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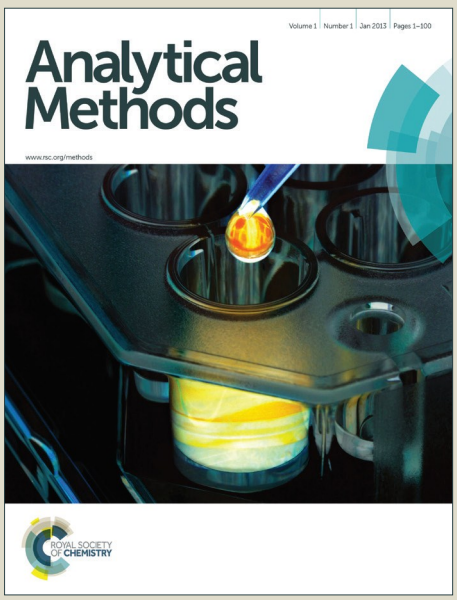

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Title Page

Concentration Addition Prediction for the Multiple-component Mixture Containing No Effect Chemicals

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Abstract The concentration addition (CA) model is usually used to assess the toxicological interaction and predict the mixture toxicity. However, when the maximum effect of a mixture is different from that of single component, it is impossible to directly use the CA to predict the mixture concentrations (C_{mix}) at some effect zones that are called as predictive blind zones (PBZone). The mixture containing no effect chemicals (NECs) is a special case of the PBZone. Aiming at this special PBZone, we assumed the EC_x of NEC to be infinity at any effects and thus readily computed various C_{mix}s in PBZone. At the same time, the combination index (CI_x) combining with its 95% observation-based confidence intervals (OCI_x) was employed to characterize the toxicological interaction. The approach was successfully used to predict the combined toxicity and assess the toxicological interactions of five chemicals, imidacloprid and metalaxyl (pesticides), 1-butylpyridinium bromide (ionic liquid), and apramycin sulfate and neomycin sulphate (antibiotics, being NECs). The results showed that except for the toxicological interactions of five mixture rays at higher effects than 25% being antagonism, all the other mixtures are additive action.

Keywords predictive blind zone, combination index, uniform design ray, combined toxicity, antagonism

Introduction

Concentration addition (CA), dose addition or Loewe additivity, is an important additive reference model in assessing toxicological interactions and predicting mixture toxicities[1-3]. The CA has been widely used to assess the combined effects of chemicals in many fields such as pharmacology and toxicology[4-6]. For a mixture of *n* components, the CA can be formulated as follows

$$\sum_{j=1}^{n} \frac{c_j}{EC_{x,j}} = 1 \quad (1)$$

where $EC_{x,j}$ refers to the concentration of *j*th (*j*=1, 2, 3,…, *n*) component that provokes x% effect when applied singly, c_j to the concentration of *j*th component in the mixture exhibiting x% effect. Eq. 1 can be rewritten as a predictive form by means of simple transformations,

$$\hat{c}_{x,mix} = 1 \Big/ \left(\sum_{j=1}^{n} \frac{p_j}{EC_{x,j}} \right) \quad (2)$$

where $\hat{c}_{x,mix}$ is the mixture concentration predicted by CA model and p_j the ratio of the molar concentration of *j*th component to the total concentration of the mixture. Thus, when p_j is fixed as a constant, the $\hat{c}_{x,mix}$s at various effects (x = 0~100) can be computed by Eq. 2 and various effective concentrations ($EC_{x,j}$) of single components. The $\hat{c}_{x,mix}$s and corresponding effects (x) form one predictive concentration-response curve (CRC_{PRE}). The prerequisite computing $\hat{c}_{x,mix}$ by CA is that all single mixture components have the same effects (x) as their mixture.

It has been shown that different chemicals have different concentration-response relationships. Most pollutants have monotonically increasing or decreasing S-shaped concentration-response curves (CRCs) where the maximum effect can reach 100% such as the

inhibition toxicities of some chemicals to *Vibrio fischeri* and green algae[7, 8]. some inorganic and organic chemicals to algae growth and bacteria luminescence have non-monotonically increasing CRCs[9-13]. In addition, some environmental pollutants have time-dependent toxicities[14-18], that is, different exposure times exhibit different toxic effects. Some chemicals such as apramycin sulfate (APR) and neomycin sulphate (NEO) even have no effects to *Vibrio qinghaiensis* sp. –Q67 (*V. qinghaiensis*) in the range of experimental concentrations. It can be predicable that the mixtures consisting of the components with different CRCs have definitely different concentration- responses, too. Thus, some mixtures do not necessarily satisfy the requirements of CA prediction (see Eq. 2) because not all the maximal effects of single components are the same as those of mixtures. In other words, not all components have definite ECx at the given effect (x%). To solve such predictive problem, new approaches such as generalized concentration addition[19, 20] and novel toxic unit extrapolation method[6] were developed. However, the techniques need to be further improved.

For convenience, we use a term, predictive blind zone (PBZone), to designate such a mixture. For example, the binary mixture system consisting of dodine and metribuzin where the maximum inhibition effect of metribuzin at the exposure time of 4 *h* on *V. qinghaiensis* is only 25%[21, 22], while the maximum effect of one mixture ray in the system is 58%, so we cannot directly use the CA to predict the mixture concentrations at the zone of effects between 25% and 58%. In other words, there is a PBZone between the effect of 25% and 58% (Fig. 1).

Fig. 1 around here

The primary purpose of this paper is to explore a special case of PBZone, a mixture which contains many mixture components with complete S-shaped CRC and at least one no effect component (NEC), and tries to address the predictive problem of effective concentrations in PBZone. We select five chemicals having different CRC profiles as mixture

components to construct the mixture system that has a PBZone. Three of five chemicals have complete S-shaped CRCs and the remaining two have no effects in the range of experimental concentrations. The uniform design ray procedure (UD-Ray)[23, 24] was used to design seven mixture rays with different mixture ratios and the microplate toxicity analysis (MTA)[25, 26] was used to determine the luminescence inhibition toxicities of the five single components and their seven mixture rays on *V. qinghaiensis*. The combination index (CI)[27-32] was used to evaluate the toxicological interaction within mixtures.

Materials and methods

Chemicals

Two antibiotics, apramycin sulfate (CAS RN 65710-07-8, APR, > 97% purity) and neomycin sulphate (1405-10-3, NEO, > 97% purity), and two pesticides, imidacloprid (138261-41-3, IMI, > 97% purity) and metalaxyl (57837-19-1, MEL, > 97% purity), were purchased from Dr. ehrenstorfer (Germany). The other ionic liquid, 1-butylpyridinium bromide (874-80-6, [bpy]Br, > 97% purity), was purchased from Acros (Belgium). Two antibiotics (APR and NEO) have not toxic to *V. qinghaiensis* in the range of experimental concentration, belonging to no effect chemicals (NECs). The essential information of the five chemicals was listed in Table S1 of the supporting information and the molecular structures were shown in Fig. S1 of the supporting information.

Toxicity test

The short-term (15 min) toxicities of five chemicals and their seven five-component mixture rays to a fresh water bacteria, *V. qinghaiensis*, were determined by the microplate toxicity analysis[25, 26]. The toxicity or effect is expressed as a percent inhibition of relative light units of *V. qinghaiensis*.

The mixture/concentration ratios (p_j) of various components in the five-component mixture ray were designed by the UD-Ray method[23, 24]. In UD-Ray design, the effective concentrations of single antibiotics, APR and NEO, were replaced by those obtained in the long-term (12 h) toxicity test because APR and NEO in the short-term (15 min) toxicity test are NECs. The mixture ratios (p_j) of various components in seven mixture rays (R1, R2, ⋯, R7) were listed in Table 1. For each mixture ray, 12 mixtures (points) with different mixture concentration levels were designed by the fixed ratio ray design (FRRD)[33, 34] (remaining p_j as a constant).

Table 1 around here

CA prediction for the mixture containing NECs

For a *n*-component mixture containing *q* NECs, the effective concentration of the mixture ($\hat{c}_{x,mix}$) at a given effect of x% can be predicted by the CA model (Eq. 3).

$$\hat{c}_{x,mix} = 1 \Big/ \left(\sum_{j=1}^{n\ \ q} \frac{p_j}{EC_{x,j}} \right) \qquad (3)$$

where the $p_j/EC_{x,j}$ contributions of NECs to the addition term (∑) approximates to zero. This is because the effects of the NECs at any concentration are equal to zero. In other words, to let the effect of a NEC reach to a specified x% (non-zero value), the concentration of the NEC must be infinity.

Combination Index (CI)

In Chou's study[27-29], the combination index (CI) of a *n*-component mixture at certain effect of x% was expressed as follows

$$CI_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1\sim n}\{(D)_j / \sum_1^n (D)\}}{(D_m)_j \{fx_j/[1-fx_j]\}^{1/m_j}} \quad (4)$$

where CI_x is the combination index of the mixture at the effect of x%, $(D_x)_{1\sim n}$ is the sum of the concentration or dose of *n* components, $\{(D)_j / \sum_1^n (D)\}$ is the mixture ratio of the *j*th components, and $(D_m)_j \{fx_j/[1-fx_j]\}^{1/m_j}$ is the concentration of the *j*th component alone that exerts the effect of x%, i.e., $EC_{x,j}$[35].

It can be proved that Eq. 4 is the sum of toxic units (TU) of various components[35]. Because $c_{x,mix}$ is the concentration of a mixture at a specific effect (x%) and the p_j is mixture ratio of the *j*th component, then $(D_x)_{1\sim n}\{(D)_j / \sum_1^n (D)\} = c_{x,mix} \cdot p_j = c_j$. Thus Eq. 4 can be rewritten as

$$CI_x = \sum_{j=1}^{n} \frac{c_j}{EC_{x,j}} \quad (5)$$

where $CI = 1$, > 1, and < 1 indicate additive action, antagonism and synergism, respectively.

For a *n*-component mixture containing *q* NECs, the combination index can be extended like CA prediction (Eq. 5) as

$$CI_x = \sum_{j=1}^{n-q} \frac{c_j}{EC_{x,j}} \quad (6)$$

In fact, any toxicity test possesses experimental error. The confidence intervals reflecting the uncertainty should be considered in the identification of toxicological interaction. In this paper, the 95% observation-based confidence intervals (OCI_x) were used to depict the uncertainty of CI_x. The OCIx come from the confidence intervals of experimental CRCs (OCIs) reported in the literatures[36,37]. If the OCI_x of CI_x are taken into account, the

upper limit of OCI_x larger than 1 and the lower limit less than 1 show an additive action. The lower limit larger than 1 indicates antagonism, and the upper limit less than 1 implies synergism.

Results and discussion

CRCs of five single components

Except for two antibiotics (APR and NEO) having no effects (15 min) in the range of experimental concentration, two pesticides (IMI and MEL) and ionic liquid ([bpy]Br) have significant monotonic S-shaped concentration-responses and their CRCs can be effectively described by non-liner functions, Logit or Weibull with location parameter (α) and shape parameter (β)[3]. The fitted α and β, determination coefficient (R^2) and root mean square error (RMSE) were listed in Table S2 and the corresponding CRCs were shown in Fig. S2 of the supporting information.

In short-term (15 min) toxicity test, two antibiotics, APR and NEO, are not toxic to *V. qinghaiensis*, belonging to NECs. To rationally design the concentrations of NECs in mixtures, we also determined the long-term (12 h) toxicity of the antibiotics. The results showed that the antibiotics have obvious long-term toxicities and their CRCs can be described by Weibull function (Fig. S2 and Table S2 of the supporting information). So, in designing the mixtures containing the NECs, we replaced the EC_xs of NECs by the EC_xs obtained in the long-term (12 h) toxicity test.

PBZone in seven mixture rays

All seven five-component mixture rays have good monotonic concentration-responses, and their CRCs can be well fitted by the Logit or Weibull function (all $R^2 > 0.98$ and RMSE < 0.03). The fitted α and β, statistics R^2 and RMSE were listed in Table 1, the experimental data

and fitted CRCs were showed in Fig. 2. The mixtures have two NECs (APR and NEO) and so have PBZones between the CRCs of mixture ray and single NEC, and CA model (Eq. 2) cannot be directly used to compute the effective concentrations of the mixtures ($c_{x,mix}$).

In the PBZone, it can be rationally thought that the concentrations of NEC components at any effects are infinite, i.e., to produce certain effect (x%), it is necessary to make the NEC's concentration reach an infinite value ($EC_x \rightarrow \infty$). Replacing the $EC_{x,NEC}$ in the original CA model (Eq. 2) by the infinity (∞), we can see the NECs have no contribution to addition term ($p_{NEC}/EC_{x,NEC} = 0$), thus Eq. 2 transforms into Eq. 3. In other words, for the prediction of the effective concentrations in the PBZone, we only need to compute the contribution of the components having obvious effects and ignore the NECs (Eq. 3). In order to inspect the concentration dependence of mixture toxicities (effects), we predicted 16 mixture effective concentrations ($EC_{x,CA}$, x=5, 10, 15, …, 80) by the CA model (Eq. 3). The effects (x%) and predictive concentrations ($EC_{x,CA}$) of each mixture ray form one predictive CRC curve (simple CRC_{PRE}). The CRC_{PRE} curves of seven mixture rays were shown in Fig. 2 (blue dash lines). From Fig. 2 (data from Table S3 of the supporting information), the CRC_{PRE}s of five rays (R1 to R5) in the high concentration levels are located above the upper limits of OCIs observed, exhibiting antagonism, while the CRC_{PRE}s in low concentration levels are located between upper and lower limits of OCIs, displaying additive actions. The CRC_{PRE}s of the other two rays (R6 and R7) are located between upper and lower limits of OCIs, being classical additive actions.

Fig. 2 around here

Quantitating toxicological interaction by CI_x

The combination index (CI_x) (Eq. 5) is often used to quantitatively evaluate toxicological interaction (synergism, additive action and antagonism) of multi-component

mixtures. We can calculate CI_xs (Eq. 6) of the mixtures containing NECs by means of a similar procedure to CA prediction. On the other hand, to reduce the effect of experimental error on the identification of toxicological interaction, we also compute the 95% observation-based confidence intervals (OCI_x) of the CI_x index. The CI_x and their OCI_x of seven mixture rays at 16 specified effects (x=5, 10, 15, ⋯, 80) were shown in Fig. 3 (detailed CI_x and OCI_x values can consult Table S4 of the supporting information).

Fig. 3 around here

As demonstrated in Fig. 3, the CI_x values of all seven rays (R1 to R7) at various effects are more than 1, they are antagonistic if ignoring OCI_x. In fact, both random error in experiments and fitted error in non-linear fitting process increase the uncertainty of CI_x. It is therefore necessary to consider the confidence intervals of CI_x. When the CI_x combining with its OCI_s is employed to identify the toxicological interaction, the rays of R1 to R5 in high effect regions (such as 0.25-0.75) are antagonism, while they in low effect regions from 0.05 to 0.10 are additive action. The other two rays, R6 and R7, are basically additive action although the lower limits of OCI_x at effect levels from 0.4 to 0.6 are a little more than 1. The toxicological interactions of seven mixture rays at several effects (0.1, 0.3, 0.5, 0.7) were summarized in Table 2.

Table 2 around here

Discussions

In this paper, we discussed a special case of PBZone in multiple-component mixtures. Howard et al. developed the generalized concentration addition (GCA) and employed the Hill function to describe the CRCs of various components and mixtures. They extended the Hill function to characterize the concentration-effect relationships in PBZone. The GCA procedure

was used to predict the joint effects of the mixtures of agonist and partial agonist[19, 20]. However, they only utilized the Hill function with slope parameter of 1 to discuss the PBZone problem, which implied that it is necessary to further examine the effects of the Hill function with various slope parameters on the mixture toxicities and introduce the other CRC functions or methodology into the PBZone study.

Moreover, our results showed that not only different mixture rays with different mixture ratios may have different toxicological interactions but also the same mixture ray at different effects or concentration levels may have different interactions. This means that it is not enough to only study the interaction of the mixtures designed by the equal effect concentration ratios (EECR) or FRRD method[38-41]. This is because the EECR or FRRD mixtures are in fact only one mixture ray of multiple-component mixture system and they cannot reflect the fact of many rays in realistic mixture system.

The assessment of toxicological interaction within mixtures has to consider the uncertainty due to the occurrence of experimental random error and fitting error. In the identification of interaction using CI_x method, only the technique combining CI_x with its OCI_x can we rationally and correctly identify whether a mixture exhibits antagonism, synergism or additive action. We can see from the computing formula of CI_x (Eq. 5), the CI_x is in fact based on concentration addition and equals to the sum of toxic units (TU)[42]. The difference between CI_x and TU method is the differences of interaction types. In TU method, apart from antagonism, synergism and additive action, there are independence and partial additive action. Because independence and partial additive action are only theoretical concepts that are difficult to and are rarely found in real mixtures, the concepts were merged into the antagonism in CI_x method.

Conclusions

This paper developed a reasonable concentration addition model to assess the combined effects of the mixtures containing at least one no-effect chemical (NEC). In the mixtures containing NEC/NECs, there is a predictive blind zone (PBZone) in which the effective concentrations cannot be directly predicted by the concentration addition. We assumed rationally that various effective concentrations (EC_x) of NEC at any effects are infinity and the EC_xs of various NECs in CA/CI prediction were replaced by the infinity. Thus, the knotty PBZone problem existing in the mixtures containing NEC/NECs was smoothly solved. The results showed that the interactions of mixtures is related to not only the mixture ratios (different mixture rays may have different interactions) but also the concentration levels of mixtures (different effect regions in the same ray may have different interactions), which implies that the assessment of toxicological interaction only at one effective concentration (such as EC_{50}) of one mixture ray (such as EECR mixtures) is not comprehensive and cannot depict the nature of interactions within a mixture system.

Acknowledgements

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Appendix A. Supplementary Material

Supplementary data associated with this article can be found, in the online version.

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Figure Captions

Figure 1 An example of predictive blind zones (PBZone). The maximum effect of metribuzin (MET) and dodine (DOD) are respectively 25% and 100% while that of their mixture ray is 58%, so there is a PBZone between the effects of 25% and 58% for the mixture ray.

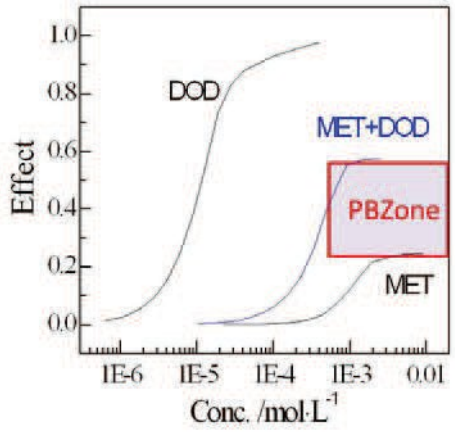
Figure 2 Concentration-response curves of seven mixture rays (○: experimental scatters at the exposure of 15 min; black solid line: fitted curves; red dot lines: 95% observation-based confidence intervals; blue dash lines: CRC predicted by CA)

Figure 3 Plot of combination index versus the effect expressed as luminescence inhibition (●: combination index; ○: 95% observation-based confidence intervals).

Table Captions

Table 1 Mixture ratios (p_j) of five components and fitted concentration-response curve (α and β) as well as statistics (R^2 and RMSE)

Table 2 Toxicological interaction (ADD represents the additive effect and ANT the antagonism) of seven mixture rays at four effect levels



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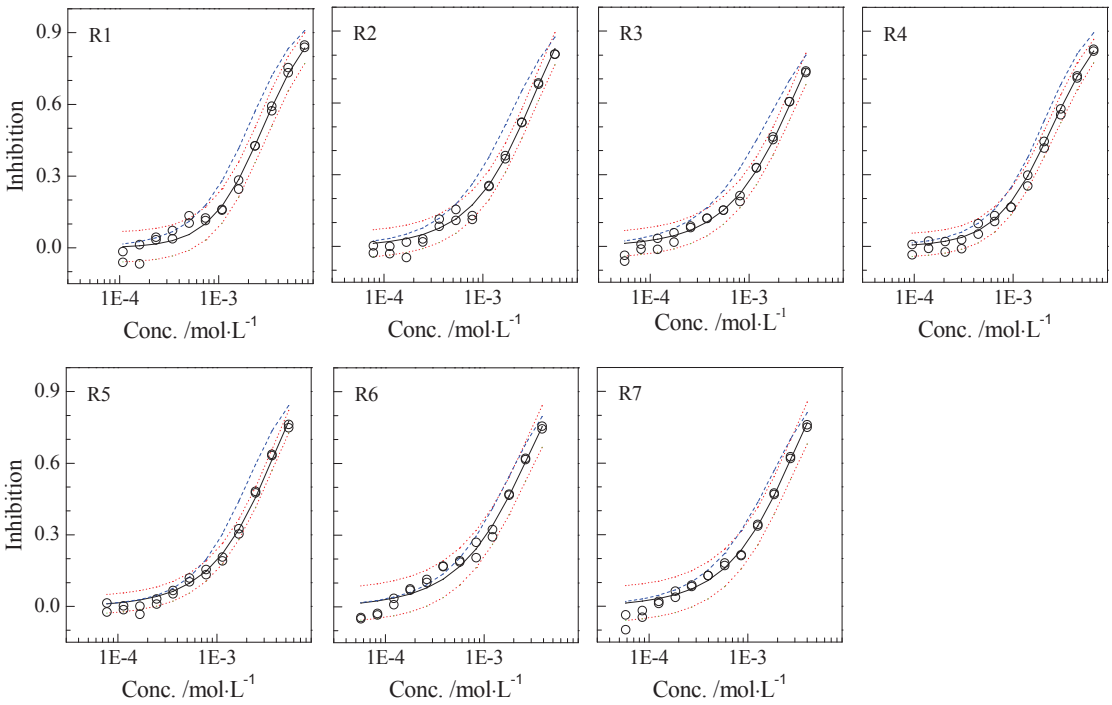

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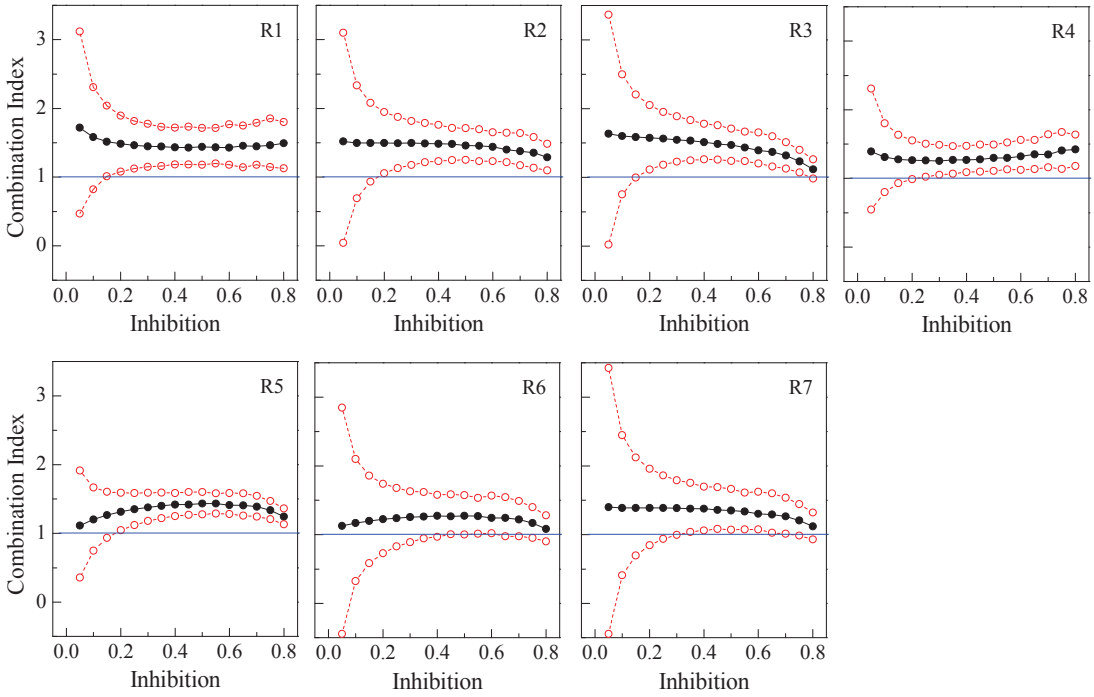


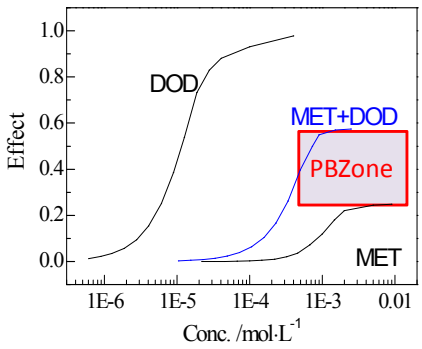
Figure 3 Plot of combination index versus the effect expressed as luminescence inhibition (●: combination index; ○: 95% observation-based confidence intervals).

Table 1 Mixture ratios (p_j) of five components and fitted concentration-response curve (α and β) as well as statistics (R^2 and RMSE)

Ray	p_j					Stock (mol/L)	Fit	α	β	R^2	RMSE
	APR	IMI	MEL	NEO	[bpy]Br						
R1	7.36830E-05	7.27172E-03	9.51183E-02	6.09713E-05	8.97475E-01	1.4824E-02	Logit	9.74	3.81	0.9922	0.0262
R2	1.53061E-04	2.39721E-02	2.49576E-01	1.61351E-05	7.26283E-01	1.0726E-02	Weibull	6.60	2.66	0.9935	0.0230
R3	3.59605E-04	8.24959E-02	1.00850E-01	1.25485E-04	8.16169E-01	7.5462E-03	Weibull	6.46	2.54	0.9923	0.0219
R4	2.65883E-04	2.13877E-03	1.49293E-01	2.49517E-05	8.48277E-01	1.3068E-02	Logit	9.25	3.55	0.9956	0.0199
R5	4.98491E-04	1.65809E-02	4.14884E-02	1.19815E-04	9.41312E-01	1.0626E-02	Weibull	6.47	2.67	0.9963	0.0160
R6	7.01685E-04	4.55705E-02	1.70002E-01	5.75716E-05	7.83668E-01	7.7430E-03	Weibull	6.20	2.43	0.9866	0.0293
R7	4.50044E-04	4.90647E-02	2.14154E-01	8.22271E-05	7.36249E-01	7.9846E-03	Weibull	6.47	2.54	0.9881	0.0295

Table 2 Toxicological interaction (ADD represents the additive action and ANT the antagonism) of seven mixture rays at four effect levels

Ray	x=0.1	x=0.3	x=0.5	x=0.7
R1	ADD	ANT	ANT	ANT
R2	ADD	ANT	ANT	ANT
R3	ADD	ANT	ANT	ANT
R4	ADD	ANT	ANT	ANT
R5	ADD	ANT	ANT	ANT
R6	ADD	ADD	ADD	ADD
R7	ADD	ADD	ADD	ADD



A concentration-effect prediction method for the mixture with a predictive blind zone (PBZone) in the mixture system containing no-effect chemicals