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RESEARCH ARTICLE

Joint toxicity of chlorpyrifos, atrazine, and cadmium at lethal concentrations to the earthworm *Eisenia fetida*

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Abstract Contaminants in the environment often occur as complex mixtures, and their combined effect may exhibit toxicity to organisms. Risk assessments based on individual components tend to underestimate the effects associated with toxic action of mixtures. Toxicity studies on chemical mixtures are urgently required to assess their potential combined toxicities. The combination index (CI)-isobologram method was used to study chemical interactions to determine the nature of toxicological interactions of two pesticides chlorpyrifos and atrazine and a heavy metal cadmium toward earthworm *Eisenia fetida* by artificial soil and filter paper acute toxicity tests. The results showed that the binary mixture of chlorpyrifos and atrazine was antagonistic toward *E. fetida* at all f_a levels in an artificial soil test. The combination of atrazine and Cd exhibited a slight degree of synergism throughout the exposure range, while chlorpyrifos plus Cd combination led to dual antagonistic/synergistic behavior. The nature of binary combinations in filter paper displayed opposite interaction to that in the artificial soil test, and the toxicity of ternary mixtures was not significantly synergistic than their binaries. The combination index (CI)-isobologram equation method could determine the interaction types for a series of effect levels of three chemicals in binary and ternary combinations in two types of acute earthworm tests. However, the nature of these interactions was not uniform along the f_a level range in any of the two tests. Bioavailability, the nature of toxicological interaction, and the test organism need to be considered for understanding exposures and chemical measures. The synergistic effect for the particular binary combination suggests that a potential risk associated with the co-occurrence of these pollutants may still exist, which may have implications in risk assessment for the terrestrial environment. The combined effects between different contaminants might be influenced by the category of chemical, as well as the bioassay procedures. More studies of combined toxicities among these contaminants in the terrestrial environment should be conducted to identify the mixtures exhibiting synergistic pattern of interactions.

Keywords Insecticide · Herbicide · Heavy metal · *Eisenia fetida* · Acute mixture toxicity · Combination index

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Background, aim, and scope

Traditional effect and risk assessment have been routinely focused on exposures to single chemicals and additive behaviors in agricultural lands (Barata et al. 2006). However, in real-life circumstances, contaminants do not appear singly and usually occur as complex mixtures, and their combined effect may exhibit toxicity to organisms (Altenburger et al. 2000). Risk assessments based on individual components tend to underestimate the effects associated with toxic action of mixtures. Therefore, toxicity studies on chemical mixtures are urgently required to assess their potential combined (additive, synergistic, or antagonistic) toxicities in agricultural soils (Schnug et al. 2014). In the last few years, there are an increasing number of studies dealing with mixtures of contaminants in environment (Jin-Clark et al. 2002; Munkegaard et al. 2008; Gomez-Eyles et al. 2009; Rodea-Palomares et al. 2010; Bjergager et al. 2012; Harabawy and Ibrahim 2014).

Pesticides and heavy metals are two types of contaminants that are commonly present in the soil environment, and pollution due to these contaminants has attracted much attention recently (Wang et al. 2012a, b). Mixtures of pesticides and the additional input of industrial chemicals (e.g., heavy metals) in agricultural fields are commonplace. Insecticides and herbicides are often coapplied together as a combined chemical to control insect pests and weeds (Choung et al. 2013). Chlorpyrifos is a typical organophosphorus insecticide, known as acetylcholinesterase (AChE) inhibitors. It can disrupt normal nervous system function due to an excessive accumulation of acetylcholine in the synapse (Taylor and Brown 1999). Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a major herbicide in the s-triazine family that has been widely used on many crops (Boundy-Mills et al. 1997; Mahía et al. 2008). It is considered to be an endocrine-disrupting chemical in amphibians and may disrupt the ecological balance (Dalton 2002; Song et al. 2009). Cadmium (Cd) is carcinogenic to both humans and wildlife (IARC 1994; Waisberg et al. 2003). It can affect demography and reproduction to earthworms and be accumulated in granules in the chloragogenous tissue surrounding the earthworm digestive tract (Fourie et al. 2007).

Studies on mixture toxicity mostly focused on aquatic species and fewer on terrestrial invertebrates in soils (Santos et al. 2010; Schnug et al. 2014). Therefore, a terrestrial species, the earthworm *Eisenia fetida*, was chosen to study the nature of combined toxicities in the present study. It is an important component of terrestrial ecosystem, which acts as decomposers in improving soil structure (Fourie et al. 2007). They are easy to handle and suitable to culture in the laboratory and are considered as suitable test organisms for ecological risk assessment in terrestrial ecosystems (OECD 1984). The main objective of the present study was to evaluate the acute toxicity of the individual and combined effects of chlorpyrifos, atrazine, and $CdCl_2 \cdot 2.5\ H_2O$ on *E. fetida* in artificial soil and filter paper tests. Mortality rate was chosen as the end point, which is the most frequent acute toxicity test used to evaluate chemical effects on organisms in laboratories (Wang et al. 2012a, b). In order to identify and quantify the nature of the ternary interactions among the chemicals, we tested ternary mixtures of the chemicals by the method of the combination index (CI) equation which has recently been used to study pollutant interactions (Rodea-Palomares et al. 2010, 2012; Rosal et al. 2010a; Boltes et al. 2012; González-Pleiter et al. 2013).

Materials and methods

Test organisms

E. fetida is an invertebrate currently used for ecotoxicological assessment of substances in soil and is the test species recommended by Organization for Economic Co-operation and Development (OECD) and International Standardization Organization (ISO) (OECD 1984, 2004; ISO 1993). Adult earthworms (weighing between 350 and 500 mg) with well-developed clitella were purchased from the Animal Sciences College, Zhejiang University, China, and cultured in the laboratory in artificial soil according to OECD guidelines (OECD 1984, 2004). Soils were mixed with decayed leaves and decomposed pig manure and kept at room temperature (20± 1 °C). The water content of the soil was measured each week, and moisture was adjusted to 35 % of the maximum water-holding capacity by adding distilled water.

Test chemicals

Chlorpyrifos (CAS-No.2921-88-2; 96 % TC) was purchased from Yangnong Agrochemical Group (Jiangsu, China). Atrazine (CAS 1912-24-9; 96 % TC) was donated by Red Sun Chemical Co. Ltd (Jiangsu, China). $CdCl_2 \cdot 2.5H_2O$ (CAS 7790-78-5; 99 % active ingredient) was purchased by Jinshanting New Chemical Industrial Group (Shanghai, China). Stock and working stock solutions of each chemical were prepared in solvent (chlorpyrifos and atrazine were dissolved in analytical-grade acetone, 99 % purity; $CdCl_2 \cdot 2.5\ H_2O$ was dissolved in distilled water) and stored in a refrigerator at 4 °C. The stock solutions were stored for up to 1 month. All working stock solutions were made immediately before use.

Toxicity test methods

Acute toxicity test in filter paper

Filter paper test was performed according to the OECD guideline (OECD 1984). A piece of filter paper was placed in a 9-cm Petri dish and treated with the test substance dissolved in 2 mL of solvent (pesticides were dissolved in acetone, $CdCl_2 \cdot 2.5\ H_2O$ was dissolved in distilled water). After the solvent was evaporated to dryness under a slow stream of filtered compressed air, the piece of filter paper was remoistened with 2 mL distilled water, and one earthworm was placed on it in order to avoid the adverse effect caused by the death of worm in the same dish. The dish was incubated in the dark at 20± 1 °C for 48 h in the dark, and mortality was recorded. Every dish as a replicate and ten replicates were set for each concentration. An earthworm was considered dead if it failed to respond to a gentle mechanical touch on the front end.

Earthworms were held on wet filter paper for 24 h at 20±1 °C in the dark to have the gut contents purged before the dose-response test.

A preliminary test was conducted to determine the desired concentration range for each chemical in which a 0–100 % mortality of the earthworms was obtained. At least five concentrations in a geometric series and a control were included for each chemical. Ten replicates were used for each concentration, and solvent was used as the control. Treated earthworms were maintained at 20±1 °C under 80–85 % relative humidity in the dark.

Acute toxicity test in artificial soil

Artificial soil used in the soil test consisted of 10 % ground sphagnum peat (<0.5 mm), 20 % kaolinite clay (>50 % kaolinite), and 70 % fine sand (OECD 1984). In toxicity tests, a small amount of calcium carbonate was added to adjust the pH to 6.0±0.5, and the water content was adjusted to 35 %. For each tested concentration, the desired amount of chemical was dissolved in 10-mL solvent and mixed into a small quantity of fine quartz sand. The sand was mixed for at least 1 h to remove the acetone by evaporation in a fume hood and then mixed thoroughly with the premoistened artificial soil in a household mixer. The final moisture contents of artificial soil were adjusted to the prescribed level by adding distilled water. A total of 0.65 kg soil (including 0.5 kg dry artificial soil and 150 mL distilled water) was placed in a 500-mL glass jar (surface area 63.6 cm^2). Ten adult earthworms were put into each jar. Controls were prepared similarly but with soils containing only 10-mL solvent without any chemicals. The jars were loosely covered with polypropylene lids, to allow exchange of air, and stored at 20±1 °C with at 80–85 % relative humidity under 400–800 lux constant light. Survival was assessed at 14 days after the treatment.

Preliminary studies were conducted to determine the range of concentrations that caused 0–100 % mortality for each chemical. Six test concentrations in a geometric series and a control were used to obtain the LC50 value. Three jars, each containing ten adult earthworms, were used for each concentration. The earthworms were preconditioned for 24 h under the same conditions described above in the untreated artificial soil before the dose-response test. The mortality in the control should not exceed 10 % at the end of any test.

Experimental design of pesticide mixtures

Solutions of chlorpyrifos, atrazine, and $CdCl_2 \cdot 2.5\ H_2O$ prepared as described above were used singly and in binary (chlorpyrifos + atrazine; chlorpyrifos + Cd; atrazine+ Cd) and three (chlorpyrifos + atrazine + Cd) combinations. Earthworms were treated with serial dilutions of each chemical individually and with a fixed constant ratio (1:1), based on the individual LC50 values, in their binary and ternary combinations. Five to six dilutions (serial dilution factor of 2) of each chemical and combination, as well as a control, were tested in three independent experiments with replicate samples.

Median effect and combination index-isobologram equations for determining individual and combined toxicities

The response to acute toxic exposure of *E. fetida* in artificial soil and filter paper tests was estimated using the median-effect equation, as described by Chou and Talalay (1984):

$$\frac{fa}{fu} = \left(\frac{D}{LC_{50}}\right)^m \tag{1}$$

where D represents the dose affecting a fraction f_a, LC50 is the dose at which 50 % mortality rate is reached, f_u is the unaffected fraction (f_a=1−f_u), and m identifies the coefficient of the shape of the dose-effect curve: m=1, m>1, and m<1 indicate hyperbolic, sigmoidal, and negative sigmoidal dose-effect curve, respectively.

The quantification of synergism or antagonism for a combination of a series of chemicals is given by CI values at 10, 50, and 90 % mortality rate:

$$(CI)_x = \sum_{j=1}^{n} \left(\frac{(D)_j}{(D_x)_j}\right) = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\left\{\frac{[D]_j}{\sum_1^n [D]}\right\}}{(D_x)_j\left\{\frac{(fax)_j}{[1-(fax)_j]}\right\}^{1/mj}} \tag{2}$$

where $^n(CI)_x$ is the combination index for n chemicals at x% mortality rate; $(D_x)_{1\text{-}n}$ is the sum of the concentrations of n chemicals causing x% mortality rate in mixture,

$[D]_j/\sum_1^n[D]$ is the proportionality of the dose of each of n chemicals causing x% mortality rate in combination; and $(D_x)_j\{(fax)_j/1-(fax)_j\}^{1/mj}$ is the concentration of each individual chemical causing x% mortality rate. CI<1, CI=1, and CI>1 indicate synergism, concentration addition, and antagonism, respectively.

Analysis of results

Computer program CompuSyn (Chou and Martin 2005) can also display a type of simplified visual graphic termed polygonogram, representing interactions between three or more compounds at a determined f_a values in a semiquantitative way. Synergism is indicated by solid lines and antagonism by broken ones, and the thickness of the lines indicates the strength of the interaction.

Results

The nature of chlorpyrifos, atrazine, and Cd interactions was evaluated both in both artificial soil and filter paper tests, based on the combination index-isobologram method. Table 1 shows the dose-effect curve parameters (D_m, m, and r) of the individual chemicals and their binary and ternary combinations, as well as the mean CI values of the mixtures. All individual chemicals (Fig. 1a, c) and their mixtures (Fig. 1b, d) fitted well to the median effect equation with common sigmoidal shape dose-response curves. D_m was the dose required to produce the 50 % mortality rate (equals to LC50 value) (Rosal et al. 2010b). The D_m values for atrazine were the lowest in both tests, while the D_m values for chlorpyrifos were an order of magnitude higher in filter paper test and the Cd D_m values were an order of magnitude higher in artificial soil test. The parameter m was the Hill coefficient used to determine the shape of the dose-effect curve, hyperbolic (m=1), sigmoidal (m>1), or negative sigmoidal (m<1). Table 1 also shows that linear regression correlation coefficients (*r* values) of the median effect plots were >0.94 in all cases, indicating the conformity of the data to the median effect principle. The individual and total concentrations of the chemicals in their binary and ternary mixtures are listed in Table 2.

Table 1 Dose-effect relationship parameters and mean combination index (CI) values of three chemicals in their binary and ternary combinations toward *Eisenia fetida* in artificial soil and filter paper acute toxicity tests

Chemicals	Dose-effect parameters			CI values		
	D_m	m	r	LC10	LC50	LC90
Artificial soil test (mg/kg)						
Chlorpyrifos	376.87	9.02	0.992	-	-	-
Atrazine	168.20	4.61	0.978	-	-	-
Cd	1442.96	9.17	0.999	-	-	-
Chlorpyrifos + Atrazine	423.38	4.14	0.973	1.34	1.58	1.88
Chlorpyrifos + Cd	1102.29	3.15	0.967	0.77	1.22	1.93
Atrazine + Cd	480.58	4.23	0.951	0.53	0.62	0.73
Chlorpyrifos + Atrazine + Cd	1285.86	3.56	0.988	1.50	1.99	2.69
Filter paper test (mg/L)						
Chlorpyrifos	1041.96	2.40	0.984	-	-	-
Atrazine	143.03	3.33	0.974	-	-	-
Cd	201.43	3.43	0.969	-	-	-
Chlorpyrifos + Atrazine	247.14	5.21	0.999	0.63	0.44	0.31
Chlorpyrifos + Cd	1152.80	2.65	0.945	1.85	1.95	2.09
Atrazine + Cd	462.17	3.12	0.951	2.54	2.68	2.83
Chlorpyrifos + Atrazine + Cd	577.75	2.91	0.999	1.30	1.32	1.36

The parameters *m*, D_m, and *r* are the antilog of x-intercept, the slope, and the linear correlation coefficient of the median effect plot, which signifies the shape of the dose-effect curve, the potency (LC50), and conformity of the data to the mass-action law, respectively (Chou and Talalay 1984; Chou 2006). EC50 and m are used for calculating the CI values (Eq. (2)); CI<1, CI=1, and CI>1 indicate synergism, additive effect, and antagonism, respectively. LC10, LC50, and LC90 are the doses required to reach a response mortality of 10, 50, and 90 %, respectively

The D_m and m values for single chemicals and for their combination mixtures were used for calculating synergism or antagonism based on the CI Eq. (2) (Chou 2006). CI values were reported at LC10, LC50, and LC90, respectively, representing the doses required to reach 10, 50, and 90 % mortality rate of the earthworms. Figures 2 and 3 show the f_a-CI plot of chemical interactions both for artificial soil and filter paper tests, respectively, demonstrating the CI value versus mortality rate by binary and ternary combinations with respect to the control. The f_a-CI plot is an effect-oriented plot that shows the types of interaction (synergism, antagonism, additive effect) as a function of the level of the effect of a particular toxicant on the tested organisms (Rodea-Palomares et al. 2010). In artificial soil test (Fig. 2), the binary mixture of chlorpyrifos and atrazine was antagonistic toward *E. fetida* at all f_a levels, with CI values slightly increased. When atrazine was mixed with Cd, a slight degree of synergism exhibited throughout the exposure range. The chlorpyrifos plus Cd combination led to dual antagonistic/synergistic behavior being synergistic at f_a values below 0.2, additive at f_a values between 0.2 and 0.4, and turned into antagonistic at f_a levels above 0.4. The ternary mixture of chlorpyrifos, atrazine, and Cd was clearly antagonistic in the whole range of f_a values.

The f_a-CI plot showed the opposite pattern of interactions in filter paper test (Fig. 3) as the atrazine plus Cd combination and chlorpyrifos plus Cd combination showed moderate antagonism at all effect levels, with CI values ranging from 2.5 to 2.9 and 1.8 to 2.2, respectively; when atrazine was mixed with chlorpyrifos, a clear synergism was observed in the whole effect range. The ternary combination was slightly antagonistic with CI values almost unaltered. Selected average CI values in both artificial soil and filter paper tests at three representative effect levels (LC10, LC50, and LC90) and the combined effects are summarized in Table 1.

Figure 4 shows the polygonograms for the binary combinations at three f_a values (0.1, 0.5, and 0.9), which shows the antagonist interaction of chlorpyrifos in combination with atrazine all over f_a values in both tests.

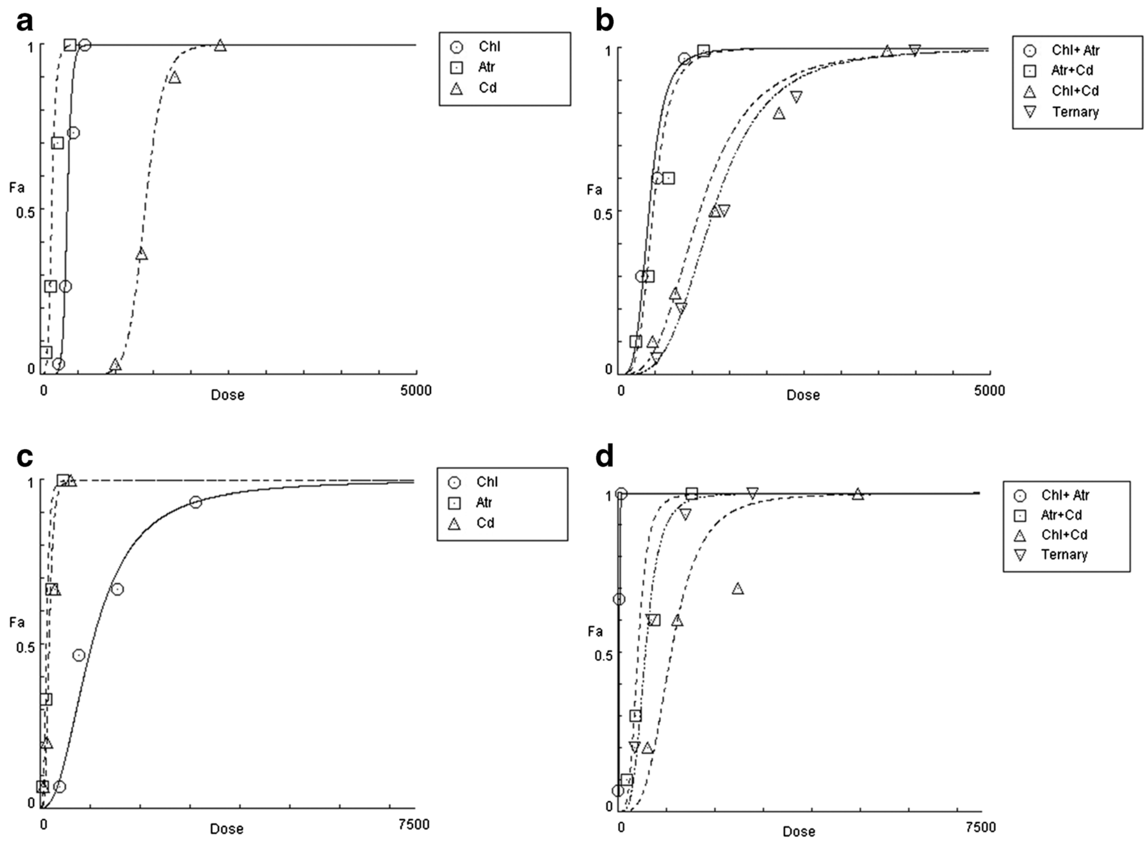

Fig. 1 Dose-response curves of chlorpyrifos (Chl), atrazine (Atr), and cadmium (Cd) toward *Eisenia fetida* in artificial soil (**a**, **b**) and filter paper (**c**, **d**) acute toxicity tests

Discussions

In the calculation of mixture toxicity predictions, the concentration addition (CA) and independent action (IA) are two traditional concepts that have been widely used (Teuschler 2007). However, these two methods have been found to have severe limitations in predicting combined effects of components with diverse dose-effect relationships (Cleuvers 2003; Fent et al. 2006). In an investigation, we demonstrated the the CI-isobologram equation method could optimize the prediction of the classical CA and IA models (Chen et al. 2014). By this method, we have been able to determine the interaction types for a series of effect levels of three chemicals in binary and ternary combinations in two types of acute earthworm tests.

However, the nature of these interactions was not uniform along the f_a level range in any of the two tests. In artificial soil test, for the atrazine plus Cd mixture, synergism predominated at all effect levels, while it showed the opposite pattern in filter paper test. The binary mixture of chlorpyrifos plus Cd exhibited moderate synergism over effect levels in filter paper test. While in artificial soil, a dual synergistic/antagonistic behavior was observed with synergism predominating at f_a values below 0.25–0.3 and antagonism below these effect values. The CI method only allows quantitative determination of synergism or antagonism, and the elucidation of the mechanism by which synergism or antagonism occurs is a separate issue that needs a different kind of approach (Rosal et al. 2010a). Due to the various abiotic and biotic modifying factors in agricultural soils, chemical measures are considered inadequate for expressing the actual exposure of earthworms. It is necessary to include chemical bioavailability in the expression of exposures. Therefore, bioavailability needs to be considered for understanding exposures, and chemical measures of bioavailability must be correlated when conducting risk assessment in soil systems (Lanno et al. 2004).

The nature of the toxicological interaction was partially influenced by the toxicological mode of action of each component (Rodea-Palomares et al. 2010). Atrazine contains five electron donor atoms that can potentially complex with Cd to form atrazine-Cd complexes that might change the toxicity characteristics of the individual compound (Meng and Carper 2000). The resulting complex might have difficulty moving into earthworms, preventing its entry into them. Therefore, the amount of atrazine and Cd decreased to earthworms in the

Table 2 Concentration of chemicals in binary and ternary mixtures toward *Eisenia fetida* in artificial soil and filter paper acute toxicity tests

Mixtures	f_a	Mixture concentrations	Individual component concentrations in mixtures		
			Chlorpyrifos	Atrazine	Cd
Artificial soil test (mg/kg)					
Chlorpyrifos + Atrazine	0.1	248.9	168.0	80.9	
	0.5	423.4	285.8	137.6	
	0.9	720.3	486.2	234.1	
Atrazine + Cd	0.1	286.0		32.3	253.7
	0.5	480.6		54.3	426.3
	0.9	807.5		91.2	716.3
Chlorpyrifos + Cd	0.1	548.2	116.5		431.7
	0.5	1102.3	234.3		868.0
	0.9	2216.3	471.1		1745.2
Chlorpyrifos + Atrazine + Cd	0.1	694.0	134.1	63.2	496.7
	0.5	1285.8	248.4	117.1	920.3
	0.9	2382.5	460.3	217.0	1705.2
Filter paper test (mg/L)					
Chlorpyrifos + Atrazine	0.1	167.4	22.3	145.1	
	0.5	230.2	30.6	199.6	
	0.9	316.8	42.1	274.7	
Atrazine + Cd	0.1	244.8		100.1	144.7
	0.5	417.3		170.7	246.6
	0.9	711.2		290.9	420.3
Chlorpyrifos + Cd	0.1	605.1	495.0		110.1
	0.5	1104.3	903.3		201.0
	0.9	2015.0	1648.3		366.7
Chlorpyrifos + Atrazine + Cd	0.1	326.0	236.7	36.5	52.8
	0.5	571.7	415.1	64.0	92.6
	0.9	1002.7	728.0	112.3	162.4

combined treatments compared with the individual conditions. On the other hand, atrazine can accelerate the biotransformation rate of chlorpyrifos to chlorpyrifos O-analog which can increase the amount of chlorpyrifos metabolites. Synergistic toxicity of chlorpyrifos would be expected when applied in combination with atrazine. Some authors (Pape-Lindstrom and Lydy 1997; Anderson and Lydy 2002) evaluated the combined effect of atrazine with organophosphorus pesticides to the aquatic organisms, and synergistic toxicities were observed for most of the binary mixtures. These findings might explain why the combined effects of atrazine plus Cd were weaker than expected from separate exposures, while chlorpyrifos plus atrazine combination exhibited synergism in filter paper test. It could be attributed to the bioavailability. The contaminants are absorbed mainly by the skin in the filter paper test, resulting in a greater diffusing through capillary into blood circle.

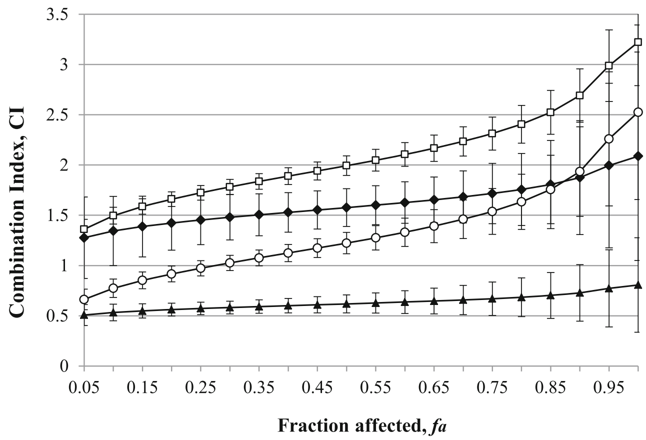

Fig. 2 Combination index plot of mixtures of compounds toward *Eisenia fetida* in artificial soil acute toxicity test. Symbol for the combinations: chlorpyrifos + atrazine (*black diamond*); chlorpyrifos + cadmium (*white circle*); atrazine + cadmium (*black triangle*); and ternary (*white square*) combinations. CI values are plotted as a function of the mortality rate (f_a) by computer simulation (CompuSyn). CI<1, = 1, and>1 indicate synergism, additive effect, and antagonism, respectively. Three independent experiments with two replicates were used. *The vertical bars* indicate 95 % confidence intervals for CI values based on sequential deletion analysis (SDA)

Whereas earthworm in artificial soil tests absorbed and digested pollutants by alimentary canal, the process was tardy. Moreover, more experiment designs of sensitive chronic test such as growth and reproduction will be adapted according to this study.

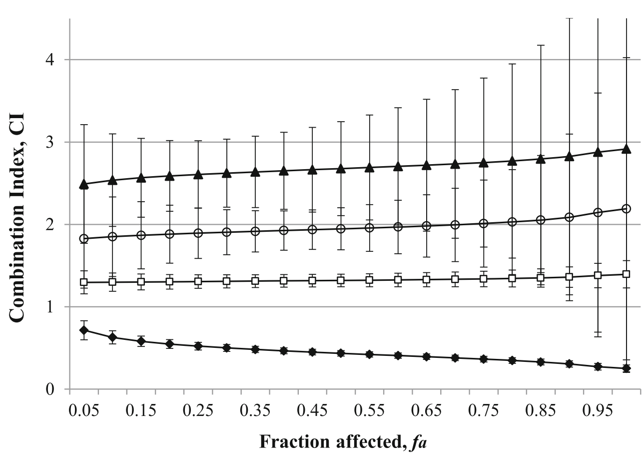

Fig. 3 Combination index plot of mixtures of compounds toward *Eisenia fetida* in filter paper acute toxicity test. Symbol for the combinations: chlorpyrifos + atrazine (*black diamond*); chlorpyrifos + cadmium (*white circle*); atrazine + cadmium (*black triangle*), and ternary (*white square*) combinations. CI values are plotted as a function of the mortality rate (f_a) by computer simulation (CompuSyn). CI<1, = 1, and>1 indicate synergism, additive effect, and antagonism, respectively. Three independent experiments with two replicates were used. *The vertical bars* indicate 95 % confidence intervals for CI values based on sequential deletion analysis (SDA)

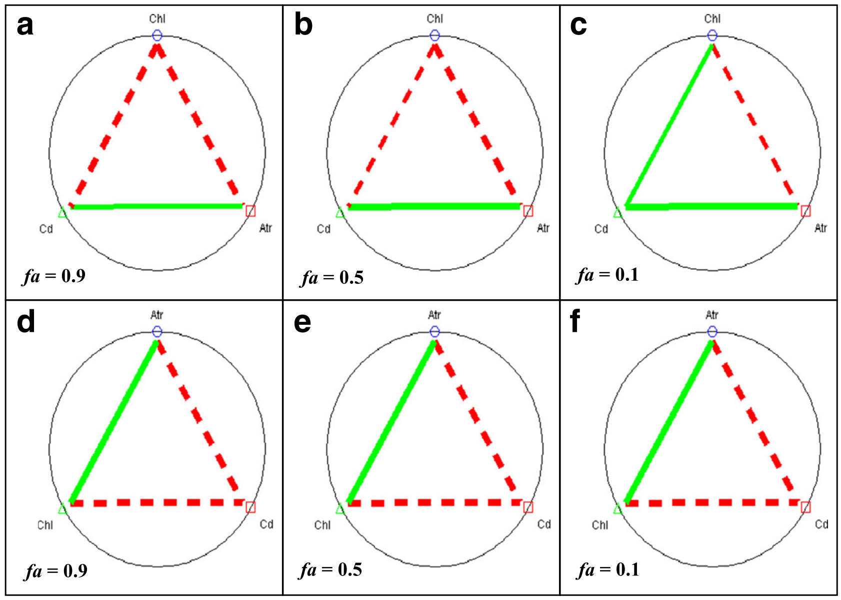

Fig. 4 Polygonograms showing the toxicological interactions of the three compounds in binary and ternary combinations. The interactions were calculated by CompuSyn in artificial soil (**a**, **b**, **c**) and filter paper (**d**, **e**, **f**) acute toxicity tests at three effect levels: f_a=0.1, 0.5, and 0.9. *Solid lines* indicate synergism, *broken lines* indicate antagonism. *The thickness of the line* represents the strength of synergism or antagonism

Therefore, toxicological interactions can also occur independently of the primary mode of action (Chou 2006) which is largely unknown and different for different organisms and trophic levels in environmentally relevant conditions (Cleuvers 2003). In the present study, ternary mixtures demonstrated antagonistic effects toward the earthworms, while some other studies found that the combined toxicity may increase in ternary mixtures with respect to their binaries. Rosal et al. (2010a, b) assessed toxic interactions of docusate sodium and chlorinated chemicals and found that synergistic behavior predominated in the ternary mixtures. Boltes et al. (2012) reported that the addition of chlorinated chemicals would induce a strong synergism for mixtures containing perfluorooctane sulfonic acid (PFOS) at all effect levels.

The combined effects of pollutants seem to also depend on the test organism. Ecotoxicity studies conducted on diverse species and trophic levels may show a completely different response to the same toxicant mixture (Rosal et al. 2010a). Cedergreen et al. (2007) stated that the reproducibility of mixture experiments can hardly be guaranteed. This will not only depend on the setup but also on the species that is tested.

Different insecticides, herbicides, and heavy metals may co-occur in the same agricultural fields. The predicted synergism in some of the mixtures indicates that the traditional evaluation of mixture toxicity from single component data when assuming additive behavior may lead to underestimation. More studies of combined toxicities among these contaminants in the terrestrial environment should be conducted to identify the mixtures exhibiting synergistic pattern of interactions.

Conclusions

The CI-isobologram equation was applied to study the nature of binary and ternary interactions of chlorpyrifos, atrazine, and Cd toward the earthworm *E. fetida*. Atrazine showed the highest toxicity in both tests. The combination of atrazine and Cd exhibited a slight degree of synergism throughout the exposure range, suggesting that a potential risk associated with the co-occurrence of the pollutants in the sediment still exists. The chlorpyrifos plus Cd combination showed both synergism and antagonism. The nature of interaction pattern was different in two types of toxicity tests which might be due to the bioavailability of chemicals in soil systems.

Perspectives

The synergistic effect for the particular binary combination suggests that a potential risk associated with the co-occurrence of these pollutants may still exist, which have implications in risk assessment for the terrestrial environment. However, the combined effects might be influenced by bioavailability and the test organism. Thus, more studies of combined toxicities among these contaminants in the terrestrial environment including soils, crops, and microorganisms should be conducted to identify the mixtures exhibiting synergistic pattern of interactions.

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