The synergistic toxicity of the multiple chemical mixtures: Implications for risk assessment in the terrestrial environment

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A R T I C L E   I N F O

Article history:
Received 9 July 2014
Received in revised form 11 November 2014
Accepted 5 January 2015
Available online xxxx

Keywords:
Insecticides
Herbicides
Heavy metal
Eisenia fetida
Acute mixture toxicity
Combination index

A B S T R A C T

The combined toxicity of five insecticides (chlorpyrifos, avermectin, imidacloprid, λ-cyhalothrin, and phoxim), two herbicides (atrazine and butachlor) and a heavy metal (cadmium) has been examined with the earthworm acute toxicity test. Toxicological interactions of these chemicals in four, five, six, seven, and eight-component mixtures were studied using the combination-index (CI) equation method. In four-component and five-component mixtures, the synergistic effects predominated at lower effect levels, while the patterns of interactions found in six, seven, and eight-component mixtures displayed synergism. The λ-CY + IMI + BUT + ATR + CPF + PHO combination displayed the most strongly synergistic interaction, with CI values ranging from 0.09 to 0.15. The nature of the interaction changes with the effect level and the relevance of synergistic effects increase with the complexity of the mixture. The CI method was compared with the classical models of concentration addition (CA) and independent action (IA) and we found that the CI method could accurately predict the combined toxicity. The predicted synergism resulted from co-existence of the pesticides and the heavy metal especially at low effect levels may have important implications in risk assessment for the real terrestrial environment.

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1. Introduction

Pesticides and heavy metals are two types of pollutants that are commonly present in the terrestrial environment (J. H. Wang et al., 2012). Insecticides and herbicides are commonly used in agricultural fields to protect crops and improve the quality and quantity of the harvest. Their co-application is commonplace where herbicides are used to control weeds and insecticides to control insect pests (Choung et al., 2013). On the other hand, the release of heavy metals into the environment, mainly as a consequence of anthropogenic activities, also constitutes a potential risk for unintended adverse health impacts to both humans and non-target wildlife (Laetz et al., 2009; Soares and Soares, 2012). These pollutants have been detected simultaneously in agricultural lands, resulting in complex exposure scenarios for soil-dwelling organisms such as earthworms.

Traditional effect and risk assessment have been routinely focused on exposures to single chemicals and additive behaviors, which may underestimate the risk associated with toxic action of mixtures (Barata et al., 2006). Assessing the combined (additive, synergistic or antagonistic) toxicity of pollutant mixtures has therefore been an enduring challenge for environmental health and ecotoxicology for the past several decades (Laetz et al., 2009). Recently, there are an increasing number of studies dealing with mixtures of contaminants (Jin-Clark et al., 2002; Munkegaard et al., 2008; Gomez-Eyles et al., 2009; Rodea-Palomares et al., 2010; Mehler et al., 2011; Bjergager et al., 2012; Harabayashi and Ibrahim, 2014). However, only few studies have been conducted on the toxicity of mixtures to soil invertebrates (e.g. Anderson and Lydy, 2002; Amorim et al., 2012; Santos et al., 2010; Schnug et al., 2014), despite soils being the primary target for pesticides applied in agriculture. Besides, there are not many reports in the literature which have studied the nature of interactions of multi-component chemical mixtures. Hence, in order to investigate the toxicity of multi-component chemical mixtures on soil-dwelling invertebrates, more studies are needed (Amorim et al., 2012).

In the present study, we investigated the ecotoxicity of the five insecticides, two herbicides, and a heavy metal to the earthworm Eisenia fetida which is an important component in improving the soil structure of the terrestrial ecosystem (Fourie et al., 2007). Atrazine (ATR) is a triazine-ring herbicide, known to act as an endocrine disruptor in frogs and fish (Hecker et al., 2005). Chlorpyrifos (CPF) and phoxim (PHO) are organophosphorus cholinesterase inhibitors after metabolism, allowing acetylcholine to accumulate at cholinergic synapses (ICPS, 1989). Imidacloprid (IMI) is a relatively new systemic insecticide that acts as an agonist at nicotinic acetylcholine receptors (nAChRs).
(Anatra-Cordone and Durkin, 2005). The pyrethroid insecticide \( \lambda \)-cyhalothrin (\( \lambda \)-CY) can interfere with sodium ion movement through the nerve membrane causing hyperactivity of the nervous system (Clark and Brooks, 1989). Butachlor (BUT) is a chloroacetanilide herbicide for the control of grasses and weeds that acts by inhibiting elongase responsible for the elongation of very long-chain fatty acids and geranylgeranyl pyrophosphate cyclisation enzymes (Böger et al., 2000). Avermectin (AVM) is used for insect control, acting as a partial agonist at a GABA-binding site. Cadmium (Cd) could be accumulated in the granules in the chloragogenous tissue surrounding the digestive tract and in the nephridia of earthworms, causing demographic and reproductive abnormalities (Fouie et al., 2007). The above herbicides and insecticides are being commonly used in agriculture in China, where herbicides are used to control weeds and insecticides are co-applied to control insect pests. Moreover, Cds are widespread occupational and environmental toxicants with high acute ecotoxicity and recognized to be the most hazardous chemicals to various ecosystems. These chemicals were selected due to their different modes of action, usage frequency, or the content in terrestrial environment.

The main objective of the present study was to evaluate the acute toxicity of the combined effects of the multi-component chemical mixtures on \( E \). fetida in an artificial soil test. In order to identify and quantify the nature of interactions among the chemicals, we tested four, five, six, seven and eight-component mixtures of these chemicals by the method of the combination index equation which has recently been used to study the nature of chemical interactions (Rodea-Palomares et al., 2010; Rosal et al., 2010; Boltes et al., 2012; Rodea-Palomares et al., 2012; González-Pleiter et al., 2013).

2. Materials and methods

2.1. Test organisms

\( E \). fetida is an invertebrate currently used for ecotoxicological assessment of substances in soil and is the test species recommended by the 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 citella were purchased from the Animal Sciences College, Zhejiang University, China, and cultured in the laboratory in artificial soil according to the 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 weekly and the moisture was adjusted to 35% of the maximum water-holding capacity by adding distilled water.

2.2. Test chemicals

Chlorpyrifos (CAS9291-88-2; 96% TC) was purchased from Yangnong Agrochemical Group (Yanzhou, Jiangsu, China). Atrazine (CAS 1912-24-9; 96% TC), imidacloprid (IMI), avermectin (AVM), phoxim (PHO) and CdCl\(_2\).2.5H\(_2\)O (Cd) prepared as described above were used in fifteen four-component mixtures (including combinations of \( \lambda \)-CY + PHO + BUT + IMI; ATR + PHO + BUT + \( \lambda \)-CY; \( \lambda \)-CY + PHO + BUT + CPF; \( \lambda \)-CY + PHO + BUT + AVM; \( \lambda \)-CY + PHO + Cd + \( \lambda \)-CY + IMI + ATR + CPF; ATR + AVM + \( \lambda \)-CY + IMI; IMI + Cd + \( \lambda \)-CY + ATR; BUT + \( \lambda \)-CY + ATR + CPF; BUT + IMI + \( \lambda \)-CY + AVM; \( \lambda \)-CY + IMI + Cd + BUT; AVM + IMI + ATR + CPF; \( \lambda \)-CY + Cd + ATR + CPF; ATR + AVM + CPF + Cd), ten five-component mixtures (including combinations of \( \lambda \)-CY + IMI + BUT + ATR + PHO, \( \lambda \)-CY + IMI + BUT + AVM + PHO, \( \lambda \)-CY + IMI + BUT + CPF + PHO, \( \lambda \)-CY + IMI + Cd + BUT + PHO, \( \lambda \)-CY + IMI + BUT + CPF + AVM, \( \lambda \)-CY + IMI + BUT + CPF + AVM), six six-component mixtures (including combinations of \( \lambda \)-CY + IMI + BUT + ATR + CPF + PHO, \( \lambda \)-CY + IMI + BUT + ATR + CPF + AVM, \( \lambda \)-CY + IMI + BUT + ATR + CPF + AVM), three seven-component mixtures (including combinations of \( \lambda \)-CY + IMI + BUT + ATR + CPF + AVM, \( \lambda \)-CY + IMI + Cd + BUT + ATR + CPF + PHO, \( \lambda \)-CY + IMI + Cd + BUT + ATR + CPF + AVM), one eight-component mixture (the combination of \( \lambda \)-CY + IMI + Cd + BUT + ATR + CPF + PHO). Earthworms were treated with serial dilutions of each chemical with a fixed equi-toxic constant mixture ratio (the same effect came from each individual chemical), based on the measured individual LC\(_{50}\) values. Five to seven dilutions (with a serial dilution factor of 2) of each chemical with their four,
five, six, seven, and eight-component combinations were tested. Besides, a control was tested in three independent experiments with replicate samples.

2.5. Chemical measurements in soil samples

2.5.1. Chlopyrifos and butachlor

Soil sample (approximately 20 g) was taken from each test container both before the organisms were added and after the organism samples were removed at the end of the test. A total of 5 g moist soil was transferred to a 50 mL polytetrafluoroethylene tube, and 5 g anhydrous MgSO₄ was then added to dry the sample. Acetonitrile (10 mL) was used for extraction, and triphenyl phosphate (1 μg mL⁻¹) was added as internal standard. The samples were shaken for 16 h and then centrifuged at 8000 g for 8 min. Subsequently, an aliquot was analyzed by gas chromatography (GC, Varian CP-3800). The limit of quantification was 0.02 μg mL⁻¹, and the average recovery for the spike levels ranged from 80% to 120%. In addition, 15 g of soil of each replicate was dried at 105 °C for 12 h to determine the water content of the samples. All concentrations were normalized by dry soil weight.

2.5.2. Atrazine, avermectin, lambda-cyhalothrin, imidacloprid and phoxim

Approximately 10 g of soil sample was collected and placed in conical flasks with glass stoppers, and the obtained samples were extracted using a 30 mL solution containing methylene chloride and acetone (6:1, v/v) for three times under mechanical vibration for 120 min. The pooled soil extracts (approximately 80 mL) were then centrifuged at 10,000 g for 5 min at 4 °C. The extract was concentrated to near dryness by rotary vacuum evaporator and recovered in acetonitrile, and its final volume was adjusted to exactly 2 mL. The extract was stored in amber glass vials at −20 °C prior to further analysis. High-performance liquid chromatography (HPLC, Agilent 1200) coupled with a reversed-phase column was used to analyze the soil extracts. Pesticides were isocratically eluted with acetonitrile and then quantified by a diode array detector at a wavelength of 200–300 nm. The recovery rate was estimated by adding 1 mL methanol containing pure pesticide (1 μg mL⁻¹) to soil. The

<table>
<thead>
<tr>
<th>Multi-component mixtures</th>
<th>Dose–effect parameters</th>
<th>CI values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D₅₀ (mg kg⁻¹)</td>
<td>m</td>
</tr>
<tr>
<td>Four-component mixtures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>λ-CY + IMI + BUT + PHO</td>
<td>52.3</td>
<td>2.65 ± 0.53</td>
</tr>
<tr>
<td>λ-CY + BUT + ATR + PHO</td>
<td>80.8</td>
<td>3.50 ± 1.17</td>
</tr>
<tr>
<td>λ-CY + BUT + CPF + PHO</td>
<td>67.3</td>
<td>2.86 ± 0.55</td>
</tr>
<tr>
<td>λ-CY + BUT + AVM + PHO</td>
<td>70.0</td>
<td>3.44 ± 0.46</td>
</tr>
<tr>
<td>λ-CY + Cd + BUT + PHO</td>
<td>105</td>
<td>3.38 ± 0.48</td>
</tr>
<tr>
<td>λ-CY + IMI + ATR + CPF</td>
<td>32.9</td>
<td>3.92 ± 0.81</td>
</tr>
<tr>
<td>λ-CY + IMI + ATR + AVM</td>
<td>113</td>
<td>2.46 ± 0.40</td>
</tr>
<tr>
<td>λ-CY + IMI + Cd + AVM</td>
<td>422</td>
<td>3.54 ± 0.65</td>
</tr>
<tr>
<td>λ-CY + IMI + BUT + ATR</td>
<td>172</td>
<td>3.13 ± 0.24</td>
</tr>
<tr>
<td>λ-CY + IMI + BUT + CPF</td>
<td>146</td>
<td>3.54 ± 0.28</td>
</tr>
<tr>
<td>λ-CY + IMI + BUT + AVM</td>
<td>233</td>
<td>2.41 ± 0.31</td>
</tr>
<tr>
<td>λ-CY + IMI + Cd + BUT</td>
<td>494</td>
<td>3.22 ± 0.33</td>
</tr>
<tr>
<td>IMI + ATR + CPF + AVM</td>
<td>98.2</td>
<td>2.92 ± 0.47</td>
</tr>
<tr>
<td>IMI + Cd + ATR + CPF</td>
<td>633</td>
<td>4.17 ± 0.24</td>
</tr>
<tr>
<td>Cd + ATR + CPF + AVM</td>
<td>396</td>
<td>3.28 ± 0.57</td>
</tr>
</tbody>
</table>

% of synergistic mixtures: 87% 87% 87% 100% 100% 100% 100% 100% 100% 100% 100% 100% 100% 100% 100% 100%

The parameters m, D₅₀ and r are 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]. CI = 1, CI > 1 indicate synergism (Syn), additive effect (Add), and antagonism (Ant), respectively. LC₁₀, LC₅₀ and LC₉₀ are the doses required to cause 10%, 50% and 90% mortalities of the earthworms, respectively.

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extracts were carried out using the abovementioned procedures, and the average recovery for the spike levels ranged from 80% to 120%.

2.5.3. Cadmium
Soil sample was homogenized and dried at 105 °C. Briefly, 1.0 g of soil sample was collected, and 3 mL solution containing HNO3 and H2O2 (2:1, v:v) was added. This mixture was gently shaken and then dried on a hot plate. After cooling, 2 mL nitric acid (0.75 M) was added, and then the sample was centrifuged. The clear digests were analyzed using direct slotted tube atom trap atomic absorption spectrometry (STAT-FAAS, AFS-9230) for cadmium. Blank analysis was carried out using the same procedure.

Average measured concentrations of the tested substances in soil both before the test start and after the completion of the test generally varied less than 20% from the nominal start concentrations of each individual chemical. Therefore, all calculations were based on nominal concentrations.

2.6. Median-effect and combination index (CI)-isobologram equations for determining individual and combined toxicities
The response to toxic exposure of *E. fetida* in artificial soil tests was estimated using the median-effect equation, as described by Chou and Talalay (1984):

\[
\frac{f_a}{f_u} = \left( \frac{D}{LC_{50}} \right)^m
\]

where \(D\) represents the dose affecting a fraction \(f_a\), \(LC_{50}\) is the dose at which 50% mortality rate is reached, \(f_u\) is the unaffected fraction \((f_u = 1 - f_a)\), 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 curves, respectively.

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

\[
(CI)_x = \sum_{j=1}^n \frac{(D_j)_x}{(D_m)_x} = \sum_{j=1}^n \frac{D_j}{(D_m)^{1/m}} \left( \frac{f_{ax}}{f_{ax}^{-1}} \right)^{1/m}
\]

where \(\sum (CI)_x\) is the combination index for \(n\) chemicals at \(x\%\) mortality rate; \((D_j)_x\) is the sum of the concentrations of \(n\) chemicals causing \(x\%\) mortality rate in mixture, \([D]_x\) is the proportionality of the dose of each of \(n\) chemicals causing \(x\%\) mortality rate in combination; and \((D_m)_x(j_{ax}^{-1} - j_{ax}^{-1})^{1/m}\) is the concentration of each individual chemical causing \(x\%\) mortality rate. CI < 1, CI = 1 and CI > 1 indicates synergism, concentration addition and antagonism, respectively.

2.7. Analysis of results
Computer program CompuSyn (Chou and Martin, 2005) was used for the calculation of dose–effect curve parameters and CI values. The \(f_a\)-CI plot represents CI versus \(f_a\) which is the fraction affected by a particular dose.

2.8. Mixture toxicity predictions based on CA, IA and CI equations
Experimental toxicities of the chemical mixtures were computed based on the predictive equations of the two most widely used definitions of additivity, concentration addition (CA) (Eq. (3)) and independent combination (CI) equations:

\[D_{x1} = \sum_{j=1}^n (D_j)_x\]

\[D_{x2} = \sum_{j=1}^n (D_j)_x \times \sum_{k=1}^n (D_k)_x\]

\[D_{x3} = \left( \sum_{j=1}^n (D_j)_x \right)^{1/n} \times \left( \sum_{k=1}^n (D_k)_x \right)^{1/n}\]

Fig. 1. Combination index plot (\(f_a\)-CI plot) for four-component mixtures of the eight chemicals for the earthworm acute toxicity test. CI values are plotted as a function of the mortality rate \((f_a)\) of the earthworms by computer simulation (CompuSyn). CI < 1, = 1 and > 1 indicates synergism, additive effect and antagonism, respectively. CPF = chlorpyrifos, ATR = atrazine, BUT = butachlor, λ-CY = λ-cyhalothrin, IMI = imidacloprid, AVM = avermectin, PHO = phoxim, Cd = cadmium.
action (IA) (Eq. (4)) (Faust et al., 2001; Altenburger et al., 2004). CA is based on the assumption that the mixture components have the same sites and similar mode of action (MOA), and is computed by equation:

\[ EC_{x,\text{mix}} = \left( \sum_{i=1}^{n} \frac{p_i}{EC_{xi}} \right)^{-1} \]  

(3)

where \( EC_{c,\text{mix}} \) is the effect concentration of the mixture eliciting \( x \%) \) effect, \( EC_{xi} \) denotes the concentration of the \( i \)th component when it exists individually and elicits the same effect (\( x \% \) ) as the mixture, \( p_i \) is the relative mass proportions of the \( i \)th component in the mixture. In the present study, for survival data, simply exchange \( EC_x \) with \( LC_{50} \) (lethal concentration 50).

IA is based on the assumption that the components in the mixture have dissimilar MOA. The following equation applies for IA.

\[ E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \]  

(4)

where \( c_{mix} \) and \( E(c_{mix}) \) are the total concentration and total effect of the mixture, respectively. \( E(c_i) \) denotes the effect of the \( i \)th component with the concentration of \( c_i \) in the mixture.

The predictive equation based on the CI values was computed as follows:

\[ EC_{x,\text{mix}} = \left( \sum_{i=1}^{n} \frac{p_i}{EC_{xi} \times CI_{\text{comp}}} \right)^{-1}. \]  

(5)

CI_{\text{comp}} is the computed combination index value for the mixture at the \( x \) level of effect (\( x \% \)) from the experimental toxicity curve of the mixture (Chou, 2006).

3. Results

3.1. Toxicological interactions of multi-component chemical mixtures

Table 1 summarizes the dose–effect curve parameters (\( D_{50}, m \) and \( r \) ) of the eight chemicals in four, five, six, seven and eight-component mixtures using the 14-d acute toxicity test towards \( E. \) fetida; 95% confidence intervals are indicated for the \( m \) parameter.

Figs. 1–2 display the \( f_a–CI \) plots for the four, five, six, seven and eight-component mixtures of the \( E. \) fetida acute toxicity tests. The \( f_a–CI \) plot depicts the CI value versus \( f_a \) (the effect level or the mortality rate of the earthworms with respect to the control) and shows the types of the interaction (synergism, antagonism or additive effect) as a function of the level of the effect of a particular mixture on the tested organism (Rodea-Palomares et al., 2010). Average CI values for three representative effect levels (\( LC_{10}, LC_{50} \), and \( LC_{90} \) ) are also shown in Table 1. The individual concentrations of eight chemicals in all multi-component mixtures can be found in the Supplemental data.

Regarding four-component mixtures, nine out of fifteen combinations showed synergistic effects in practically the whole range of \( f_a \) values. The \( \lambda-CY + \) BUT + CPF + PHO combination was the most strongly synergistic (with CI values ranging from 0.06 to 0.16 as effect level increased) in the whole range. Four combinations resulted in dual synergistic/antagonistic on the \( f_a–CI \) behaviors: at low and mean effect levels, the mixtures were synergistic, but the synergism significantly decreased with increasing \( f_a \) levels until it approached an additive effect at \( f_a \) levels between 0.85 and 0.9 and turned into slight antagonism at \( f_a \) values >0.95 (e.g., \( \lambda-CY + IMI + ATR + AVM \) was synergistic at \( f_a \) levels below 0.8, additive at \( f_a \) values between 0.8 and 0.9 and dominated by antagonism above 0.9). Two combinations exhibited increasingly antagonistic effects along with the \( f_a \) range, in which the \( \lambda-CY + IMI + Cd + ATR \) combination showed the most strongly antagonistic interaction, with the CI values ranging from 1.69 to 4.26.

In the ten five-component mixtures, eight combinations showed synergistic effects in practically the whole range of \( f_a \) values. The

Fig. 2. Combination index plot \( (f_a–CI \) plot) for five-component (a, b), six-component (c), seven, and eight-component (d) mixtures of the eight chemicals for the earthworm acute toxicity test. CI values are plotted as a function of the mortality rate \( (f_a) \) of the earthworms by computer simulation (CompuSyn). CI < 1, = 1 and > 1 indicates synergism, additive effect and antagonism, respectively.
Fig. 3. Experimental toxicity values (Exp) and predicted dose–response curves of four-component mixtures based on concentration addition (CA), independent action (IA) and combination index (CI) models for the earthworm acute toxicity test.
Fig. 4. Experimental toxicity values (Exp) and predicted dose–response curves of five-component mixtures based on CA, IA and CI models for the earthworm acute toxicity test.
The λ-CY + IMI + BUT + AVM + PHO combination was the most strongly synergistic (with the CI values ranging from 0.07 to 0.24 as the effect level increased) in the whole range. Two combinations led to dual synergistic/antagonistic behaviors (e.g., λ-CY + IMI + BUT + ATR + AVM mixture was synergistic at effect levels below 0.5, additive between 0.5 and 0.8 and turned into antagonism above 0.8).

All of the six six-component mixtures presented strong synergistic effects along with the $f_m$ range, in which the λ-CY + IMI + BUT + ATR + CPF + PHO combination displayed the most strongly synergistic interaction, with CI values ranging from 0.09 to 0.15 as the effect level increased. Similarly, the synergism of the three seven-component and one eight-component mixtures was evident throughout the entire effect level ranges but decreased with increasing effect levels. The results suggest that the co-existence of the pesticides and the heavy metal represents an enhanced risk to terrestrial ecosystems.

### 3.2. Experimental and predicted toxicity of the mixtures of chemicals under CA, IA and CI methods

To validate the toxicological interactions of the multi-component mixtures predicted by the combination index (CI) equation, the predicted dose–response curves of the mixtures were generated based on the classical CA and IA models. The predicted dose–response curves of the mixtures based on the CI values were also generated at the different $f_m$ levels of the mixtures. Figs. 3–6 demonstrate the predicted dose–response curves under CA, IA and CI models together with the experimental values for four, five, six, seven and eight-component mixtures in earthworm acute toxicity tests. Generally, when comparing the experimental and predicted toxicities of all the multi-component mixtures based on the three models (CA, IA and CI), the predicted toxicity of the mixtures according to CI method is closer to the experimental toxicity values than that using CA or IA in nearly all of the multi-component mixtures. For example, when analyzing the toxicity of the λ-CY + IMI + BUT + PHO mixture, neither the CA nor the IA model predicted the observed synergistic interaction, while it was accurately predicted by the CI method (Fig. 3a). The CI method improved the predictive power of the classical CA and IA models, allowing us to achieve more accurate predictions of combined toxicity in synergistic mixtures.

### 4. Discussion

The CA and IA are two traditional concepts that have been widely utilized in predicting mixture toxicities (Altenburger et al., 2003;
Backhaus et al., 2003; Jonker et al., 2004, 2005; Loureiro et al., 2009). These two concepts provide a reference structure to which the experimental mixture toxicity could be compared (Backhaus et al., 2004). However, the classic models for the prediction of the mixture toxicity are based on simple assumptions on the mode of toxic action (Rodea-Palomares et al., 2012). Chemicals with known mechanisms of action, once released in the environment, might have diverse dose–response relationships. CA had severe limitations when the dose–response curves of the individual chemicals were not identical (Cleuvers, 2003). In an extensive statistical evaluation on the accuracy of the predictive models, only a half of the experimental toxicities of the mixtures could be correctly predicted by either CA or IA models (Cedergreen et al., 2007). Recently, Rosal et al. (2010) reported an environmental application of the median-effect/combination index (CI)-isobologram equation originally developed by Chou (2006), which allows quantitative determinations of chemical interactions at different concentrations and effect levels. Besides, knowledge on the component–component type of interaction is not required to assess the overall interaction of the mixture (Chou, 2006). In the present study, we determined the interaction types for a series of effect levels of eight chemicals in multi-component mixtures in earthworm acute toxicity test by applying this method, which improved the predictive power of the CA and IA models.

An important feature of the observed multi-component mixture toxicity is that the nature of the interaction changes with the effect level. The percentage of mixtures representing synergism at three representative effect levels (LC10, LC50 and LC90) was 87%, 87%, 60%, respectively, in four-component mixtures, while this figure reached 100%, 87%, and 87%, respectively, in five-component mixtures. The synergistic effects predominated at the lower effect levels. González-Pleiter et al. reported an antagonistic effect of the multi-component (four and five-antibiotics) mixtures at very low to low effect levels, with synergism clearly predominating at higher effect levels (González-Pleiter et al., 2013).

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), which might reduce the amount of exposed atrazine and Cd to the earthworms. In the present study, two mixtures containing atrazine and Cd showed additive or antagonistic effects. However, the patterns of interactions found in the rest of the multi-component (six, seven, and eight-component) mixtures cannot be easily explained in terms of the known mechanisms of action. These mixtures exhibited clearly synergistic toxicities in the whole range of the effect levels. The results point out that the complexity of the mixture tends to increase the relevance of synergistic effects. Koutsasitis and Aoyama (2007) examined the toxicity of the mixtures of antimicrobial biocides on the brine shrimp Artemia salina and synergism was observed in the quaternary combination. Rodea-Palomares et al. (2010) investigated a complex mixture including pharmaceuticals and a real wastewater sample consisting of thirty micropollutants on the cyanobacterium Anabaena CPB4337. They concluded that synergism was the predominant interaction in a wide range of the effect level. Petersen et al. (2014) noted that the combined effects of pharmaceuticals, personal care products, biocides and organic contaminant multi-compound mixtures on the marine algae Skeletonema pseudocostatum conformed to the concentration addition concept. Taking into account that CA may underestimate the actual risk, synergistic effect could still be expected. Yang et al. (2008) studied the combined effects of 12 antibiotic agents on the green alga Pseudokirchneriella subcapitata finding that the synergistic effects predominated at low antibiotic concentrations.

Different insecticides, herbicides, and heavy metals may co-occur in the same terrestrial environment. The predicted synergism in the majority of the mixtures especially at the low effect levels indicated a
potential ecotoxicological risk associated with the co-occurrence of these chemicals at environmentally relevant concentrations. This may have important implications in the terrestrial environment.

5. Conclusions

The combination index (CI)-isobologram equation was applied to study the nature of the multi-component interactions of five insecticides, two herbicides, and a heavy metal on the earthworm *E. fetida* with acute toxicity test. In the four-component and five-component mixtures, the synergistic effects predominated at the lower effect levels. The patterns of interactions found in the rest of the multi-component displayed strong synergism. The relevance of synergistic effects increases with the complexity of the mixture. The CI method could improve the predictive power of the mixtures when compared with the classical CA and IA models. The predicted synergism resulted from co-existence of the pesticides and the heavy metal represents an enhanced risk to the terrestrial ecosystem, which may have important implications in risk assessment for the real terrestrial environment.

Acknowledgments

The research was supported by the National Natural Science Foundation of China (Grant No. 31401767). Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences, and the Innovation Project of the Zhejiang Academy of Agricultural Sciences (2014CX010).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2015.01.014.

References


