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Study on the variation rules of the joint effects for multicomponent mixtures containing cyanogenic toxicants and aldehydes based on the transition state theory

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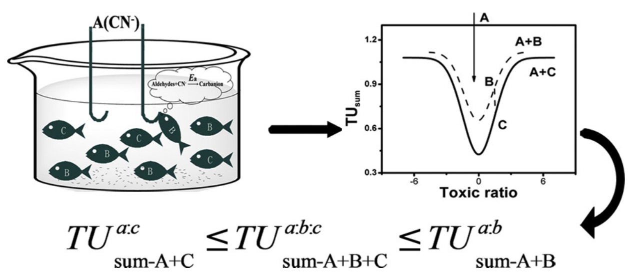
HIGHLIGHTS

- The fishing hypothesis was proposed based on the transition state theory.
- The hypothesis was used to reveal variation rules of joint effects of mixtures.
- Joint effects of multicomponent mixtures are among those of binary mixtures.
- Answer to the question why joint effect of binary equitoxic mixtures is the strongest.
- Joint effects of multicomponent mixtures can be predicted using the hypothesis

GRAPHICAL ABSTRACT



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ABSTRACT

Although the study of the variation rules of the joint effects for multicomponent mixtures has gained increasing attention, it still remains unclear how the variation occurs and what the relationships between the joint effects of multicomponent mixtures and their corresponding binary mixtures are. To explain how the variation occurs, this study first proposes a hypothesis on the variation rules of the joint effects using the well-known transition state theory. The hypothesis concluded that the joint effect of multicomponent mixtures is among the joint effects of the corresponding binary mixtures. This hypothesis was named the fishing hypothesis because there is a similarity between the action process of the joint effects and the fishing process. Next, the hypothesis was validated by use of the experimental data by evaluating the joint effects of binary, ternary and quaternary mixtures containing cyanogenic toxicants and aldehydes on *Photobacterium phosphoreum*. The application of the fishing hypothesis can explain the rule as to how the joint effects of a multicomponent-mixture vary with its number of components and their ratios. This study provides a good method to predict the joint effects of multicomponent mixtures using the joint effects of their corresponding binary mixtures. An improvement in the fishing hypothesis will be needed in our future studies due to the approximate assumptions used in the deduction of the hypothesis

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1. Introduction

Organisms in real environments are seldom exposed to single chemicals but are frequently exposed to multicomponent chemical mixtures [1,2]. However, current environmental quality standards and ecological risk assessments are mostly based on the experimental toxicity data of a single component [3]. As is well known, interactions among components in a mixture might cause intricate and substantial variations in the apparent properties of its constituents; thus, the joint effects of mixtures cannot be predicted by only using the toxicities of single components [4–6]. Furthermore, it is impossible for us to detect all of the toxicities for all of the possible mixtures. Consequently, the prediction of the toxicities of the mixtures is important and considerable research has focused on this issue. For example, Faust et al. assessed the joint toxicities of 16 biocides using independent action (IA) models [7]. Rosal et al. employed the combination index (CI) to assess the interactions of mixtures [8,9]. Our previous work predicted the joint effects of binary mixtures using Quantitative Structure Activity Relationship (QSAR) models [10]. However, although these methodologies fostered the assessment of the joint effects of mixtures, there is still a lack of general methodologies to identify or predict the occurrence of the toxicities of mixtures, as noted by Altenburger et al. [11,12]. This is because these methodologies usually focus on certain types of mixtures rather than reveal the variation rules of the joint effects for mixtures in general, i.e., the rules that govern how the joint effect of a multicomponent-mixture will vary with its number of components and their ratios. It is therefore necessary to reveal the various joint effect rules, which will provide a theoretical basis for prediction of the joint effects for multicomponent mixtures.

In the field of environmental toxicology, the study on the variation rules of the joint effects for multicomponent mixtures is becoming a hot issue [13–17]. A pioneering work proposed a hypothesis to explain the variation in the toxicities of equitoxic mixtures of nonspecific toxicants (narcotics) and found that the synergistic or antagonistic effects (interactions) will weaken as the number of components in the mixtures containing narcotic chemicals increased. The figure showing this effect seems to resemble a funnel, and therefore, it was named the funnel hypothesis (Fig. A1, see Appendix A in the supporting information) [18].

A climax hypothesis was then proposed in our previous work to reveal the variation of the joint effects for mixtures of reactive toxicants (Fig. 1) [19]. It was demonstrated that the joint effect at equitoxic ratios was the strongest, i.e., a climax was seen at the equitoxic ratios in the figures with TU plotted vs the toxic ratios. In this hypothesis, each figure has a climax, and therefore, it was named the climax hypothesis.

However, the hypothesis only discussed the climax phenomenon that states that the joint effects of binary mixtures at equitoxic ratios are stronger than that at non-equitoxic ratios. It was still impossible to predict the joint effects of multicomponent mixtures. By now, the joint effects of binary mixtures can be readily obtained and abundant data have been obtained. This raised the following questions: can the abundant binary mixture data be used to predict the joint effects of multicomponent mixtures; what is the relationship between the joint effects of binary mixtures and multicomponent mixtures; and do some variation rules exist within the relationships? If it was found that some variation rules exist, this study strove to answer the question as to why do such rules exist? These were the questions that we strove to answer in the present study.

As is well-known, transition state theory (TST) is usually used to estimate the reaction rate constant and explain how chemical reactions occur [20–22]. Many previous studies have demonstrated that the application of TST could provide an approach to revealing the essence of a chemical reaction process [23,24]. Li et al. studied the atmospheric reactions of nitrogen dioxide with different aldehydes (formaldehyde, acetaldehydes, propanal and butyraldehyde) in the environment using TST [20]. The calculated rate constants using the TST were consistent with the experimental results, which concluded that the rate constant of butyraldehyde was smaller than for the other aldehydes. This indicates that the TST can be employed to investigate the variation rules of chemical reactions of multicomponent mixtures. Our previous study found that the joint effects between cyanogenic compounds and aldehydes are very interesting; their joint effects vary from addition to synergism and antagonism [25]. In addition, we further revealed that these interesting results were due to the intracellular chemical interactions between the individual chemicals [17,26,27]; cyanogenic compounds were hydrolyzed to release CN^-, and then, CN^- reacted with the aldehydes and generated a cyanohydrin carbanion intermediate (Fig. 2). Consequently, the TST can also be employed to explain how these intracellular chemical reactions occur and to reveal the joint toxicological mechanism and the variation rules of the joint effects for these mixtures.

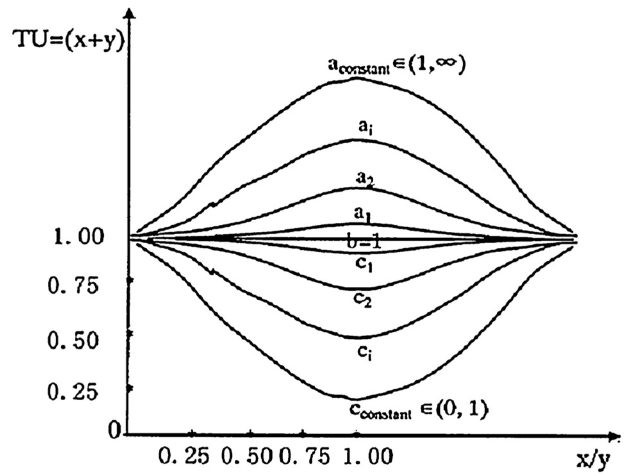

Fig. 1. Schematic of the climax hypothesis (from Lin et al. [19]).

Furthermore, it was also found based on these studies that the chemical interaction process for mixtures was similar to a fishing process. For example, for a ternary mixture containing one cyanogenic compound (A) and two aldehydes (B and C), the joint effect of the mixture was related to its intracellular chemical reaction [10]. In the intracellular chemical reaction, the cyanogenic compound in the mixture was first hydrolyzed to release cyanide ions (CN^-) under the action of enzymes. Then, the CN^- reacted with the aldehyde that possessed the stronger reactivity. It was assumed that the reactivity of aldehyde C was stronger than that of aldehyde B. After a while, the reaction of CN^- with aldehyde C weakened and the reaction of CN^- with aldehyde B was triggered. If CN^- (or the cyanogenic compound) was observed as a fishhook, aldehydes B and C could be observed as different types of fish. The reactivity between CN^- and the aldehydes was seen as the bait in Fig. 3. The amount of the fish hooked should be related to the amounts of the different types of fish and the attractiveness of the bait to the different types of fish. The fish with a larger population or a stronger attraction to the bait will be hooked first. Obviously, the fishing process is similar to the action process of the mixture toxicity. Based on their similarity, the present study proposed a fishing hypothesis to further explain the variation rules of the joint effects for mixtures containing cyanogenic compounds and aldehydes.

The purposes of the present study were therefore to use TST to investigate the process of the intracellular chemical reactions of mixtures containing cyanogenic compounds and aldehydes by a proposed fishing hypothesis, to find the variation rules of the

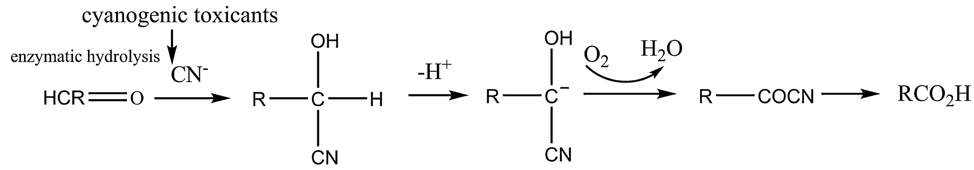

Fig. 2. Intracellular chemical reaction of cyanogenic compounds and aldehydes (from Lin et al. [10]).

joint effects for the multicomponent non-equitoxic mixtures and to provide a good way to predict the joint effects for multicomponent mixtures.

2. Theory

The premise of the fishing hypothesis (Fig. 3) is as follows: (1) a fishhook can only catch one fish, and (2) there is competition among different types of fish for the same hook but no competition among different types of fish, i.e., there is a competition between different aldehydes for the same CN^-, but no competition among different aldehydes.

Previous studies revealed that the joint effects of binary equitoxic mixtures containing cyanogenic compounds and aldehydes (TU_{sum}) were related to their intracellular chemical reaction [10]. Based on the intracellular chemical reaction, it can be deduced that there was a relationship between the joint effects of binary mixtures and their reaction rates.

$$TU_{sum} = f(v) \tag{1}$$

For a chemical reaction (A + B → product), the reaction rate (v) can be quantified based on the principle of reaction kinetics according to Eq. (2),

$$v = kC_AC_B \tag{2}$$

where k is a reaction rate constant, and C_A and C_B are the concentrations of the reactants. The reaction rate constant can be obtained using the Arrhenius equation,

$$k = A\ \exp\left(-\frac{E_a}{RT}\right) \tag{3}$$

where A is a pre-exponential factor, E_a is the activation energy, R is the Boltzmann constant, and T is the absolute temperature in Kelvin.

The basic functional relationship between the joint effects of a binary mixture of cyanogenic compounds and aldehydes (TU_{sum}) and the reaction rate was obtained by combining the three equations listed above to give the following equation:

$$TU_{sum} = f(v) = f(Ae^{-E_a/RT}C_AC_B) \tag{4}$$

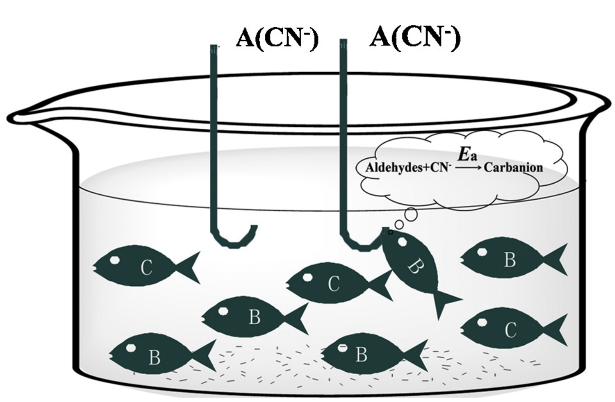

Fig. 3. Schematic of the fishing hypothesis.

3. Materials and instrumentation

Malononitrile was obtained from the Sinopharm Chemical Reagent Company (Shanghai, China). The other chemicals in this study were purchased from the Sigma–Aldrich Company (St Louis, MO, USA). All of these chemicals were of analytical reagent grade. The instrument for the toxicity testing (chemiluminescent immunoassay analyzer BH9507) was supplied by the Beijing Hamamatsu Company (Beijing, China). The freeze-dried marine bacterium, *Photobacterium phosphoreum* (T_3 mutation), was supplied by the Institute of Soil Science at the Chinese Academy of Sciences (Nanjing, China) and was reconstituted and maintained on agar slants at 4 °C.

3.1. Toxicity experiment

Bioluminescence assays were performed using the diluted bacteria, which had been cultured at 20 °C in a yeast-tryptone-salt-glycerol broth for 12–14 h [27]. The toxicity was measured by quantifying the decrease in the light emission from the luminous bacteria as a result of exposure to a 3% NaCl solution of the test chemicals or mixtures with the duration of 15 min [28]. The decrease in the light emission was measured at different concentrations and each concentration was tested in triplicate. The EC_{50} value was then calculated using a probit model based on the relationship between the decreases in the light emissions and the corresponding concentrations [29].

As soon as the EC_{50} of a single chemical was obtained, mixtures with different ratios of the chemicals were prepared and were immediately tested [30]. The test method for mixtures was conducted in a similar manner to the method used for the single chemical tests [27]. The joint effect of a mixture at the median inhibition (TU_{sum}, the sum of the toxic units at the median inhibition) was calculated by Eq. (5).

$$TU_{sum} = \frac{C_A}{EC_{50\text{-}A}} + \frac{C_B}{EC_{50\text{-}B}} \tag{5}$$

C_A and C_B are the concentrations of components A and B in the mixtures at the median inhibition. $EC_{50\text{-}A}$ and $EC_{50\text{-}B}$ are the median effective concentrations of the single chemicals A and B, respectively.

3.2. Calculation of the transition state

All of the calculations were performed using Gaussian 09 software (Gaussian, Inc.). The optimizations of the minima as well as the transition structures were carried out using the hybrid density functional theory (DFT) method at the B3LYP/6-31G level. The frequency calculations were performed to identify the stationary points as local minima or transition structures. The intrinsic reaction coordinate (IRC) calculations were employed to ensure that all of the transition states had the desired reaction coordinates.

3.3. Statistical analysis

Data analysis was conducted using SPSS 18.0 software (SPSS Inc., Chicago IL). A *t*-test was employed to perform a statistical analysis,

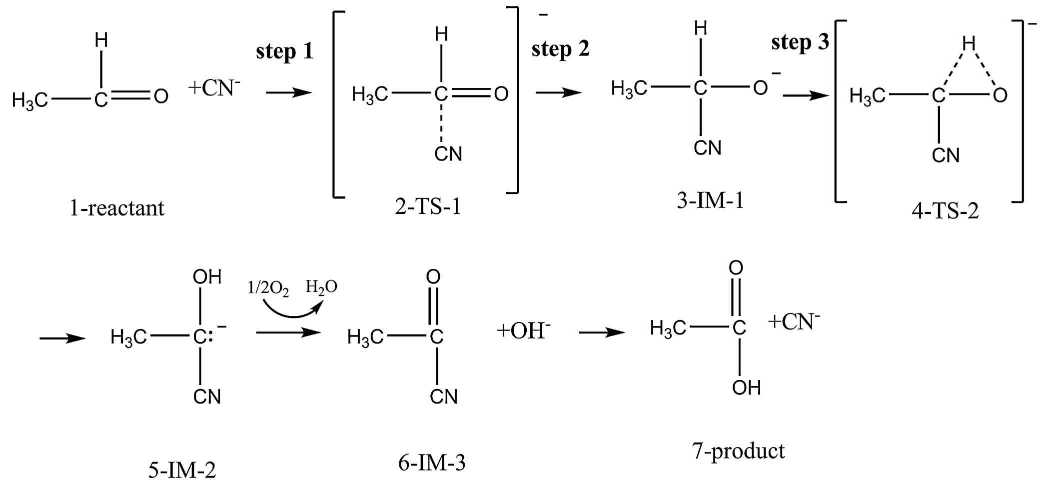

Fig. 4. Reaction process between CN^- and acetaldehyde.

and the obtained significance level (*P*) was used to assess whether there was a significant difference between the joint effects of two mixtures. A value of $P < 0.05$ indicated that the difference was significant, and a value of $P > 0.05$ indicated that the difference was insignificant [31].

4. Results and discussion

4.1. The proposal of the fishing hypothesis

4.1.1. Calculation of the transition state

Based on the intracellular chemical reaction (Fig. 2), the Gibbs free energies (ΔG) of the reactants, products, transition states and intermediates were calculated. The potential energy curves of the reactions were plotted accordingly, and the results for the mixtures containing acetaldehyde are shown in Figs. 4 and 5[b]. The other results are shown in the supplementary information in Figs. A2–A11.

It can be shown from Figs. 4 and 5 that the activation energies of the reactions of CN^- with different aldehydes varied. The activation energy, regarded as the bait in the fishing hypothesis, is related to the reactivity of the aldehydes with CN^-. However, the calculated E_a did not directly correlate to the corresponding joint effects of the cyanogenic compounds and aldehydes at equitoxic ratios. This is because the concentrations of different cyanogenic compounds and aldehydes at the equitoxic ratios differed. This phenomenon demonstrates that the joint effects are related not only to activation energies but also to concentrations of the reactants. This deduction qualitatively validates the basic relationship between the joint effects of binary mixtures and the reaction rates (Eq. (4)). The following section will validate the relationship from a quantitative point of view.

4.1.2. Validation of the relationship between the joint effects of binary mixtures and the reaction rates

To validate the relationship between the joint effects of binary mixtures and the reaction rates, the individual toxicities of individual chemicals were first determined, and then, the joint effects of binary mixtures containing cyanogenic compounds (A) and aldehydes (B) were determined based on their individual toxicities. The results are shown in the supplemental information (Tables A1 and A2). The plotted curves of the toxic ratios vs the joint effects of these binary mixtures follow normal distributions (as shown in Fig. 6). The curves can be divided into two sections when taking the zero point on the *x* axis as a reference: the L curve (left) and the R curve (right). In the L curve, the concentrations of the cyanogenic compounds changed continuously while the concentrations of the aldehydes remained constant. In the R curve, the concentrations of the cyanogenic compounds remained constant and the concentrations of the aldehydes changed continuously. It should be noted that, in the present study, we only discuss the parts of the curves that represent the joint effects that are less than 1 and do not discuss the parts of the curves that represent additive joint effects.

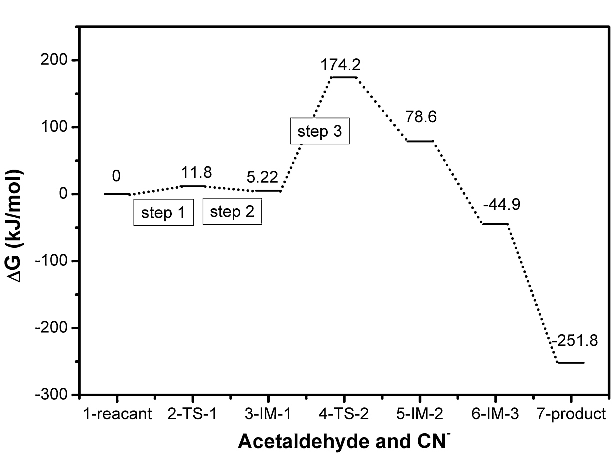

Fig. 5. Potential energy curve of the reaction between CN^- and acetaldehyde.

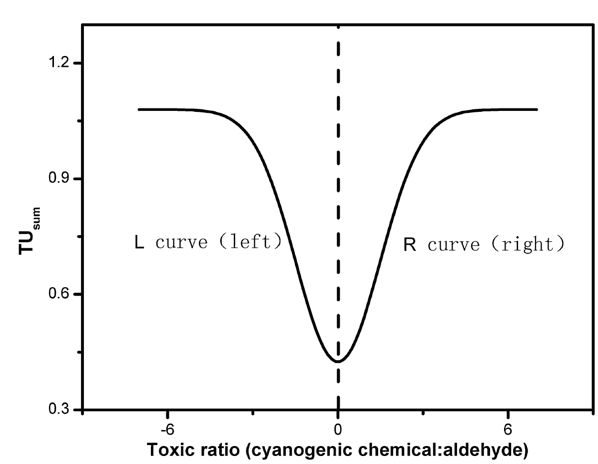

Fig. 6. Illustration of the toxic ratios vs the joint effects in binary mixtures.

The total joint effects of the binary mixtures were closely related to step 1 and step 3 in Figs. 4 and 5 and thus can be described by the following equations:

$$TU_{sum} = f(\nu_{step\text{-}1}) + f(\nu_{step\text{-}3}) \quad (6)$$

$$f(\nu_{step\text{-}1}) = f(A_1 e^{-E_{a\text{-}1}/RT} C_A C_B) \quad (7)$$

$$f(\nu_{step\text{-}3}) = f(A_3 e^{-E_{a\text{-}3}/RT} C_{3-IM\text{-}1}) \quad (8)$$

For mixtures on the L curve, the concentrations of the cyanogenic compounds changed continuously while the concentrations of the aldehydes remained constant. The CN^- ion could regenerate at the end of the reaction, and therefore, the concentration of the intermediate 3-IM-1 ($C_{3\text{-}IM\text{-}1}$) mainly depended on the concentrations of the aldehydes. The concentrations of the aldehydes in the L curve remained constant, and therefore, Eq. (8) can be rewritten as follows:

$$f(\nu_{step\text{-}3}) = b_1 \ (\text{where } b_1 \text{ is a fixed value}) \quad (9)$$

Combining Eqs. (9) and (6) yields the following equations:

$$TU_{sum} = f(e^{-E_{a\text{-}1}/RT} C_A C_B) + b_1 \quad (10)$$

$$\frac{TU_{sum}}{C_A} = f(-aE_{a\text{-}1} + b_2) \quad (11)$$

For mixtures on the R curve, the concentrations of the cyanogenic compounds remained constant and the concentrations of the aldehydes changed continuously. The CN^- ion could regenerate at the end of the reaction, and the concentration of the intermediate 3-IM-1 ($C_{3\text{-}IM\text{-}1}$) depended on concentrations of the aldehydes (C_B). So, Eq. (8) can be rewritten as follows:

$$f(\nu_{step\text{-}3}) = f(A_3 e^{-E_{a\text{-}3}/RT} C_{3-IM\text{-}1}) = f(A_3 e^{-E_{a\text{-}3}/RT} C_B) \quad (12)$$

Combining Eq. (12) with Eqs. (6) and (7) yields the following equation:

$$TU_{sum} = f(A_1 e^{-E_{a\text{-}1}/RT} C_A C_B) + f(A_3 e^{-E_{a\text{-}3}/RT} C_B) \quad (13)$$

Because $E_{a\text{-}1}$ is far less than$E_{a\text{-}3}$, the contribution of the total joint effects from step 1 is far less than the contribution from step 3:

$$f(\nu_{step\text{-}1}) \ll f(\nu_{step\text{-}3}) \quad (14)$$

Thus, the joint effects for the mixtures on the R curve can be obtained as follows:

$$TU_{sum} = f(A_3 e^{-E_{a\text{-}3}/RT} C_B) \Rightarrow \frac{TU_{sum}}{C_B} = f(-aE_{a-3} + b) \quad (15)$$

To validate the relationship between the joint effects of these mixtures and the reaction rates (Eqs. (11) and (15)), the joint effects of these mixtures and the activation energies (E_a) were obtained and can be found in the supplementary information (Table A2 and Figs. A2–A11).

For mixtures on the L curve, there is a correlation between the joint effects (TU_{sum}) and the concentrations of the aldehydes (C_B), and the results are shown in Table 1.

$$k_B = \frac{TU_{sum}}{C_B} + \frac{b_3}{C_B} \quad (16)$$

The relationship between k_B (the ratio of TU_{sum} to C_B) and the activation energies in step 3 ($E_{a\text{-}3}$) can also be obtained. These results validate the fact that there is a basic functional relationship between the joint effects and the reaction rates for mixtures on L curves.

$$\ln E_{a\text{-}3} = 0.00002 \times k + 5.122, \quad n = 6, \quad r = 0.797 \quad (17)$$

For mixtures on the R curve, the relationship between TU_{sum} and the concentrations of cyanogenic chemicals (C_A) can be obtained (Table 2).

$$k_A = \frac{TU_{sum}}{C_A} + \frac{b_2}{C_A} \quad (18)$$

The relationship between k_A (the ratio of TU_{sum} to C_A) and the activation energies in step 1 ($E_{a\text{-}1}$) can also be obtained. These results validate that there is a basic functional relationship between the joint effects and the reaction rates for mixtures on the R curves.

$$\ln E_{a\text{-}1} = 0.012 \times k_B - 1.404, \quad n = 6, \quad r = 0.897 \quad (19)$$

In general, the above experimental data validates that there is a basic functional relationship between the joint effects and the reaction rates for mixtures on both the L and R curves. The basic functional relationship between the joint effects and the reaction rates can be concluded as follows:

$$TU_{sum} = f(Ae^{-E_a/RT} C_A C_B) \quad (20)$$

Specifically, for mixtures on the R curve, the concentrations of the cyanogenic compounds remained constant, and the concentrations of the aldehydes changed continuously. This basic functional relationship (Eq. (20)) can be rewritten as follows:

$$TU_{sum} = f(A_1 e^{-E_{a-1}/RT} C_A C_B) \quad (C_B \text{ is a fixed value}). \quad (21)$$

For mixtures on the L curve, the basic functional relationship (Eq. (20)) can be rewritten as follows:

$$TU_{sum} = f(A_3 e^{-E_{a-3}/RT} C_A C_B) \quad (C_A \text{ is a fixed value}). \quad (22)$$

4.1.3. The development of the fishing hypothesis

For a ternary mixture (cyanogenic compound A + aldehyde B + aldehyde C), its joint effect can be defined as $TU_{sum\text{-}A+B+C}$. The three components consisted of the corresponding binary mixtures, including mixture A + B and mixture A + C. The joint effects of the two binary mixtures were defined as $TU_{sum\text{-}A+B}$ and $TU_{sum\text{-}A+C}$. It was assumed that the joint effect of mixture A + C was stronger than that of mixture A + B, i.e., $TU_{sum\text{-}A+C} < TU_{sum\text{-}A+B}$ (Fig. 7). The purpose of the fishing hypothesis was to reveal the relationship between the joint effect of the ternary mixture ($TU_{sum\text{-}A+B+C}$) and the joint effects of the corresponding binary mixtures ($TU_{sum\text{-}A+B}$, $TU_{sum\text{-}A+C}$).

4.1.3.1. Relationship between $TU_{sum\text{-}A+B+C}$ and $TU_{sum\text{-}A+C}$. Because the joint effect of mixture A + C is stronger than that of mixture A + B ($TU_{sum\text{-}A+C} < TU_{sum\text{-}A+B}$), the following inequality can be obtained based on the basic functional relationship between the joint effects and reaction rates:

$$f(A_B e^{-E_a/RT} C_{CN^-} C_B) > f(A_C e^{-E_{a\text{-}c}/RT} C_{CN^-} C_C) \quad (23)$$

The joint effect of the ternary mixture A + B + C equals the sum of the contributions of the joint effects of the binary mixtures A + B and A + C according to Eq. (24).

$$TU_{sum\text{-}A+B+C} = f(\nu) = f(\nu_{C\text{-}(0\to t_1)}) + f(\nu_{B\text{-}(t_1\to t_2)}) \quad (24)$$

In the ternary mixture, CN^- first reacted with aldehyde C, which possessed a stronger reactivity with CN^- ($t = 0$, t denotes the reaction time). When the effective concentration of aldehyde C (C_C) was reduced to zero ($t = t_1$), the reaction between CN^- and aldehyde B started, and the reaction between CN^- and aldehyde C ceased. At this time, the concentration of the cyanogenic compound (C_{CN}') was obtained by the following equation:

$$C'_{CN} = C_{CN} - C_C \quad (25)$$

Table 1
Relationship between TU_{sum} and C_B (the concentrations of the aldehydes on the L curve).

Binary mixture	Fitting equation ($TU_{sum} = k_B C_B + b_3$)	Fitting parameter
Malononitrile + acetaldehyde	$TU_{sum} = 220.9\,C_B + 0.033$	$n = 9$, $r^2 = 0.999$
Malononitrile + propanal	$TU_{sum} = 383.9\,C_B + 0.066$	$n = 8$, $r^2 = 0.997$
Malononitrile + valeraldehyde	$TU_{sum} = 1479\,C_B + 0.178$	$n = 9$, $r^2 = 0.965$
Malononitrile + heptanal	$TU_{sum} = 7696\,C_B + 0.199$	$n = 12$, $r^2 = 0.981$
Malononitrile + benzaldehyde	$TU_{sum} = 2171\,C_B + 0.169$	$n = 9$, $r^2 = 0.981$
Malononitrile + *p*-nitrobenzaldehyde	$TU_{sum} = 17850\,C_B + 0.050$	$n = 14$, $r^2 = 0.997$

Table 2
The relationship between TU_{sum} and C_A (the concentrations of the cyanogenic chemical on the R curve).

Binary mixtures	Fitting equation ($TU_{sum} = k_A C_A + b_2$)	Fitting parameter
Malononitrile + acetalehyde	$TU_{sum} = 342.5\,C_A + 0.033$	$n = 9$, $r = 0.999$
Malononitrile + propanal	$TU_{sum} = 331.5\,C_A + 0.066$	$n = 8$, $r = 0.997$
Malononitrile + valeraldehyde	$TU_{sum} = 275.4\,C_A + 0.190$	$n = 9$, $r = 0.979$
Malononitrile + heptanal	$TU_{sum} = 282.0\,C_A + 0.206$	$n = 12$, $r = 0.980$
Malononitrile + benzaldehyde	$TU_{sum} = 280.3\,C_A + 0.178$	$n = 9$, $r = 0.977$
Malononitrile + *p*-nitrobenzaldehyde	$TU_{sum} = 335.8\,C_A + 0.052$	$n = 14$, $r = 0.998$

When the reaction between aldehyde B and CN^- ceased ($t = t_2$), the total joint effect can be described by the sum of the contributions of the reactions of CN^- with aldehyde B and aldehyde C.

$$TU_{\text{sum-A+B+C}} = f(v_C) + f(v_B) = f(A_C e^{-E_a/RT} C_{CN^-} C_C) + f(A_B e^{-E_a/RT} C'_{CN^-} C_B) \quad (26)$$

Based on the functional relationships between the joint effects and the reaction rates, the following relationship can be deduced:

$$0 < f(A_B e^{-E_a/RT} C_{CN^-} C_B) < f(A_B e^{-E_a/RT} C'_{CN^-} C_B) \quad (27)$$

Combining Eq. (26) with Inequality (27) yields the following equations:

$$TU_{\text{sum-A+B+C}} - TU_{\text{sum-A+C}} = TU_{\text{sum-A+B+C}} - f(A_C e^{-E_a/RT} C_{CN^-} C_C) = f(A_B e^{-E_a/RT} C'_{CN^-} C_B) > 0 \quad (28)$$

$$TU_{\text{sum-A+B+C}} - TU_{\text{sum-A+C}} > 0 \Rightarrow TU_{\text{sum-A+B+C}} > TU_{\text{sum-A+C}} \quad (29)$$

The result shows that the joint effect of the ternary mixture ($TU_{\text{sum-A+B+C}}$) is weaker than the joint effect of the binary mixture $TU_{\text{sum-A+C}}$.

4.1.3.2. Relationship between $TU_{\text{sum-A+B+C}}$ and $TU_{\text{sum-A+B}}$. According to the basic functional relationship between the joint effects and the reaction rates, Eq. (25) can be rewritten as follows:

$$f(A_B e^{-E_a/RT} C'_{CN^-} C_B) = f(A_B e^{-E_a/RT} (C_{CN^-} - C_C) C_B) = f(A_B e^{-E_a/RT} C_{CN^-} C_B) - f(A_B e^{-E_a/RT} C_C C_B) \quad (30)$$

Combining Eq. (30) with Eq. (26) yields the following equations:

$$TU_{\text{sum-A+B+C}} = f(A_C e^{-E_a/RT} C_{CN^-} C_C) + f(A_B e^{-E_a/RT} C_{CN^-} C_B) - f(A_B e^{-E_a/RT} C_C C_B) \quad (31)$$

$$TU_{\text{sum-A+B+C}} - TU_{\text{sum-A+B}} = TU_{\text{sum-A+B+C}} - f(A_B e^{-E_a/RT} C_{CN^-} C_B) = f(A_C e^{-E_a/RT} C_{CN^-} C_C) - f(A_B e^{-E_a/RT} C_C C_B) \quad (32)$$

Because the joint effect of mixture A + C is stronger than that of mixture A + B, the following inequality can be deduced:

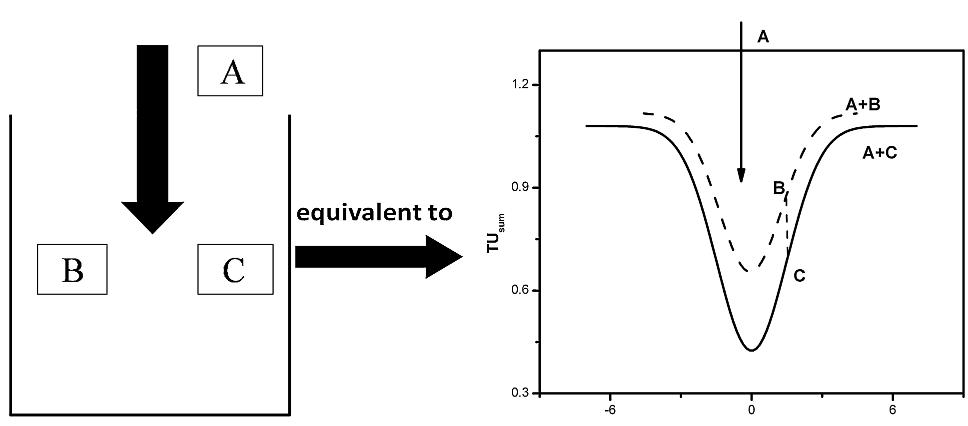

Fig. 7. The fishing hypothesis in ternary mixtures.

$$f(A_C e^{-E_a/RT} C_{CN^-} C_C) < f(A_B e^{-E_a/RT} C_{CN^-} C_B) < f(A_B e^{-E_a/RT} C_C C_B) \quad (33)$$

Combining Eq. (32) with Inequality (33) yields the following inequality:

$$TU_{\text{sum-A+B+C}} - TU_{\text{sum-A+B}} < 0 \Rightarrow TU_{\text{sum-A+B+C}} < TU_{\text{sum-A+B}} \quad (34)$$

The result shows that the joint effect of the ternary mixture A + B + C ($TU_{\text{sum-A+B+C}}$) is stronger than that of the binary mixture A + B ($TU_{\text{sum-A+B}}$).

Combining Inequalities (29) and (34) yields the following inequality:

$$TU_{\text{sum-A+C}} < TU_{\text{sum-A+B+C}} < TU_{\text{sum-A+B}} \quad (35)$$

This inequality can be employed for mixtures not only at equitoxic ratios but also for mixtures at non-equitoxic ratios.

$$TU^{a:c}_{\text{sum-A+C}} < TU^{a:b:c}_{\text{sum-A+B+C}} < TU^{a:b}_{\text{sum-A+B}} \quad (36)$$

The superscript (a, b and c) indicates the toxic ratios of chemicals A, B and C in the mixtures ($a = C_A/EC_{50\text{-A}}$, $b = C_B/EC_{50\text{-B}}$ and $c = C_C/EC_{50\text{-C}}$). For example, for a mixture at a toxic ratio of 10:2:1, its corresponding binary mixtures are a mixture A + B at a ratio of 10:2 and mixture A + C at a ratio of 10:1. The result shows that the total joint effect of the ternary mixture (A + B + C) is between the joint effects of the corresponding binary mixtures (A + B and A + C).

In the mixture, cyanogenic compound A was observed as the bait, and aldehydes B and C were observed as the fish. The concentrations of the individual aldehydes were observed as the amount of fish. The reactivity of the cyanogenic compound with the individual aldehydes was observed as the attraction of the fish to the bait, which was related to the activation energy E_a and the concentrations of the reactants. When the concentration of the bait remained constant, the amount of the fish that were caught depended on the amount of fish and the attraction of the fish to the bait. When the amount of fish remained constant, the amount of the fish caught depended on the amount of the fishhooks and the attraction of the fish to the bait. The fishing hypothesis can therefore be summarized as follows: the total amount of the fish caught is between the amounts of the fish caught by a single fishhook, i.e., the joint effects of multicomponent mixtures depend on the concentrations of the components, the reactivity between the components and should be among the joint effects of the corresponding binary mixtures.

4.2. Validation of the fishing hypothesis

The joint effects of binary, ternary and quaternary mixtures containing cyanogenic compounds and aldehydes were determined to validate the fishing hypothesis, and the results are shown in Fig. 8.

It can be shown from Fig. 8 that the joint effects of the ternary mixtures ($TU_{\text{sum-A+B+C}}$) are between the joint effects of the corresponding binary mixtures ($TU_{\text{sum-A+B}}$ and $TU_{\text{sum-A+C}}$). For example, the joint effect of the ternary mixture containing malononitrile (A), benzaldehyde (B) and *p*-nitrobenzaldehyde (C) at an equitoxic ratio ($TU_{\text{sum-A+B+C}}$) was 0.20 as seen in Fig. 8(II). The joint effects of the corresponding binary mixtures were 0.12 ($TU_{\text{sum-A+C}}$) and 0.38 ($TU_{\text{sum-A+B}}$), respectively. Other mixtures at non-equitoxic ratios in these figures also agree well with the fishing hypothesis. The results are consistent with the fishing hypothesis.

It should be noted that, when the concentration of a component is too large, the joint effect of the binary mixture might be predominant in the ternary mixture. For example, the ratio of the mixture A + B + C is 1:100:1 in Fig. 8(II). The concentration of component B is extremely large, and thus, the joint effect of the binary mixture containing the component (A + B) might equal the joint effect of the ternary mixture (A + B + C). This is consistent with the previous results reported by Rodea-Palomares et al. [9]. Accordingly, the boundary conditions of Eq. (36) can be revised as shown in Eq. (37).

$$TU^{a:c}_{\text{sum-A+C}} \le TU^{a:b:c}_{\text{sum-A+B+C}} \le TU^{a:b}_{\text{sum-A+B}} \quad (37)$$

Similarly, for a quaternary mixture, the joint effect of the quaternary mixture is also among the joint effects of the corresponding binary mixtures (Fig. 9).

It can be shown from Fig. 9 that the total joint effects of quaternary mixtures ($TU_{\text{sum-A+B+C+D}}$) are among the joint effects of the binary mixtures ($TU_{\text{sum-A+B}}$, $TU_{\text{sum-A+C}}$, $TU_{\text{sum-A+D}}$), which is consistent with the fishing hypothesis. This demonstrates that the fishing hypothesis can be employed not only for ternary mixtures but also for quaternary mixtures.

4.3. Application of the fishing hypothesis

4.3.1. Explanation of the reason why the joint effects of binary mixtures at equitoxic ratios are stronger than that at non-equitoxic ratios

Our previous study found that the joint effects of binary mixtures at equitoxic ratios were stronger than at other ratios [17,19]. However, the reason why the joint effects at equitoxic ratios were the strongest still remained unclear. The fishing hypothesis can be employed to explain the rule.

Based on the obtained basic functional relationship (Eq. (20)), the reaction rate of a binary mixture can be represented as follows:

$$v = A \times e^{-E_a/RT} \times C_A \times C_B \quad (38)$$

The concentrations of the individual chemicals in the binary mixture, C_A and C_B, can be described using their single toxicities, $EC_{50\text{-A}}$ and $EC_{50\text{-B}}$, in Eq. (39).

$$C_A = a \times EC_{50\text{-A}}, \quad C_B = b \times EC_{50\text{-B}} \quad (39)$$

In Eq. (39), a and b are the toxic ratios of chemicals A and B. Combining Eq. (38) with Eq. (37) yields the following equation:

$$\begin{aligned} v &= A \times e^{-E_a/RT} \times C_A \times C_B \\ &= A \times e^{-E_a/RT} \times a \times EC_{50\text{-A}} \times b \times EC_{50\text{-B}} \\ &= (A \times e^{-E_a/RT} \times EC_{50\text{-A}} \times EC_{50\text{-B}}) \times (a \times b) \end{aligned} \quad (40)$$

To obtain the strongest joint effect, the reaction rate of the chemicals in the binary mixture should reach a maximum value. In the above equation, only the product of the toxic ratios ($a \times b$) is variable. To obtain the maximum product of $a \times b$, Eq. (38) is combined with Eq. (1) and the following equation is obtained:

$$\begin{aligned} TU_{\text{sum}} &= \frac{C_A}{EC_{50\text{-A}}} + \frac{C_B}{EC_{50\text{-B}}} \\ &= \frac{a \times EC_{50\text{-A}}}{EC_{50\text{-A}}} + \frac{b \times EC_{50\text{-B}}}{EC_{50\text{-B}}} \\ &= a + b \end{aligned} \quad (41)$$

The product of a and b can be rewritten as the following equation:

$$\begin{aligned} a \times b &= a \times (TU_{\text{sum}} - a) = a \times TU_{\text{sum}} - a^2 \\ &= -\left(a - \frac{TU_{\text{sum}}}{2}\right)^2 + \left(\frac{TU_{\text{sum}}}{2}\right)^2 \end{aligned} \quad (42)$$

It can be deduced from Eq. (41) that the product of a and b could reach a maximum value when the mixture is at the equitoxic ratio

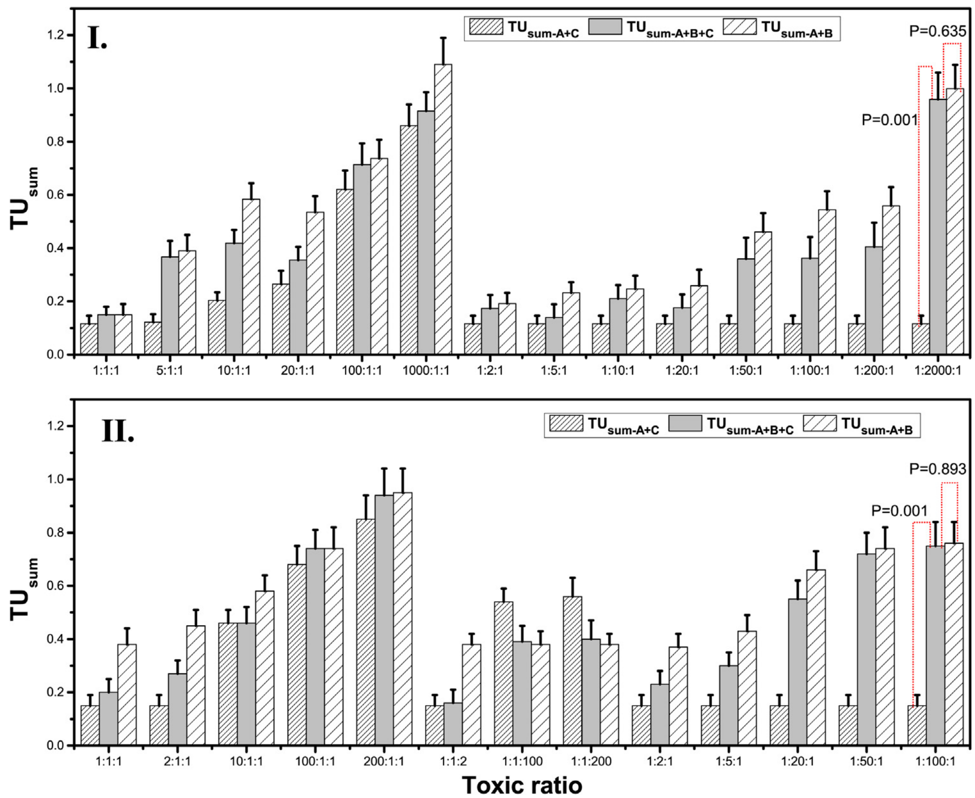

Fig. 8. Validation of the fishing hypothesis using ternary mixtures. I: mixtures containing malononitrile (A), *p*-nitrobenzaldehyde (B) and acetaldehyde (C). II: mixtures containing malononitrile (A), benzaldehyde (B) and *p*-nitrobenzaldehyde (C). P denotes the significance level between the multicomponent mixtures and the binary mixtures.

($a = b = TU_{\mathrm{sum}}/2$). Consequently, the joint effects of binary mixtures at equitoxic ratios are stronger than that at non-equitoxic ratios.

$$TU^{1:1}_{\text{sum-A+}B} \le TU^{a:b}_{\text{sum-A+B}} \tag{43}$$

This deduction demonstrates that the fishing hypothesis can be employed to explain the reason why the joint effects at equitoxic ratios are stronger than that at non-equitoxic ratios.

4.3.2. Application of the fishing hypothesis to elucidate the relationship of joint effects between multicomponent non-equitoxic mixtures and binary equitoxic mixtures

The fishing hypothesis concluded that, for a ternary mixture A + B + C, the joint effect of the multicomponent non-equitoxic mixture was between the joint effects of the two binary mixtures A + B and A + C ($TU_{\text{sum-A+C}} < TU_{\text{sum-A+B}}$). For the mixture A + B, its joint effect approaches the additive effect as its toxic ratio varies from the equitoxic ratio to non-equitoxic ratios [17].

$$\lim_{a:b\to\infty} TU^{a:b}_{\text{sum-A+B}} = 1 \tag{44}$$

For the mixture A + C, its joint effect at equitoxic ratios is stronger than at non-equitoxic ratios (Inequality (42)). Combining Eq. (43) with Inequalities (42) and (37) yields the following inequality:

$$TU^{1:1}_{\text{sum-A+C}} \le TU^{a:b:c}_{\text{sum-A+B+C}} \le 1 \tag{45}$$

This demonstrates that the joint effects of multicomponent non-equitoxic mixtures are related to the joint effects of binary equitoxic mixtures. Using ternary mixtures containing

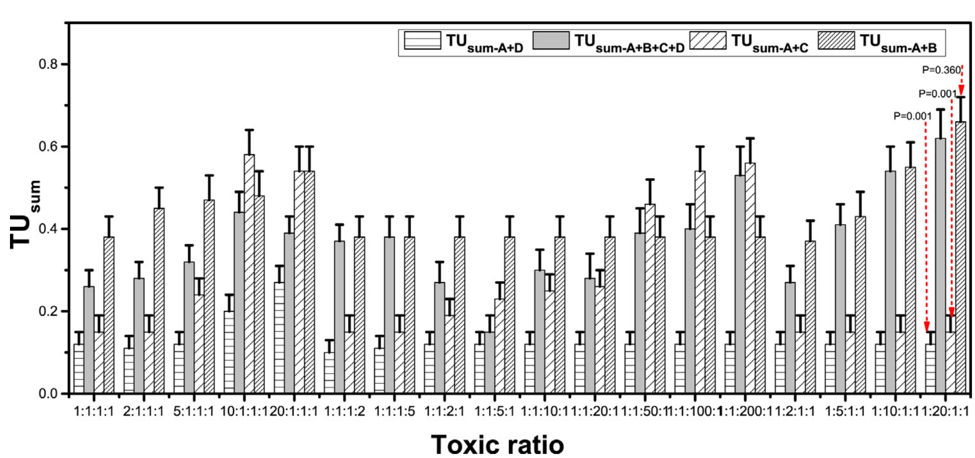

Fig. 9. Validation of the fishing hypothesis using quaternary mixtures containing malononitrile (A), benzaldehyde (B), *p*-nitrobenzaldehyde (C) and acetaldehyde (D). *P* denotes the significance level between the multicomponent mixtures and the binary mixtures.

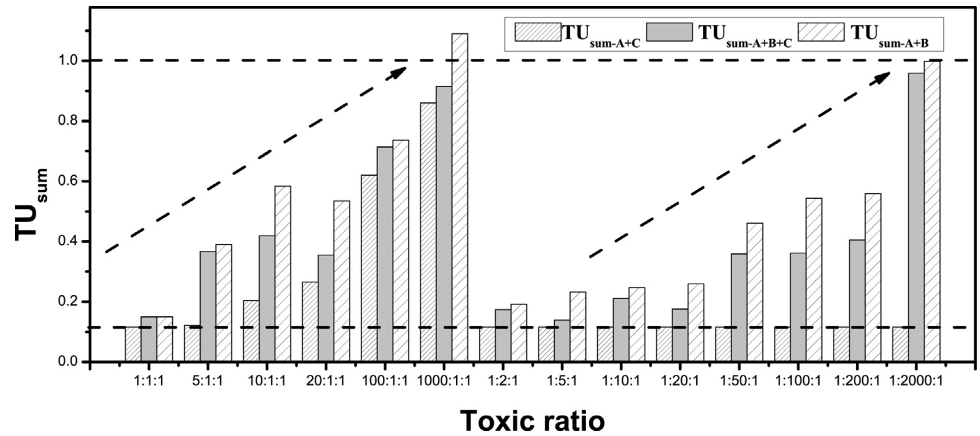

Fig. 10. Relationships between the joint effects of ternary mixtures and binary equitoxic mixtures (malononitrile (A), *p*-nitrobenzaldehyde (B) and acetaldehyde (C)).

malononitrile, acetaldehyde and *p*-nitrobenzaldehyde, the relationship was validated, and the results are shown in Fig. 10. The two dotted lines denote the strongest joint effect of the binary equitoxic ratio ($TU_{sum\text{-}A+C}$ = 0.12) and the additive effect (TU_{sum} = 1.00), respectively.

Fig. 10 shows that the joint effects of multicomponent mixtures are always weaker than the joint effects of the binary mixture A + C. As the toxic ratios vary from equitoxic ratios to non-equitoxic ratios, the joint effects approach the additive effect. This demonstrates that the fishing hypothesis can elucidate the relationship of joint effects of multicomponent mixtures and binary equitoxic mixtures, which is beneficial for the prediction of the joint effects of multicomponent mixtures. Specifically, most current literature has focused on the joint effects of binary equitoxic mixtures, while in the real environment organisms usually encounter multicomponent non-equitoxic mixtures. Using Inequality 45, we can predict the joint effects of multicomponent non-equitoxic mixtures in real environments based on the reported joint effects of binary equitoxic mixtures in the literature.

4.3.3. Application of the fishing hypothesis to mixtures containing other reactive compounds

The fishing hypothesis was proposed based on mixtures containing cyanogenic compounds and aldehydes. However, the fishing hypothesis can also be applied to other reactive toxicants, such as mixtures containing allyl chlorides or allyl alcohols (Fig. 11).

It can be seen from Fig. 11 that the joint effects of ternary mixtures ($TU_{sum\text{-}A+B+C}$) are stronger than those of binary mixtures containing malononitrile and allyl alcohol ($TU_{sum\text{-}A+C}$) and weaker than those of binary mixtures containing

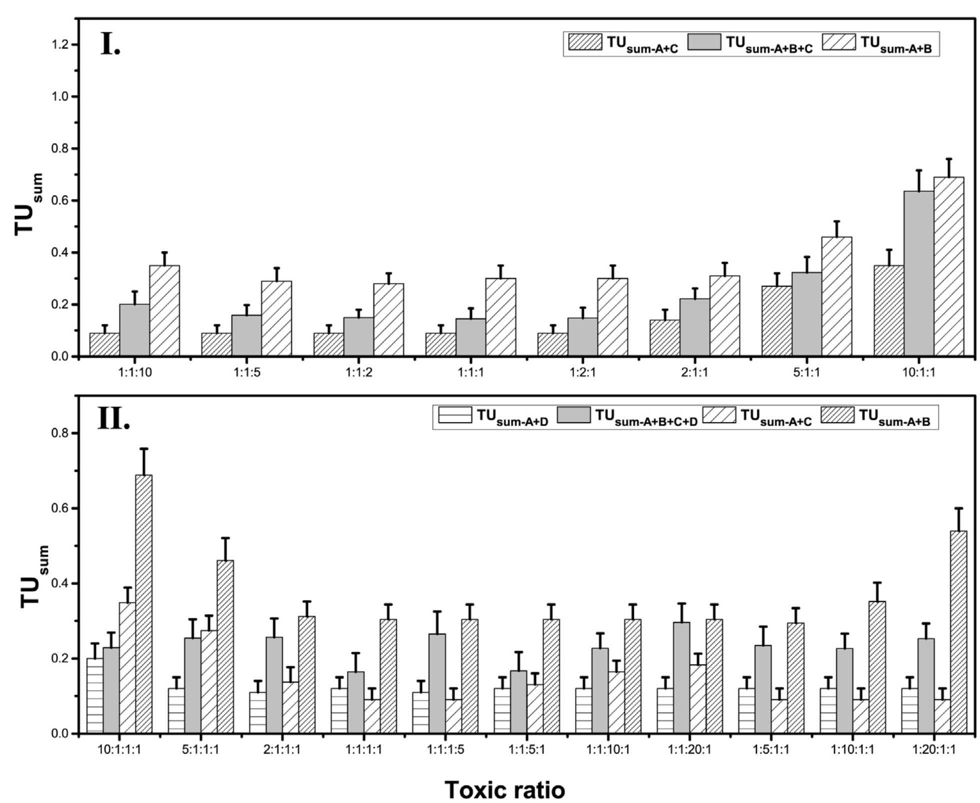

Fig. 11. Application of the fishing hypothesis to other mixtures. I: ternary mixtures containing malononitrile (A), allyl chloride (B) and allyl alcohol (C). II: quaternary mixtures containing malononitrile (A), acetaldehyde (B), allyl alcohol (C) and allyl chloride (D).

malononitrile and allyl chloride ($TU_{sum\text{-}A+B}$). The joint effects of quaternary mixtures ($TU_{sum\text{-}A+B+C+D}$) are also among the joint effects of the corresponding binary mixtures ($TU_{sum\text{-}A+B}$, $TU_{sum\text{-}A+C}$, $TU_{sum\text{-}A+D}$). This demonstrates that the fishing hypothesis, derived from mixtures containing cyanogenic compounds and aldehydes, can also be applied to mixtures containing other reactive compounds.

4.4. Limitations of the fishing hypothesis

It should be noted that, in the deduction of fishing hypothesis (Section 2), we assumed that the cyanogenic toxicant (A) first reacted with an aldehyde (C) that possessed a stronger reactivity than the other aldehyde (B). After a while, the concentration of C decreased, and its reaction rate also decreased. When the reaction rate of A + C decreased to a lower value than that of mixture A + B, the reaction of A and B was triggered. This assumption is only a theoretical assumption. In the process of a real chemical reaction, simultaneous reactions between A and B as well as A and C may occur, but the fraction of the final products would definitely depend on the concentrations of B and C and on their reaction rates with A. However, if we wanted to describe the process by using an exact mathematical equation, it would be extremely complex and would need many parameters that may be still unknown to our knowledge. Therefore, to describe the process mathematically, we assumed that the cyanogenic toxicant (A) first reacted with the aldehyde (C) that possessed the stronger reactivity.

Despite the approximate assumptions used in the deduction of the fishing hypothesis, our present study results should not be denied because the purpose of the study was only to reveal how the joint effects of multicomponent mixtures occur. Our previous study revealed that the joint effect of a ternary mixture at an equitoxic ratio depended on the joint effect of the binary mixture containing the dominate components (the individual chemicals that can yield a much stronger interaction) [17]. This phenomenon indicated that approximate assumptions in the deduction of the fishing hypothesis would not affect the joint effects of multicomponent mixtures, and therefore would not deny the results in our present study.

However, some questions will arise as to whether the assumption will give rise to uncertainty in the fishing hypothesis when the hypothesis is used beyond this study, and how we can improve the fishing hypothesis. These will be studied in our future work.

5. Conclusions

In this study, a basic functional relationship between the joint effects of binary mixtures and their reaction rates was derived based on TST, and as a result, the fishing hypothesis was proposed. The fishing hypothesis concluded that the joint effects of multicomponent mixtures depended on the concentrations of the components and the reactivity between the cyanogenic compounds and the aldehydes. The total joint effects of multicomponent mixtures were between the joint effects of the corresponding binary mixtures. Moreover, the fishing hypothesis elucidated why the joint effects of binary mixtures at equitoxic ratios were stronger than the effects at non-equitoxic ratios. This study provided insights into the variation rules of joint effects for multicomponent mixtures at non-equitoxic ratios and will be beneficial for the prediction of the joint effects for mixtures containing reactive toxicants at non-equitoxic ratios. However, it should be noted that before the fishing hypothesis can be used confidently beyond this study, it will need to be improved in our future studies due to the approximate assumptions used in the deduction of the hypothesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2013.12.035.

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