic, while chlorpyrifos was classified as very toxic to organisms at 48-h interval.

Table 1 Individual and combined toxicities with contact filter paper test of atrazine and three insecticides to *Eisenia fetida*; additive index as an indicator of chemical synergy

Chemical	Time (h)	LC_{50} (95 % CI)[c] μg a.i. cm^{-2}	Additive index[a] (95 % CI)[c]
Individual pesticides			
Atrazine	24	22.75 (12.74–100.11)	
	48	4.93 (3.76–6.35)	
Chlorpyrifos	24	178.11 (92.64–301.92)	
	48	32.00 (23.82–42.03)	
Lambda-cyhalothrin	24	31.18 (16.22–52.85)	
	48	4.89 (3.52–6.38)	
Imidacloprid	24	0.18 (0.11–0.94)	
	48	0.05 (0.041–0.058)	
Binary mixtures			
Atrazine–chlorpyrifos	24	61.38 (42.83–137.26)	−2.04 (−11.25 to 0.75)
	48	5.04 (3.85–8.09)	−0.18 (−1.49 to −0.18)
Atrazine–*lambda*-cyhalothrin	24	5.61 (4.14–9.53)	1.34 (−0.34 to 7.32)
	48	1.75 (1.16–2.24)	0.41 (−0.23 to 0.57)
Atrazine–imidacloprid	24	0.12 (0.089–0.15)	0.58 (−0.37 to 9.73)
	48	0.07 (0.042–0.089)	−0.56 (−1.62 to −0.21)
Ternary mixtures			
Atrazine–chlorpyrifos–*lambda*-cyhalothrin	24	3.54 (2.61–5.75)	2.47 (0.15–10.95)[b]
	48	0.30 (0.15–0.54)	6.69 (2.13–9.94)[b]
Atrazine–chlorpyrifos–imidacloprid	24	0.20 (0.13–0.26)	−0.10 (−1.49 to 6.27)
	48	0.07 (0.009–0.11)	−0.43 (−2.16 to 2.71)
Atrazine–*lambda*-cyhalothrin–imidacloprid	24	0.18 (0.13–0.24)	0.03 (−1.25 to 7.79)
	48	0.11 (0.043–0.16)	−1.32 (−3.45 to −0.12)
Quaternary mixture			
Atrazine–chlorpyrifos–*lambda*-cyhalothrin–imidacloprid	24	0.16 (0.11–0.45)	0.12 (−3.24 to 7.19)
	48	0.11 (0.078–0.19)	−1.33 (−4.28 to −0.25)

[a] An additive index greater than zero indicates greater than additive toxicity

[b] Significant chemical synergy interactions between pesticides

[c] *CI* confidence index

Binary mixture toxicity

The LC_{50} values of different binary mixtures at 24 and 48-h intervals were determined to understand the interaction of the herbicide atrazine and each insecticide in the acute toxicity toward *E. fetida* (Table 1). For all of the tested binary mixtures (atrazine–chlorpyrifos, atrazine–*lambda*-cyhalothrin and atrazine–imidacloprid), the calculated additive indexes at 24 and 48-h intervals ranged from −2.04 (−11.25 to 0.75) to 1.34 (−0.34 to 7.32) and from −0.56 (−1.62 to −0.21) to 0.41 (−0.23 to 0.57), respectively, suggesting antagonistic response and greater than additive toxicity. However, additive indexes were increased when the exposure period was increased for the binary mixture of atrazine–chlorpyrifos, indicating a positive correlation between the mixture toxicity and exposure time.

Ternary and quaternary mixture toxicities

A significant synergistic effect was observed from the ternary mixture of atrazine–chlorpyrifos–*lambda*-cyhalothrin, with additive indexes of 2.47 (0.15–10.95) and 6.69 (2.13–9.94) at 24 and 48-h intervals, respectively. In contrast, the interaction was antagonistic response for the ternary mixture of atrazine–chlorpyrifos–imidacloprid, with additive indexes of −0.10 (−1.49 to 6.27) and −0.43 (−2.16 to 2.71) at 24 and 48-h intervals, respectively (Table 1). Interestingly, similar toxic effects were observed from the ternary mixture of atrazine–*lambda*-cyhalothrin–imidacloprid and the quaternary mixture of atrazine–chlorpyrifos–*lambda*-cyhalothrin–imidacloprid, and both of them exhibited greater than additive toxicity and antagonistic response at 24 and 48-h intervals, respectively.

Artificial soil toxicity

Individual pesticide toxicity

The acute toxicities of four tested pesticides to *E. fetida* from artificial soil test are shown in Table 2. Each pesticide exhibited significantly different levels of toxicity to the worms. At 7-day interval, imidacloprid showed the highest toxicity, followed by atrazine and chlorpyrifos. In contrast, *lambda*-cyhalothrin exhibited the lowest toxicity to the worms. The toxicity of imidacloprid was 249.0-fold higher than that of *lambda*-cyhalothrin to *E. fetida*. The average acute toxicity for the four tested pesticides could be ranked in a descending order as follows: imidacloprid>atrazine> chlorpyrifos>*lambda*-cyhalothrin. At 14-day interval, imidacloprid still showed the highest toxicity to *E. fetida*. Compared with 7-day interval, a similar decreasing order of the average acute toxicity was detected at 14-day interval. By comparing the efficacy of imidacloprid with the other pesticides according to their toxicity values at LC_{50}, we clearly showed that the toxicity level of imidacloprid was about 64.0-, 136.5- and 198.7-fold compared with atrazine, chlorpyrifos and *lambda*-cyhalothrin, respectively.

Binary mixture toxicity

The additive indexes of atrazine–*lambda*-cyhalothrin mixture at 7-day and 14-day intervals were 4.15 (2.66–6.49) and 4.43 (2.91–7.24), respectively, suggesting a significant synergistic effect. In contrast, the other two binary mixtures of atrazine–chlorpyrifos and atrazine–imidacloprid exhibited additive indexes of −1.53 (−5.49 to −0.53) and

Table 2 Individual and combined toxicities with artificial soil test of atrazine and three insecticides to *Eisenia fetida*; additive index as an indicator of chemical synergy

Chemical	Time (d)	LC_{50} (95 % CI)[c] mg a.i. kg^{-1}	Additive index[a] (95 % CI)[c]
Individual pesticides			
Atrazine	7	204.8 (179.9–236.3)	
	14	180.4 (158.4–204.5)	
Chlorpyrifos	7	421.3 (380.7–501.9)	
	14	384.9 (353.5–440.3)	
Lambda-cyhalothrin	7	784.5 (619.7–1209.9)	
	14	560.3 (475.9–718.5)	
Imidacloprid	7	3.15 (2.86–3.71)	
	14	2.82 (2.61–3.17)	
Binary mixtures			
Atrazine–chlorpyrifos	7	216.1 (174.0–250.6)	−0.57 (−1.05 to −0.084)
	14	163.6 (117.3–198.8)	−0.33 (−0.82 to −0.19)
Atrazine–*lambda*-cyhalothrin	7	31.49 (26.35–38.13)	4.15 (2.66–6.49)[b]
	14	25.16 (19.33–30.39)	4.43 (2.91–7.24)[b]
Atrazine- Imidacloprid	7	7.85 (5.57–18.27)	−1.53 (−5.49 to −0.53)
	14	4.28 (3.61–5.71)	−0.54 (−1.22 to −0.16)
Ternary mixtures			
Atrazine–chlorpyrifos–*lambda*-cyhalothrin	7	18.47 (12.91–34.56)	5.35 (1.96–9.98)[b]
	14	10.81 (6.45–18.61)	8.32 (3.77–17.13)[b]
Atrazine–chlorpyrifos–imidacloprid	7	4.39 (3.69–5.89)	−0.43 (−1.11 to −0.018)
	14	2.56 (2.14–3.01)	0.077 (−0.18 to 0.45)
Atrazine–*lambda*-cyhalothrin–imidacloprid	7	4.71 (3.91–6.56)	−0.52 (−1.34 to −0.074)
	14	2.92 (2.49–3.47)	−0.41 (−0.36 to 0.25)
Quaternary mixture			
Atrazine–chlorpyrifos–*lambda*-cyhalothrin–imidacloprid	7	4.61 (3.83–6.32)	−0.51 (−1.27 to −0.059)
	14	2.41 (1.88–2.98)	0.14 (−0.18 to 0.64)

[a] An additive index greater than zero indicates greater than additive toxicity

[b] Significant chemical synergy interactions between pesticides

[c] *CI* confidence index

−0.33 (−0.82 to −0.19) at 7 and 14-day intervals, respectively, suggesting a significant antagonistic effect (Table 2). However, additive indexes were increased when the exposure time was increased for all of the tested binary mixtures.

Ternary and quaternary mixture toxicities

Similar to the results of contact toxicity test (Table 1), the ternary mixture of atrazine–chlorpyrifos–*lambda*-cyhalothrin still exhibited a significant synergistic effect, with additive indexes of 5.35 (1.96–9.98) and 8.32 (3.77–17.13) at 7 and 14-day intervals, respectively. However, the other two ternary mixtures of atrazine–chlorpyrifos–imidacloprid and atrazine–*lambda*-cyhalothrin–imidacloprid showed additive indexes of −0.52 (−1.34 to −0.074) and 0.077 (−0.18 to 0.45) at 7 and 14-day intervals, suggesting significant antagonistic effect and additive toxicity, respectively. For the quaternary mixture of atrazine–chlorpyrifos–*lambda*-cyhalothrin–imidacloprid, the calculated additive indexes were −0.51 (−1.27 to −0.059) and 0.14 (−0.18–0.64) at 7 and 14-day intervals, indicating significant antagonistic effect and additive toxicity, respectively (Table 2). Moreover, additive indexes were increased when the exposure period was increased for all of the tested ternary and quaternary mixtures.

Discussion

Results from single toxicity tests showed that imidacloprid was the most toxic among four selected pesticides. The results of imidacloprid with soil toxicity test from the present study were consistent with Luo et al. (1999). The results from soil toxicity test showed that atrazine was a chemical of very low toxicity. Similar results have been obtained by Mosleh et al. (2003) and Pizl (1988) when they compared the toxicity of atrazine with other tested pesticides on certain species of earthworms. The estimated 14-day LC_{50} of chlorpyrifos in the present study (384.9 mg a.i. kg^{-1}) was within the previously reported range of 129 mg a.i. kg^{-1} and 1174 mg a.i. kg^{-1} (Ma and Bodt 1993). Results from this study showed that *lambda*-cyhalothrin was the least toxic to *E. fetida* among the selected four pesticides in soil toxicity test. It has been demonstrated that most of the pyrethroids are non-toxic to earthworms under soil toxicity test in other studies (Inglesfield 1984; Roberts and Dorough 1984).

Joint toxicity of chemicals could be evaluated by combining chemicals with either equitoxic ratio or equivalent concentrations, which are the two most commonly methods (Phyu et al. 2011; Stepić et al. 2013). Although some of selected pesticides with equitoxic ratio have been tested in our previous work (Chen et al. 2015; Wang et al. 2015a, b), the joint toxicity of them was tested using the chemicals with equivalent concentrations in the present study. We aimed to clarify if the joint toxicity of combined pollutants was closely linked with the ratio of their concentrations in the mixture. Therefore, our current study is the supplementation and improvement of our previous work (Chen et al. 2015; Wang et al. 2015a, b). In addition, our findings provided a solid scientific basis for accurately and comprehensively evaluating the risk of combined contaminations.

Laboratory bioassays under controlled conditions using relevant test organisms constitute a first approach to evaluate pesticide risks in soil compartment (Heimbach 1984; Wang et al. 2012b; Piola et al. 2013). However, a reasonable correlation between the results of acute toxicity tests and the effects observed in the field has been reported (Heimbach 1998). Several protocols using earthworms have been developed to test the toxic potential of chemicals and contaminated soil (Heimbach 1984; Luo et al. 1999; Wang et al. 2012b). Among these methods, most attention has been paid to the contact filter paper test and artificial soil test, and they have been adopted by OECD (1984, 2004), European Economic Community (EEC 1985), and International Standards Organization (ISO 1993). Contact filter paper test is an initial screening technique to assess the relative toxicity of chemicals to earthworms, in which the pesticides are mainly absorbed by the skin. However, it poorly reflects the situations in the soil ecosystem (Grumiaux et al. 2010; Tripathi et al. 2010). In contrast, artificial soil test mimics the natural environment of earthworms, and the pesticides are mainly absorbed by gut in this method (De Silva and van Gestel 2009; Udovic and Lestan 2010).

Pesticides are rarely found as single compounds in natural environment (Jin-Clark et al. 2008; Bjergager et al. 2012; Saxena et al. 2014). In contrast, they often exist as mixtures (Phyu et al. 2011). Atrazine, one of the widely used agricultural pesticides in China, functions as photosynthesis inhibitor through blockage of photosystem II in target plants. Kao et al. (1995) observed that atrazine induces cytochrome P450 and general esterase activities in insects. These induced enzymes break down pesticides, with the effect of either increasing or decreasing the toxicity of other pesticides depending on toxicities of their resulting metabolites (Anderson and Zhu 2004). As the most commonly and widely used organophosphate insecticide worldwide, chlorpyrifos can be activated into chlorpyrifos-oxon, leading to rapid acetylcholinesterase (AChE) phosphorylation in animals (Pérez et al. 2013). Such a process ultimately blocks the function of AChE, an important enzyme involved in neurotransmission, causing rapid muscular twitching and paralysis in the affected animals (Yen et al. 2011). The pyrethroid insecticide *lambda*-cyhalothrin is extensively used worldwide for agricultural pest control due to its higher efficiency, relatively shorter half-life in soil and lower mammalian toxicity (Narahashi 2000). Pyrethroids can modify the kinetics of voltage-sensitive sodium channels, resulting in abnormal nerve function in insects (Bloomquist 1996). Previous studies have shown that most pyrethroids are not toxic to earthworms (Inglesfield 1984; Roberts and Dorough 1984; Wang et al. 2012a). Our findings revealed that *lambda*-cyhalothrin was the least toxic to *E. fetida* in artificial soil test. This result also further indicated the rational use of these compounds in agriculture to avoid damage to the soil ecosystem. The neonicotinoid insecticide imidacloprid, functions as competitive inhibitor on nicotinic acetylcholine receptors (nAChRs) in the central nervous system (Tomizawa and Casida 2003; Elbert et al. 2008). Our study indicated that imidacloprid was the most toxic one to *E. fetida* among the four tested pesticides. Considering its high efficacy against target organisms such as sucking pests, environmental managers should carefully evaluate the use of imidacloprid in integrated pest management (IPM) programs to avoid serious damage to earthworms (Jeschke and Nauen 2008). Since atrazine, chlorpyrifos, *lambda*-cyhalothrin and imidacloprid are all widely used in China, they are likely to co-exist in soil and surface water (Choung et al. 2011). Results from this study also show that the simultaneous use of these pesticides in agriculture could cause harm to beneficial soil organisms such as earthworms because of synergistic effects.

Currently, the eco-toxicological assessment of soil quality is based on the internationally accepted test protocols with earthworms, collembolans and enchytraeids (Daam et al. 2011). The test protocols and guidelines with earthworms are designed to estimate the effects of chemicals on the survival and reproduction of adult individuals (EEC 1985; OECD 1984, 2004). However, some studies indicated that juvenile earthworms are often more sensitive to pollutants than adults (Zhou et al. 2008).

In addition to the antagonistic and greater than additive responses, we also described synergistic responses for herbicide-insecticide mixtures in the current study. Extremely synergistic responses were found from the binary mixture of atrazine–*lambda*-cyhalothrin and the ternary mixture of atrazine–chlorpyrifos–*lambda*-cyhalothrin. Little evidence of atrazine/*lambda*-cyhalothrin interaction is available in the literature. Hayes et al. (2006) reported that pesticide mixture containing atrazine and *lambda*-cyhalothrin at low dosage exerts significant adverse effects on Leopard frog (*Rana pipiens*). Other researchers have found similar results that atrazine can potentiate the effects of organophosphate insecticides (Anderson and Lydy 2002). Lydy and Linck (2003) found that atrazine increases the toxicity of chlorpyrifos to the earthworm *E. fetida* by 7.5-fold. These increased toxicities of pesticide mixtures can lead to adverse effects on earthworm populations, threatening the normal function of soil ecosystem. Therefore, experiments using single pesticide do not reflect field situations, where multiple pesticides or pesticide mixtures are used (Thompson 1996; Choung et al. 2011; Bjergager et al. 2012).

In the present study, our data were obtained based on the acute toxicity. However, sublethal endpoints seemed to be more sensitive indicators compared with lethality (Hackenberger et al. 2008). Moreover, various biomarkers were also used in sublethal endpoints, such as lysosomal membrane stability and genetic effects (Jensen et al. 2007; Fuchs et al. 2011). Molecular biomarkers are able to show alternations in the physiological status of organisms, and they are sensitive to serve as early warning signs of pollution or stress conditions to organisms (Sánchez-Hernández 2006; Tripathi et al. 2010). Additionally, biomarker responses may be sensitive indicators of chemical stress before sublethal effects, such as inhibition of growth or reproduction (Scott-Fordsmand and Weeks 2000). Therefore, in order to appropriately assess the ecological risk, more studies should be focused on long-term effects of pesticide mixtures at low concentrations on earthworms.

Conclusions

Imidacloprid is the most toxic of all the tested pesticides under both bioassay systems. The binary mixture of atrazine–*lambda*-cyhalothrin exhibited a significant synergistic effect on the earthworms under the soil toxicity bioassay. The ternary mixture of atrazine–chlorpyrifos–*lambda*-cyhalothrin displayed a significant synergistic effect on the earthworms under both bioassay systems. Taken together, experiments using single pesticide do not reflect field situations, and toxicity data obtained from single pesticide may underestimate the effects of pesticide mixtures on soil invertebrate populations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards Any studies in this paper using earthworms were conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

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