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RESEARCH ARTICLE

Joint acute toxicity of the herbicide butachlor and three insecticides to the terrestrial earthworm, *Eisenia fetida*

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Abstract The herbicide butachlor and three insecticides phoxim, chlorpyrifos, and *lambda*-cyhalotrhin are widely used pesticides with different modes of action. As most previous laboratory bioassays for these pesticides have been conducted solely based on acute tests with a single compound, only limited information is available on the possible combined toxicity of these common chemicals to soil organisms. In this study, we evaluated their mixture toxicity on the terrestrial earthworm, *Eisenia fetida,* with binary, ternary, and quaternary mixtures. Two different types of bioassays were employed in our work, including a contact filter paper toxicity test and a soil toxicity test. Mixture toxicity effects were assessed using the additive index method. For all of the tested binary mixtures (butachlor-phoxim, butachlor-chlorpyrifos, and butachlor-*lambda*-cyhalothrin), significant synergistic interactions were observed after 14 days in the soil toxicity assay. However, greater additive toxicity was found after 48 h in the contact toxicity bioassay. Most of the ternary and quaternary mixtures exhibited significant synergistic effects on the worms in both bioassay systems. Our findings would be helpful in assessing the ecological risk of these pesticide mixtures to soil invertebrates. The observed synergistic interactions underline the necessity to review soil quality guidelines, which are likely underestimating the adverse combined effects of these compounds.

Keywords Soil organism · Ecotoxicity · Combined toxicity · Pesticide

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Background, aim, and scope

The usage of pesticides has significant economic, environmental, and public health benefits, by which food production is increased and vector-borne diseases are reduced (Snelder et al. 2008; Dabrowski et al. 2014). Pesticides are either directly applied to soil to control soil-borne pests or deposited on soil as runoff from foliar applications (Piola et al. 2013). In some cases, the concentrations of their residues have been shown to be sufficiently high to affect many non-target species, including beneficial soil organism, such as earthworms (Frampton et al. 2006; Daam et al. 2011). Earthworms are commercially valuable soil invertebrates, representing 60–80 % of the total animal biomass in soil (Edwards and Bohlen 1992). They maintain structure, texture, and fertility of soil by increasing aeration and drainage through their burrowing, feeding, and casting activities (Sánchez-Hernández 2006). These organisms have been broadly used as model organisms in toxicity tests since they are often affected by pesticide application (Reinecke and Reinecke 2007; Calisi et al. 2011).

Although numerous ecotoxicological studies using earthworms have been carried out in recent years, most of them focused on effects of single pesticides (Hackenberger et al. 2008; Jin-Clark et al. 2008; Ellis et al. 2010; Choung et al.

2011; Liu et al. 2011; Bjergager et al. 2012; Wang et al. 2012b; Alves et al. 2013). However, pesticides are rarely found as single compounds in natural environments. In contrast, they are often found as mixtures (Thompson 1996). The combined toxic effects of multiple pesticides or pesticide mixtures have become an important safety concern in ecotoxicology because pesticide mixtures can have a greater negative impact than their individual constituents (Phyu et al. 2011; Bjergager et al. 2012). The results obtained from a toxicity test conducted with a single pesticide may underestimate the ecological risk of compound mixtures that are actually present in natural environment (Zhou et al. 2011). Nevertheless, data on mixture toxicity of pesticides are scarce for earthworms.

Toxicity quantification of pesticide mixtures in the environment is crucial when performing risk assessments and evaluating environmental quality (Anderson and Zhu 2004; Boillot and Perrodin 2008). Recent evidence suggested that the herbicide butachlor and three insecticides, phoxim, chlorpyrifos, and *lambda*-cyhalothrin, are often present in the same soil samples, where butachlor is used to control weeds and the abovementioned three insecticides are co-applied to control insect pests (Chen et al. 2014). Therefore, there is a growing concern about their interactive toxicity to soil organisms, including earthworms. In the present work, we aimed to examine the joint acute effects of butachlor and phoxim, chlorpyrifos, and *lambda*-cyhalothrin on the earthworm, *Eisenia fetida*. Our findings are hypothesized to enable a more realistic assessment of the potential effects of pesticide mixtures on earthworms.

Materials and methods

Test organisms

As one of the favorite worm species for composting and organic gardening, the earthworm *E. fetida* (Oligochaeta, Lumbricidae) is frequently used as a biological indicator to evaluate the effects of contaminants on soil biota. It is also the earthworm test species recommended by Organisation for Economic Co-operation and Development (OECD) (OECD 1984, 2004). Adult earthworms (weighing 350–500 mg) with well-developed clitella were purchased from the College of Animal Sciences, Zhejiang University, China. They were maintained at room temperature (20 ± 1 °C) in artificial soil according to OECD guidelines (OECD 1984) under laboratory conditions. The soil was composed of (dry weight) 10 % ground sphagnum peat (<0.5 mm), 20 % kaolinite clay (>50 % kaolinite), and 70 % fine sand (OECD 1984, 2004). The pH of the soil was adjusted to 6.0 ± 0.5 using a small amount of calcium carbonate. Soil was mixed with decayed leaves and decomposed pig manure. Soil water content was monitored every week, and a 35 % maximum water-holding capacity was maintained by the addition of distilled water as needed. Additional control tests were carried out using chloroacetamide as a toxic reference standard.

Test chemicals

Four pesticides were tested in this study, including one chloroacetanilide herbicide, butachlor, two organophosphate insecticides, phoxim and chlorpyrifos, and one pyrethroid insecticide, *lambda*-cyhalothrin (Table 1). The selected pesticides are widely used in agriculture worldwide. Butachlor [95 % technical product (TC)] was supplied by Hangzhou Qingfeng Chemical Industrial Group (Hangzhou, Zhejiang, China). Phoxim (98%TC) was provided by Lianyungang Liben Agrochemical Group (Liangyungang, 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.

Table 1 Selected physicochemical properties of butachlor, phoxim, chlorpyrifos, and *lambda*-cyhalothrin and their mode of action

Chemical	CAS number	Molecular weight (g mol^{-1})	Purity (%)	Water solubility (mg L^{-1}, 20 °C)	Log *Kow*
Butachlor	23184-66-9	311.85	95	20	4.50
Phoxim	14816-18-3	298.30	98	7.0	4.39
Chlorpyrifos	2921-88-2	350.59	96	2.0	4.70
Lambda-cyhalothrin	68085-85-8	449.85	97	0.005	7.0

Toxicity test methods

Filter paper contact test

Earthworms were exposed to pesticides using the filter paper contact test method as previously described (OECD 1984). 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 quantitative) 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, the worms' mortality was recorded. An earthworm was considered dead if it failed to respond to a gentle mechanical touch on the front end. Moreover, the mortality in controls should not exceed 10 % at the end of any test.

A preliminary test was conducted to determine the desired concentration range of the test chemicals, in which 0–100 % mortality of the earthworm was obtained. To establish the concentration-mortality relationship, earthworms were exposed to six concentrations increasing with a geometrical ratio of twofold and a control for each chemical. A total of 10 replications were performed for each concentration.

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

Artificial soil test

The artificial soil test was performed using OECD soil (OECD 1984) with two exposure periods: 7 and 14 days. In the toxicity test, the water content was adjusted to 35 % of the dry weight. 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 lux of constant light. Mortality was assessed after treatment for 7 and 14 days. Besides, the mortality in controls should not exceed 10 % at the end of any test.

The concentrations corresponding to 0–100 % mortality were determined using a range of concentrations, including 0, 0.1, 1.0, 10, 100, and 1000 mg kg^{-1} artificial soil. To obtain LC_{50}, 5–6 test concentrations in a geometric series and a control were used for each pesticide. Three jars, each containing 10 adult earthworms, were used for each concentration. The earthworms were pre-conditioned for 24 h under the above-mentioned conditions in the untreated artificial soil before the dose-response test.

Mixture toxicity test

Interaction of mixture has been previously classified as additive, synergistic, and antagonistic (Jin-Clark et al. 2008). Mixture experiments of the herbicide butachlor and three insecticides to *E. fetida* were conducted following a fixed equivalent concentration ratio design. For the equivalent dose mixture treatments, the initial concentration (1X) of each chemical tested was equivalent to 6.25 % of the LC_{50} of the most toxic pesticide 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 binary, ternary, and quaternary mixture components was kept constant (1:1, 1:1:1, or 1:1:1:1), whereas the total concentration of the mixture systematically varied.

Statistical analysis

The acute toxicity of pesticides to *E. fetida* was assessed by a probit analysis using a program developed by 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 (Prabhaker et al. 2011). The synergy of pesticide mixtures was determined on the basis of an additivity index and 95 % confidence interval from LC_{50} data according to the method of Marking (1985). This method defines an additivity index for the combined effect of a mixture of chemicals. The biological activity (S) of test compounds A, B and C was determined by the equation as follows:

$$S = (A\mathrm{m}/A\mathrm{i}) + (B\mathrm{m}/B\mathrm{i}) + (C\mathrm{m}/C\mathrm{i}),$$

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;

and S is the sum of the biological activity. The calculated S values were then substituted into appropriate formulas to determine the additivity index (AI). The AI was calculated using the following equations:

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

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 additivity index value, the greater the chemical synergy.

Results

Tables 2 and 3 summarize the joint acute toxicity of the herbicide butachlor and three insecticides to the earthworm, *E. fetida*. A concentration-dependent response was observed from all assessed compounds by lethal toxicity tests, and the survival rate was negatively correlated with the pesticide concentration (Figs. 1 and 2).

Contact toxicity

Individual pesticide toxicity

Table 2 lists the results of filter paper contact test. The results demonstrated that different pesticides widely varied in their contact toxicities to *E. fetida*. Among the four selected

Table 2 The joint acute toxicity with contact filter paper test of butachlor, phoxim, chlorpyrifos, and *lambda*-cyhalothrin to *Eisenia fetida*; additivity index as an indicator of chemical synergy

Chemical	Time (h)	LC_{50} (95 % CI) μg a.i. cm^{-2}	Additivity index [a] (95 % CI)
Individual pesticides			
Butachlor	24	43.11 (32.04~74.61)	
	48	20.88 (15.85~27.51)	
Phoxim	24	20.96 (11.35~153.68)	
	48	3.67 (2.71~4.75)	
Chlorpyrifos	24	178.1 (92.64~301.92)	
	48	32.01 (23.82~42.03)	
Lambda-cyhalothrin	24	31.18 (16.22~52.85)	
	48	4.89 (3.52~6.38)	
Binary mixtures			
Butachlor-Phoxim	24	49.41 (28.25~70.57)	−2.50 (−19.36~0.78)
	48	6.92 (4.99~14.82)	−1.22 (−5.41~−0.23)
Butachlor-Chlorpyrifos	24	59.97 (36.70~152.20)	−0.73 (−5.39~0.63)
	48	6.68 (4.83~16.04)	0.89 (−0.69~2.44)
Butachlor-*Lambda*-cyhalothrin	24	9.01 (5.93~24.53)	1.01 (−1.28~4.22)
	48	4.58 (3.24~7.48)	−0.16 (−1.59~0.60)
Ternary mixtures			
Butachlor-Phoxim-Chlorpyrifos	24	6.79 (4.67~22.44)	0.92 (-1.92~8.22)
	48	1.82 (1.09~2.55)	0.55 (−0.21~2.40)
Butachlor-Phoxim-*Lambda*-cyhalothrin	24	1.96 (1.19~2.77)	3.95 (0.99~20.58) [b]
	48	0.64 (0.03~1.19)	1.99 (0.17~71.16) [b]
Butachlor-Chlorpyrifos-*Lambda*-cyhalothrin	24	3.22 (2.35~4.73)	4.10 (1.04~10.92) [b]
	48	1.61 (0.87~2.22)	1.19 (0.16~4.27) [b]
Quaternary mixture			
Butachlor-Phoxim-Chlorpyrifos- *Lambda*-cyhalothrin	24	0.86 (0.64~1.30)	9.60 (3.02~35.98) [b]
	48	0.49 (0.30~0.67)	2.66 (0.96~6.68) [b]

[a] An additivity indices greater than zero indicates greater than additive toxicity

[b] Significant chemical synergy interactions between pesticides

Table 3 The joint acute toxicity with artificial soil test of butachlor, phoxim, chlorpyrifos, and *lambda*-cyhalothrin to *Eisenia fetida*; additivity index as an indicator of chemical synergy

Chemical	Time (d)	LC_{50} (95 % CI) mg a.i. kg^{-1}	Additivity index[a] (95 % CI)
Individual pesticides			
Butachlor	7	1709.7 (1282.4~3016.4)	
	14	1197.8 (993.8~1614.3)	
Phoxim	7	1083.2 (960.4~1305.2)	
	14	901.5 (821.3~1016.7)	
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)	
Binary mixtures			
Butachlor-Phoxim	7	50.78 (42.62~62.32)	11.90 (7.81~20.38) [b]
	14	37.50 (30.26~45.21)	12.71 (8.95~19.62) [b]
Butachlor-Chlorpyrifos	7	281.4 (238.1~333.2)	0.21 (-0.14~0.52)
	14	170.6 (125.6~206.8)	0.71 (0.26~1.75) [b]
Butachlor-*Lambda*-cyhalothrin	7	444.7 (375.9~521.2)	0.21 (-0.25~1.29)
	14	244.8 (179.5~296.7)	0.56 (0.085~1.77) [b]
Ternary mixtures			
Butachlor-Phoxim-Chlorpyrifos	7	33.18 (26.28~39.72)	6.75 (4.66~11.31) [b]
	14	23.45 (15.97~29.61)	8.39 (5.68~15.16) [b]
Butachlor-Phoxim-*Lambda*-cyhalothrin	7	50.47 (41.86~62.54)	6.14 (3.66~11.42) [b]
	14	32.88 (26.29~39.16)	7.16 (4.91~11.70) [b]
Butachlor-Chlorpyrifos-*Lambda*-cyhalothrin	7	23.46 (19.42~28.77)	9.07 (5.92~15.34) [b]
	14	15.43 (11.91~18.68)	11.42 (8.02~18.61) [b]
Quaternary mixture			
Butachlor-Phoxim-Chlorpyrifos-*Lambda*-cyhalothrin	7	22.83 (18.67~28.16)	7.50 (4.86~12.68) [b]
	14	14.22 (10.64~17.39)	10.11 (7.04~16.85) [b]

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

[b] Significant chemical synergy interactions between pesticides

pesticides, 24h-LC_{50} values ranged from 20.88 (15.85~27.51) to 178.11 (92.64~301.92) μg a.i. cm^{-2}, and 48h-LC_{50} values ranged from 3.67 (2.71~4.75) to 32.01 (23.82~42.03) μg a.i. cm^{-2}. After 24 h, phoxim exhibited the highest toxicity with an LC_{50} value of 20.96 (11.35~153.68) μg a.i. cm^{-2}. Meanwhile, *lambda*-cyhalothrin and butachlor showed relatively less toxicity with LC_{50} values of 31.18 (16.22~52.85) and 43.11 (32.04~74.61) μg a.i. cm^{-2}, respectively. In contrast, chlorpyrifos displayed the lowest toxicity against *E. fetida* with an LC_{50} value of 178.11 (92.64~301.92) μg a.i. cm^{-2}. After 48 h, phoxim and *lambda*-cyhalothrin showed the highest intrinsic toxicity with LC_{50} values of 3.67 (2.71~4.75) and 4.89 (3.52~6.38) μg a.i. cm^{-2}, respectively. Butachlor demonstrated relatively less toxicity with an LC_{50} value of 20.88 (15.85~27.51) μg a.i. cm^{-2}, and chlorpyrifos exhibited the lowest toxicity with an LC_{50} value of 32.01 (23.82~42.03) μg a.i. cm^{-2} to the animals. The toxicity for the four tested pesticides could be ranked in a descending order as follows: phoxim, *lambda*-cyhalothrin > butachlor and chlorpyrifos. The toxicity of phoxim was 8.72-fold higher compared with chlorpyrifos after exposure for 48 h.

According to the classification of Robert and Dorough (1984), chlorpyrifos was categorized as slightly toxic, whereas butachlor, phoxim, and *lambda*-cyhalothrin were moderately toxic to worms after 24 h based on the resulting LC_{50} values for *E. fetida* exposed to impregnated papers. However, phoxim and *lambda*-cyhalothrin were classified as highly toxic, whereas butachlor and chlorpyrifos were classified as moderately toxic to organisms after exposure for 48 h.

Binary mixture toxicity

The LC_{50} values of different binary mixtures after exposure for 24 and 48 h were determined to understand the interaction of the herbicide butachlor and each insecticide in the joint acute toxicity toward *E. fetida*. For

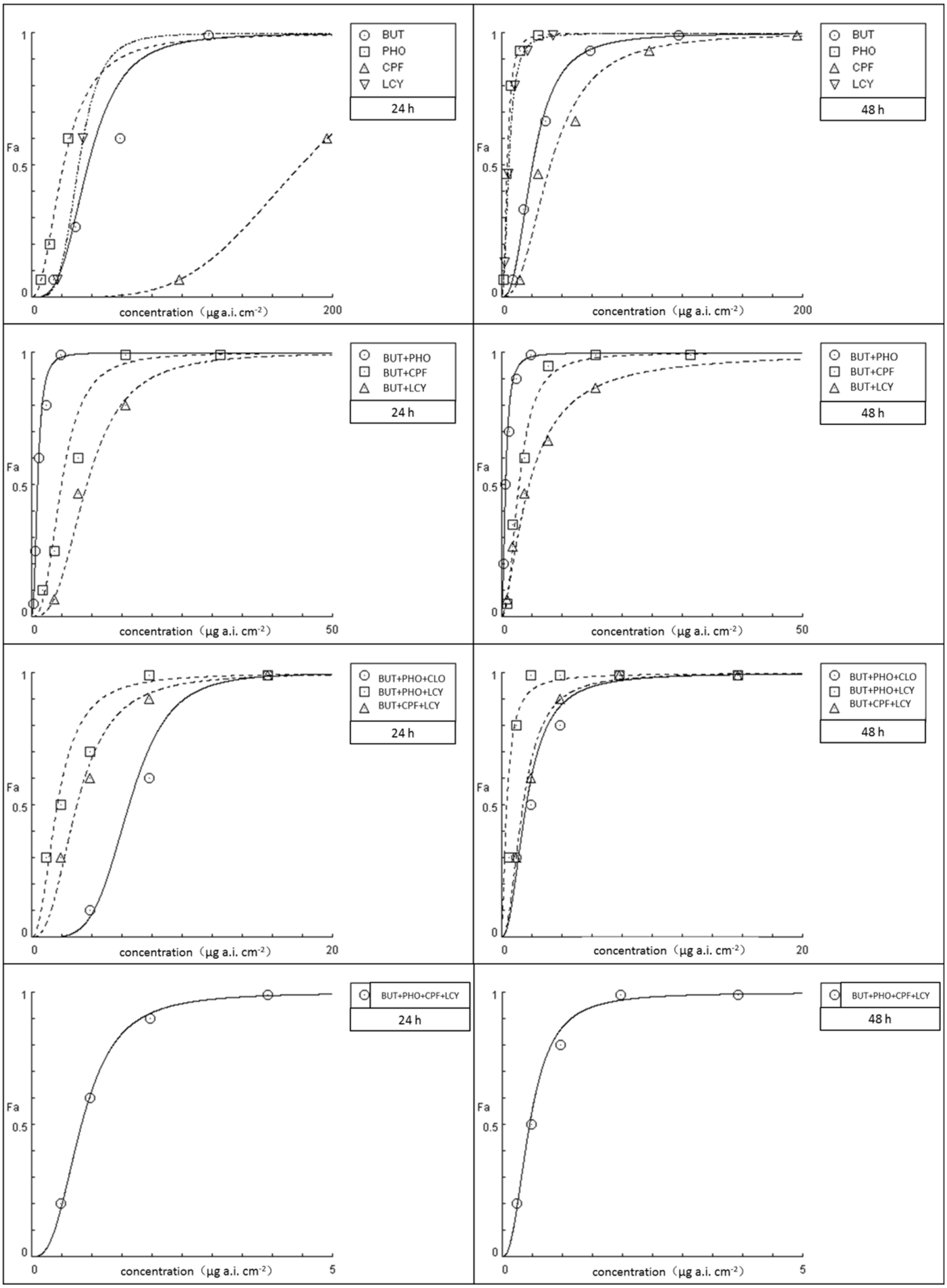

Fig. 1 Dose-response curves of butachlor (*BUT*), phoxim (*PHO*), chlorpyrifos (*CPF*), and *lambda*-cyhalothrin (*LCY*) and their mixtures for the mortality rate of *Eisenia fetida* in filter paper test

all of the tested binary mixtures (butachlor-phoxim, butachlor-chlorpyrifos, and butachlor-*lambda*-cyhalothrin), the calculated additivity indices ranged from −2.50 (−19.36~0.78) to 1.01 (−1.28~4.22) after 24 h and from −1.22 (−5.41~−0.23) to 0.89 (−0.69~2.44) after 48 h, respectively, suggesting

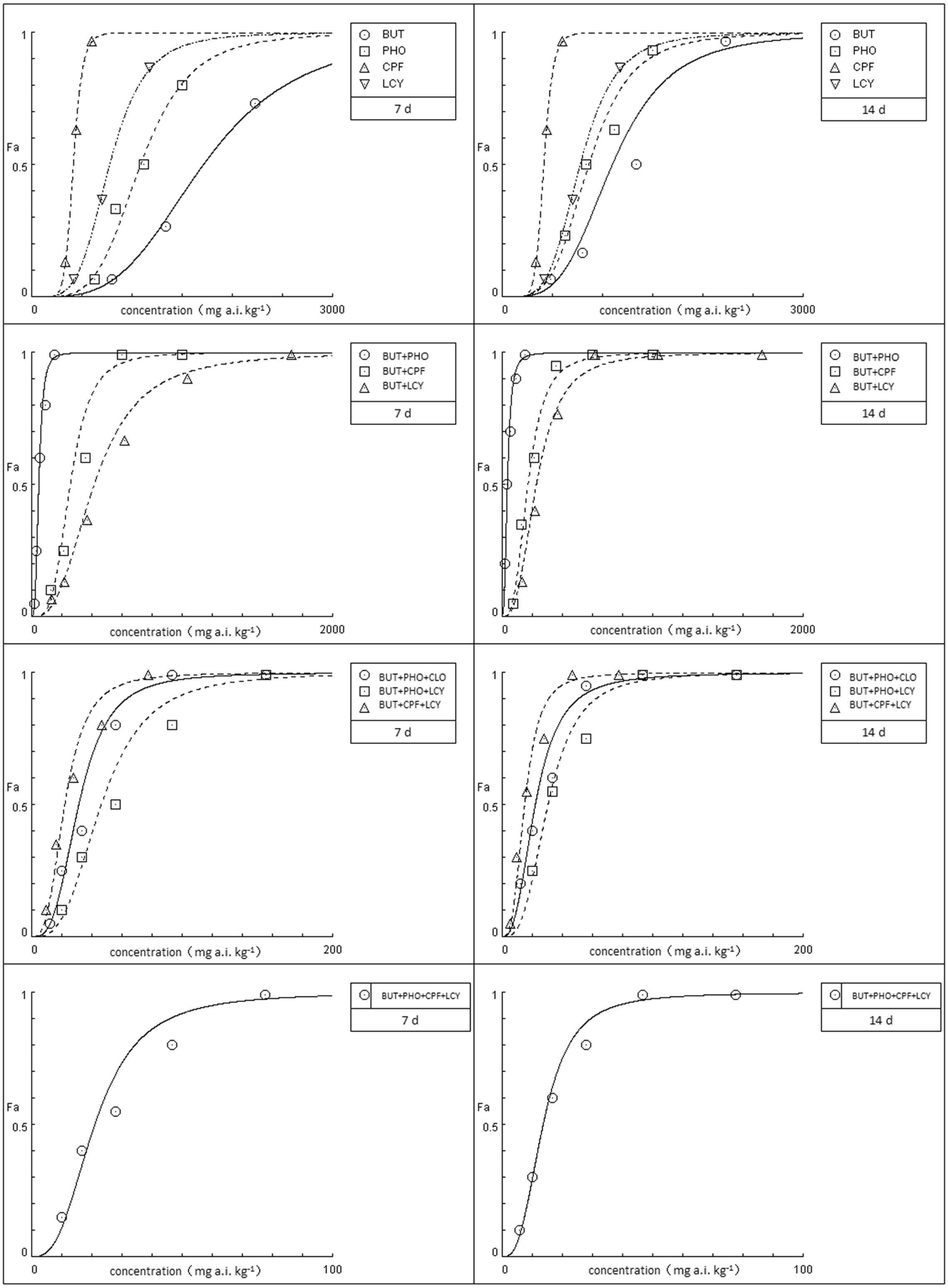

Fig. 2 Dose-response curves of butachlor (*BUT*), phoxim (*PHO*), chlorpyrifos (*CPF*), and *lambda*-cyhalothrin (*LCY*) and their mixtures for the mortality rate of *Eisenia fetida* in artificial soil test

antagonistic response to greater than additive toxicity. However, additivity indices were increased when the exposure period was increased for the two binary mixtures of butachlor-phoxim and butachlor-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 two ternary mixtures. The additivity index of butachlor-phoxim-*lambda*-cyhalothrin mixture after exposure for 24 and 48 h was 3.95 (0.99~20.58) and 4.10 (1.04~10.92), and that of the butachlor-chlorpyrifos-lambda-cyhalothrin mixture was 1.19 (0.16~4.27) and 1.99 (0.17~71.16), respectively. In contrast, the interaction was greater than additive toxicity for the ternary mixture of butachlor-phoxim-chlorpyrifos, with additivity indexes of 0.92 (−1.92~8.22) and 0.55 (−0.21~2.40) after exposure for 24 and 48 h, respectively. The quaternary mixture (butachlor-phoxim-chlorpyrifos-*lambda*-cyhalothrin) also exhibited a significant synergistic effect, with additivity indices of 9.60 (3.02~35.98) and 2.66 (0.96~6.68) after exposure for 24 and 48 h, respectively.

Soil toxicity

Individual pesticide toxicity

Table 3 shows the acute toxicities to *E. fetida* of the four tested pesticides using artificial soil test. Similar to the results of contact toxicity test, each pesticide exhibited different levels of toxicity to animals. After 7 days, chlorpyrifos showed the highest toxicity with an LC_{50} value of 421.3 (380.7~501.9) mg a.i. kg^{-1}. Meanwhile, *lambda*-cyhalothrin and phoxim demonstrated relatively less toxicity with LC_{50} values of 784.5 (619.7~1209.9) and 1083.2 (960.4~1305.2) mg a.i. kg^{-1}, respectively. In contrast, butachlor exhibited the lowest toxicity to worms with an LC_{50} value of 1709.7 (1282.4~3016.4) mg a.i. kg^{-1}. The average acute toxicity for the four tested pesticides could be ranked in a descending order as follows: chlorpyrifos > *lambda*-cyhalothrin > phoxim ≥ butachlor. After 14 days, chlorpyrifos still showed the highest toxicity with an LC_{50} value of 384.9 (353.5~440.3) mg a.i. kg^{-1}, followed by *lambda*-cyhalothrin with an LC_{50} value of 560.3 (475.9~718.5) mg a.i. kg^{-1}. Similar toxicity was detected from phoxim and butachlor, which was the lowest toxicity against *E. fetida* with LC_{50} values of 1083.2 (960.4~1305.2) and 1709.7 (1282.4~3016.4) mg a.i. kg^{-1}, respectively. Based on their LC_{50} values, the toxicity for the four tested pesticides could be ranked in a descending order as follows: chlorpyrifos > *lambda*-cyhalothrin > phoxim, butachlor. The toxicity of chlorpyrifos was 4.06-fold higher compared with butachlor after exposure for 14 days.

Binary mixture toxicity

The additivity index of butachlor-phoxim mixture after exposure for 7 and 14 days was 11.90 (7.81~20.38) and 12.71 (8.95~19.62), respectively, suggesting a significant synergistic effect. For the two binary mixtures of butachlor-chlorpyrifos and butachlor-*lambda*-cyhalothrin, their additivity indices ranged from 0.21 (−0.25~1.29) to 0.71 (0.26~1.75) after exposure for 7 and 14 days, suggesting greater than additive toxicity or synergistic effect. However, additivity indices were increased when the exposure time was increased for all of the tested binary mixtures.

Ternary and quaternary mixture toxicities

For all of the tested ternary mixtures (butachlor-phoxim-*lambda*-cyhalothrin, butachlor-phoxim-chlorpyrifos and butachlor-chlorpyrifos-*lambda*-cyhalothrin), their additivity indices ranged from 6.14 (3.66~11.42) to 9.07 (5.92~15.34) after exposure for 7 days and from 7.16 (4.91~11.70) to 11.42 (8.02~18.61) after exposure for 14 days, respectively, suggesting significant synergistic effects. Similar to the results of contact toxicity test, the quaternary mixture (butachlor-phoxim-chlorpyrifos-*lambda*-cyhalothrin) also exhibited a significant synergistic effect, with additivity indices of 7.50 (4.86~12.68) and 10.11 (7.04~16.85) after exposure for 7 and 14 days, respectively. Moreover, additivity indices were increased when the exposure period was increased for all of the tested ternary and quaternary mixtures.

Discussions

There were no acute toxicity data in the literature for butachlor, phoxim, and *lambda*-cyhalothrin with which to compare with our study. The pesticides are absorbed mainly by the skin of earthworm in the filter paper test method (Wang et al. 2012b). In contrast, the pesticides are absorbed mainly by the gut in the soil toxicity method. Chlorpyrifos had a strong toxic effect on the earthworm because it mainly exerted its toxic action by way of the gut absorption. The 14d-LC_{50} of chlorpyrifos estimated in the present study (384.9 mg a.i. kg^{-1}) is within the range of values of reported previously between 129 and 1174 mg a.i. kg^{-1} (Ma and Bodt 1993). Alshawish et al. (2004) tested the effect of chlorpyrifos, cypermethrin, dicofol, mancozeb, and haloxyfopetotyl on their chronic toxicity on the earthworm, *Aporrectodea caliginosa* in laboratory cultures. They concluded that chlorpyrifos was the most toxic pesticide, which can produce significant impacts on earthworm fecundity at 50 mg/kg dry soil, while cypermethrin at the same dosage was the least toxic, which produced 20 % reductions in cocoon viability and no effects on hatchlings development.

In the current study, we reported that herbicide-insecticide mixtures possessed strong synergism compared with their individual constituents. A very great synergism response was found for most of the ternary and quaternary mixtures with both bioassay systems. A possible underlying mechanism could be that when organophosphate-pyrethroid mixtures coexisted, organophosphate insecticides might bind to monooxygenases, first resulting in the activation of molecules and then preventing the binding and subsequent degradation of pyrethroid insecticide by monooxygenase enzymes (Kulkarni and Hodgson 1980; Martin et al. 2003). When binding to the organophosphate insecticide, these enzymes could also lead to the formation non-toxic metabolites through the process of hydroxylation of either oxon or thioate forms, ultimately causing the degradation via oxidative ester cleavage (Gunning et al. 1999). In this way, the binding of monooxygenase enzymes with organophosphate insecticides would prevent or delay the degradation and enhance the toxicity of pyrethroid insecticide via competitive substrate inhibition (Espinosa et al. 2005). Previously, it has been assumed that organophosphates, when used in a combination with pyrethroids, inhibit the enzymes (monooxygenases and/or esterases) responsible for metabolic detoxification in different organisms (Maklakov et al. 2001; Bielza et al. 2007).

Our results demonstrated that the mixture of herbicide butachlor and insecticides phoxim, chlorpyrifos, and *lambda*-cyhalothrin exerted significant synergistic toxicity, and therefore, it might pose a greater than expected threat to terrestrial organism (Choung et al. 2011). The mechanisms of chemical synergy for mixtures remain poorly understood. The most popular theories include an increase in uptake rates of chemical into the organisms through biological membranes, formation of toxic metabolites, reduction of excretion, alteration of distribution, and inhibition of detoxification systems (Anderson and Zhu 2004; Jin-Clark et al. 2008; Pérez et al. 2013). In the present study, we showed that synergistic interactions among pesticides after 14 days were greater than those after 7 days in the artificial soil test. The pesticide synergy was positively correlated with the exposure time, suggesting that earthworms chronically exposed to pesticides in natural environments maybe subjected to greater chemical synergy than that found in this study.

Most tests of pesticide toxicity to terrestrial ecosystems are based on the exposure of organisms to a single pesticide (Hackenberger et al. 2008; Saxena et al. 2014). However, earthworms are continually exposed to a complex mixture of pesticides, and which can greatly intensify the toxic effects of individual substances (Wang et al. 2012a). Our acute toxicity tests were conducted under laboratory conditions, and the pesticide concentrations employed in our study were higher than those typically found in terrestrial environments (Ferrari et al. 2003; Scholtz and Bidleman 2007). Therefore, we planned to investigate the effects of these pesticides using a microcosmic system in the future study, which can simulate conditions similar to the natural environment.

Our investigation suggested that prediction on the individual pesticide characteristics may not provide information on mixture outcomes, although these data are limited in nature. As natural soil often contains complex mixtures of pesticides, evaluations based on toxicity data obtained from single pesticide assays are not enough to accurately assess the ecological risk of toxicants in a practical environment (Zhou et al. 2011; Lee et al. 2015). There are few reports on joint toxicity of herbicide and insecticide to terrestrial organisms (Choung et al. 2011). Normally, two basic hypotheses, i.e., concentration addition and independent action, are used to explore the mechanism of joint action of toxicants to organisms (Phyu et al. 2011). Further investigation is warranted for pesticide mixtures. The use of a rapid, simple, and cost-effective toxicity test will have great benefit in providing data on pesticide interactions and applications in monitoring ecosystems (Loureiro et al. 2009). This study also demonstrated the value of earthworms in determining joint toxicity. Additional research is needed using this and similar tests (e.g., microcosmic system) on actual and simulated environments under various conditions to better understand the ecotoxicology of pesticide mixtures.

Conclusions and perspectives

Chlorpyrifos was the most toxic among the four pesticides tested in the solid toxicity bioassay, but it was the least toxic in the filter paper bioassay. Most of the ternary and quaternary mixtures exhibited significant synergistic effects on the worms. The binary mixture of butachlor-phoxim indicated a significant synergistic effect on the worms with soil toxicity. Consequently, using toxicity data obtained from single pesticide in evaluating eco-toxicological risk may underestimate the effects of pesticide mixtures on soil invertebrate populations. Given that several classes of pesticides may coexist in soil ecosystem, it is crucial to examine the pesticide interactions. Therefore, more attention should be paid to mixture effects when defining standards for soil environment quality and risk-assessment procedures. Taken together, 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.

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References

Alshawish SA, Mohamed AI, Nair G (2004) Prolonged toxicity of sublethal dosages of chemical pesticides on the body mass and cocoons of *Aporrectodea caliginosa* (Savigny 1826) (Oligochaeta: Lumbricidae) inhabiting Benghazi, Libya. Proc Natl Acad Sci India Section B (Biol Sci) 74(Part 2):123–133

Alves PRL, Cardoso EJBN, Martines AM, Sousa JP, Pasini A (2013) Earthworm ecotoxicological assessments of pesticides used to treat seeds under tropical conditions. Chemosphere 90:2674–2682

Anderson TD, Zhu KY (2004) Synergistic and antagonistic effects of atrazine on the toxicity of organophosphorodithioate and organophosphorothioate insecticides to *Chironomus tentans* (Diptera: Chironomidae). Pestic Biochem Physiol 80:54–64

Bielza P, Espinosa PJ, Quinto V, Abellán J, Contreras J (2007) Synergism studies with binary mixtures of pyrethroid, carbamate and organophosphate insecticides on *Frankliniella occidentalis* (Pergande). Pest Manag Sci 63:84–89

Bjergager MB, Hanson ML, Solomon KR, Cedergreen N (2012) Synergy between prochloraz and esfenvalerate in *Daphnia magna* from acute and subchronic exposures in the laboratory and microcosms. Aquat Toxicol 110–111:17–24

Boillot C, Perrodin Y (2008) Joint-action ecotoxicity of binary mixtures of glutaraldehyde and surfactants used in hospitals: use of the toxicity index model and isoblogram representation. Ecotoxicol Environ Saf 71:252–259

Calisi A, Lionetto MG, Schettino T (2011) Biomarker response in the earthworm Lumbricus terrestris exposed to chemical pollutants. Sci Total Environ 409:4456–4464

Chen C, Wang YH, Zhao XP, Qian YZ, Wang Q (2014) Combined toxicity of butachlor, atrazine and λ-cyhalothrin on the earthworm *Eisenia fetida* by combination index (CI)-isobologram method. Chemosphere 112:393–401

Chi H (1997) Computer program for the probit analysis. National Chung Hsing University, Taichung

Choung CB, Hyne RV, Stevens MM, Hose GC (2011) Toxicity of the insecticide terbufos, its oxidation metabolites, and the herbicide atrazine in binary mixtures to *Ceriodaphnia* cf *dubia*. Arch Environ Contam Toxicol 60:417–425

Daam MA, Leitão S, Cerejeira MJ, Paulo Sousa J (2011) Comparing the sensitivity of soil invertebrates to pesticides with that of *Eisenia fetida*. Chemosphere 85:1040–1047

Dabrowski JM, Shadung JM, Wepener V (2014) Prioritizing agricultural pesticides used in South Africa based on their environmental mobility and potential human health effects. Environ Int 62:31–40

Edwards CA, Bohlen PJ (1992) The effects of toxic chemicals on earthworms. Rev Environ Contam Toxixol 125:23–99

Ellis SR, Hodson ME, Wege P (2010) The soil-dwelling earthworm *Allolobophora chlorotica* modifies its burrowing behaviour in response to carbendazim applications. Ecotoxicol Environ Saf 73:1424–1428

Espinosa PJ, Contreras J, Quinto V, Grávalos C, Fernández E, Bielza P (2005) Metabolic mechanisms of insecticide resistance in the western flower thrips, *Frankliniella occidentalis* (Pergande). Pest Manag Sci 61:1009–1015

Ferrari F, Trevisan M, Capri E (2003) Predicting and measuring environmental concentration of pesticides in air after soil application. J Environ Qual 32:1623–1633

Frampton GK, Jansch S, Scott-Fordsmand JJ, Römbke J, Van den Brink PJ (2006) Effects of pesticides on soil invertebrates in laboratory studies: a review and analysis using species sensitivity distributions. Environ Toxicol Chem 25:2480–2489

Gunning RV, Moores GD, Devonshire AL (1999) Esterase inhibitors synergise the toxicity of pyrethroids in Australian *Helicoverpa armigera* (Lepidoptera: Noctuidae). Pestic Biochem Physiol 63: 52–62

Hackenberger BK, Jarić-Perkušić D, Stepić S (2008) Effect of temephos on cholinesterase activity in the earthworm *Eisenia fetida* (Oligochaeta, Lumbricidae). Ecotoxicol Environ Saf 71:583–589

Jin-Clark Y, Anderson TD, Zhu KY (2008) Effect of alachlor and metolachlor on toxicity of chlorpyrifos and major detoxification enzymes in the aquatic midge, *Chironomus tentans* (Diptera: Chironomidae). Arch Environ Contam Toxicol 54:645–652

Kulkarni AP, Hodgson E (1980) Metabolism of insecticides by mixed function oxidase systems. Pharmacol Ther 8:379–475

Lee WM, Yoon Y, An YJ (2015) Combined toxicities of methyl tert-butyl ether and its metabolite tert-butyl alcohol on earthworms via different exposure routes. Chemosphere 128:191–198

Liu WY, Wang CY, Wang TS, Fellers GM, Lai BC, Kam YC (2011) Impacts of the herbicide butachlor on the larvae of a paddy field breeding frog (*Fejervarya limnocharis*) in subtropical Taiwan. Ecotoxicology 20:377–384

Loureiro S, Jorim M, Campos B, Rodrigues SMG, Soares AMVM (2009) Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environ Pollut 157:625–636

Ma WC, Bodt J (1993) Differences in toxicity of the insecticide chlorpyrifos to six species of earthworms (Oligochaeta, Lumbricidae) in standardized soil tests. Bull Environ Contam Toxicol 50:864–870

Maklakov A, Ishaaya I, Freidberg A, Yawetz A, Horowitz AR, Yarom I (2001) Toxicological studies of organophosphate and pyrethroid insecticides for controlling the fruit fly *Dacus ciliatus* (Diptera: Tephritidae). J Econ Entomol 94:1059–1066

Marking LL (1985) Toxicity of chemical mixtures. In: Petroceli S, Rand G (eds) Fundamentals of aquatic toxicology. Hemisphere Publishing Corporation, Washington DC, pp 164–176

Martin T, Ochou OG, Vaissayre M, Fournier D (2003) Organophosphorus insecticides synergize pyrethroids in the resistant strain of cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) from West Africa. J Econ Entomol 96:468–474

OECD (1984) OECD Guideline for Testing of Chemicals, Earthworm Acute Toxicity. OECD, Paris, France. No. 207

OECD (2004) OECD Guideline for Testing of Chemicals, Earthworm Reproduction Test (*Eisenia fetida* / *Eisenia andrei*). OECD, Paris, France. No. 222

Pérez J, Monteiro MS, Quintaneiro C, Soares AM, Loureiro S (2013) Characterization of cholinesterases in *Chironomus riparius* and the effects of three herbicides on chlorpyrifos toxicity. Aquat Toxicol 144–145:296–302

Phyu YL, Palmer CG, Warne MS, Hose GC, Chapman JC, Lim RP (2011) A comparison of mixture toxicity assessment: examining the chronic toxicity of atrazine, permethrin and chlorothalonil in mixtures to *Ceriodaphnia* cf. *dubia*. Chemosphere 85:1568–1573

Piola L, Fuchs J, Oneto ML, Basack S, Kesten E, Casabé N (2013) Comparative toxicity of two glyphosate-based formulations to *Eisenia andrei* under laboratory conditions. Chemosphere 91:545–551

Prabhaker N, Castle SJ, Naranjo SE, Toscano NC, Morse JG (2011) Compatibility of two systemic neonicotinoids, imidacloprid and thiamethoxam, with various natural enemies of agricultural pests. J Econ Entomol 104:773–781

Reinecke SA, Reinecke AJ (2007) The impact of organophosphate pesticides in orchards on earthworms in the Western Cape, South Africa. Ecotoxicol Environ Saf 66:244–251

Roberts BL, Dorough HW (1984) Relative toxicities of chemicals to the earthworm *Eisenia foetida*. Environ Toxicol Chem 3:67–78

Sánchez-Hernández JC (2006) Earthworm biomarkers in ecological risk assessment. Rev Environ Contam Toxicol 188:85–126

Saxena PN, Gupta SK, Murthy RC (2014) Comparative toxicity of carbaryl, carbofuran, cypermethrin and fenvalerate in *Metaphire posthuma* and *Eisenia fetida*—a possible mechanism. Ecotoxicol Environ Saf 100:218–225

Scholtz MT, Bidleman TF (2007) Modelling of the long-term fate of pesticide residues in agricultural soils and their surface exchange with the atmosphere: part II. Projected long-term fate of pesticide residues. Sci Total Environ 377:61–80

Snelder DJ, Masipiqueña MD, de Snoo GR (2008) Risk assessment of pesticide usage by smallholder farmers in the Cagayan Valley (Philippines). Crop Prot 27:747–762

Thompson HM (1996) Interactions between pesticides: a review of reported effects and their implications for wildlife risk assessment. Ecotoxicology 5:59–81

Wang JH, Zhu LS, Meng Y, Wang J, Xie H, Zhang QM (2012a) The combined stress effects of atrazine and cadmium on the earthworm *Eisenia fetida*. Environ Toxicol Chem 31:2035–2040

Wang Y, Wu S, Chen L, Wu C, Yu R, Wang Q, Zhao X (2012b) Toxicity assessment of 45 pesticides to the epigeic earthworm *Eisenia fetida*. Chemosphere 88:484–491

Zhou SP, Duan CQ, Michelle WHG, Yang FZ, Wang XH (2011) Individual and combined toxic effects of cypermethrin and chlorpyrifos on earthworm. J Environ Sci 23:676–680