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Combined Toxicity of the Mixtures of Phenol and Aniline Derivatives to *Vibrio qinghaiensis* sp.-Q67

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Abstract To test whether the dose addition and independent action models can predict the combined toxicity of the mixtures of phenol and aniline derivatives, six phenolic and two aniline derivatives were selected as the test components. The inhibition toxicity of the derivatives and their mixtures to *Vibrio qinghaiensis* sp.-Q67 indicated that all dose–response relationships could be effectively described by the Weibull function with correlation coefficients greater than 0.99. The combined toxicity of two equivalent-effect concentration ratio mixtures and eight uniform design concentration ratio mixtures could be predicted successfully by the dose addition model within 95% confidence intervals. However, it was also well predicted by the independent action model, especially at lower concentrations.

Keywords Phenol derivative · Aniline derivative · *Vibrio qinghaiensis* · Combined toxicity

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Every year an estimated 1,000 new compounds are produced, which makes the toxicological evaluation of chemical mixtures an extremely important and challenging area of toxicology (Zhou et al. 2004). Many methods and models have been introduced to evaluate the toxicity of mixtures of different compounds, such as the toxicity units (TU), the addition index (AI) and the mixture toxicity index (MTI) approaches (Lin et al. 2002; Altenburger et al. 2003). However, the majority of these methods and models are based on two different concepts (Bödeker et al. 1992), the dose addition (DA) and independent action (IA) concepts. It has been shown in a number of studies that the DA model has good predictive capabilities for the combined toxicity of compounds having a similar mode of action (MOA) (Faust et al. 2003; Backhaus et al. 2004; Altenburger et al. 2005; Junghans et al. 2006; Yang et al. 2010).

Phenols and anilines are important compounds in the manufacture of dyes, pharmaceuticals, polymers and synthetic resins. Phenolic compounds are highly toxic to all organisms. Toxic phenol can strongly inhibit the growth of bacteria, algae and mollusks (Gao et al. 2006). Anilines can cause skin ferrihemoglobin diseases (Thomulka et al. 1996). Phenol, *m*-cresol, 2,4-dichlorophenol, 2,4,

6-trichlorophenol, pentachlorophenol, *p*-nitrophenol, aniline, 4-nitroaniline, 2,4-nitroaniline, and 2,6-dichloro-4-nitroaniline have been listed as priority pollutants (Zhou et al. 1990).

The use of tests with photoluminescent bacteria has received attention because of their simplicity, speed, sensitivity, and low cost. It has been found that the Qinghai Q67 bacteria can grow well in water and be luminous (Zhang et al. 2009, 2011; Liu et al. 2009). Therefore, the inhibition toxicities of the six phenol and two aniline compounds as well as those of their mixtures to *Vibrio qinghaiensis* sp.-Q67 were determined by using the microplate toxicity test procedure (Zhang et al. 2009, 2011; Liu et al. 2009). To effectively explore how combined toxicity varies with varying concentrations of specific compounds in a mixture, we employed the uniform design concept that allocates experimental points that are uniformly scattered in the experimental region (space) to design the test mixtures. To validate whether the DA model or the IA model can predict the combined toxicity of a mixture of phenolic and aniline derivatives, it is necessary to inspect whether the dose–response curves (DRCs) predicted by the DA or IA models locates within the 95% confidence intervals of the experimental DRCs.

Materials and Methods

The properties of six phenolic derivatives, *p*-chlorophenol (P1), *o*-chlorophenol (P2), 2,4-dichlorophenol (P3), *m*-cresol (P4), *p*-cresol (P5) and *o*-nitrophenol (P6), and two aniline derivatives, *p*-methlaniline (A1) and *o*-nitroaniline (A2), used in the test are listed in Table 1. Phenolic and aniline derivatives (PADs) stock solutions were prepared in distilled water and stored at 4°C.

The test organism is a novel freshwater photobacterium, *Vibrio-qinghaiensis* sp. Q67 (Q67), purchased from East China Normal University. Details of the culture media and the culture condition of Q67 were given in our previous works (Zhang et al. 2009, 2011; Liu et al. 2009). The culture medium consists of 13.6 mg KH_2PO_4, 35.8 mg $Na_2HPO_4{\cdot}12H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.61 g $MgCl_2{\cdot}6H_2O$, 33.0 mg $CaCl_2$, 1.34 g $NaHCO_3$, 1.54 g NaCl, 5.0 g yeast extract, 5.0 g tryptone, 3.0 g glycerin, and 1,000 mL distilled water, and is adjusted to pH 8.8–9.0. By adding 2% agar to the above solution, it becomes a solid culture medium. 50 mL culture medium was added into 100 mL conical flasks, which were occluded with a brown paper, then sterilized with high pressure steam for 20 min at 121°C. Before each test, the bacteria were inoculated from a stock culture, which is maintained on Q67 culture medium agar at 4°C, to a fresh agar and were cultured at 22 ± 1°C for 24 h. The cells were further grown in liquid culture medium by shaking (120 r/min) at 22 ± 1°C for 18 h (Zhang et al. 2009; Liu et al. 2009).

The toxicity tests of PADs and their mixtures were performed using a Veritas™ luminometer with a 96-well microplate (Turner BioSystems Inc., USA). For each of the eight PADs, 12 concentration gradients were designed based on our previous microplate toxicity test procedure (Zhang et al. 2009; Liu et al. 2009). In 12 wells of the first row in a microplate, 100 μL Milli-Q water was added as 12 controls. In 12 wells of the second row, 12 different toxicant solutions were added, derived from an appropriate dilution factor to provide a response (inhibition of chemiluminescence) ranging from 1% inhibition to maximum inhibition. The total volume was brought up to 100 μL with Milli-Q water. In the same way as the in second row, the test solutions were prepared in 12 wells of the third, fourth, and fifth rows (replications of the second row). The bacterial suspension (100 μL) was then added into each test well to make a final test volume of 200 μL. Each microplate test was repeated three times.

A mixture consisting of many PADs was regarded as a pseudo-toxicant whose concentration was the sum of the concentrations of various components in the mixture. Then, the microplate toxicity test of a mixture can be run by the same procedure as that of an individual toxicant.

The relative light units (RLUs) produced by Q67 photobacteria exposed to various treatments (PAD or mixture)

Table 1 Some physical properties of eight PADs

No	Name	CAS·RN*	Purity (%)	Model	$RMSE_x$	*R*	α	β	pEC_{50}
P1	*p*-chlorophenol	106-48-9	99.0	Weibull	0.00897	0.9996	5.57	1.73	3.43
P2	*o*-chlorophenol	95-57-8	98.0	Weibull	0.03120	0.9907	4.63	1.78	2.81
P3	2,4-dichlorophenol	120-83-2	98.0	Weibull	0.01709	0.9986	6.14	1.78	3.66
P4	*m*-cresol	108-39-4	98.0	Weibull	0.01605	0.9983	5.16	1.95	2.83
P5	*p*-cresol	106-44-5	98.0	Weibull	0.01913	0.9984	5.28	1.89	2.99
P6	*o*-nitrophenol	88-75-5	98.0	Weibull	0.02897	0.9940	4.98	1.67	3.20
A1	*p*-methlaniline	106-49-0	99.0	Weibull	0.00926	0.9993	4.45	1.91	2.52
A2	*o*-nitroaniline	88-74-4	98.5	Weibull	0.01894	0.9971	6.14	1.78	3.65

and controls (without any toxicant) were determined. The toxicity of a treatment to Q67 was expressed as an inhibition ratio (E or x), which is calculated as follows:

$$E = x = \frac{I_0 - I}{I_0} \times 100\% \quad (1)$$

where I_o is an average of the RLUs of the controls and I an average of the RLUs of the treatments with an identical concentration.

To compare with the classical method, two mixtures were constructed by using an equivalent-effect concentration ratio (EECR) method. Here, the concentration ratios of each of the PADs in two mixtures named as EECR-1 and EECR-2 are the proportions of EC_{10}, and EC_{50} to the total concentration (C_{mix}) of the mixture, respectively (Table 2). It is well known that the concentration compositions in EECR mixtures are limited into a very narrow space in the experimental region. To effectively expand the space, therefore, we employed a uniform design (UD) concept that allocates experimental points that are uniformly scattered on the region to design the uniform design concentration ratio (UDCR) mixtures. UD is especially suitable to examine the combined toxicity that varies with the concentration compositions in the whole mixture region with a minimal number of experiments. In this paper, eight 8-component mixtures (denoted as UDCR-1, UDCR-2, UDCR-3, UDCR-4, UDCR-5, UDCR-6, UDCR-7 and UDCR-8) were designed (Table 2). The mixtures were considered as the special "single" substance whose total concentration is the sum of single concentration of substituted benzene. The dose–effect data of every mixture was determined by the toxicity experiment. Appropriate test concentrations for each test were chosen by the pretreatment experiments, and there were no uniform dilution factors for mixtures.

The selected model parameters of R, $RMSE_x$, the EC_x values of mixtures, and predictions based on DA and IA were determined by the computer program APTox® designed by our laboratory. Experimental data were plotted with Origin software.

Results and Discussion

The best simulated dose–effect curves of the eight species of substituted benzene were obtained by using a non-linear least squares fit to the experimental data (Table 1). The results showed that the two-parameter Weibull function described the dose–effect curves of the eight PADs on the photobacterium. The analytical formulas for the Weibull function and its inverse function are given in Eqs. 2 and 3. The fitting parameters, i.e., the root mean square error (RMSE), α, β and the correlation coefficient (R) are also shown in Table 1. By inserting α and β into the equation for the inverse function of Weibull, the concentrations at each effect level (i.e., EC_x, the concentration that inhibits chemiluminescence by $x\%$) can be determined.

$$x = 1 - \exp(-\exp(\alpha + \beta \cdot \lg(EC_x))) \quad (2)$$

$$EC_x = 10^{\wedge}\left((\ln(-\ln(1-x)) - \hat{\alpha})\Big/\hat{\beta}\right) \quad (3)$$

The results showed that the concentration-toxicity data of the eight compounds can be well fitted by Weibull model function, and all the correlation coefficients, R, were greater than 0.99 (Table 1). The Weibull models with R values greater than 0.99 and RMSE values less than 0.032 exhibited not only good calibration ability but high stability. Various effect concentrations (EC_x) were calculated from the fitted concentration–response curve (CRC) models. The pEC_{50} values (i.e., $-\log EC_{50}$ values) for the eight individual PADs ranged from 2.52 for *p*-methylaniline to 3.66 for 2,4-dichlorophenol (Table 1). The order of toxicity was 2,4-dichlorophenol > *o*-nitroaniline > *p*-chlorophenol > *o*-nitrophenol > *p*-cresol > *m*-cresol > *o*-chlorophenol > *p*-methlaniline.

Table 2 The effect concentrations (levels) of eight PADs in ten mixtures

Mixture	P1	P2	P3	P4	P5	P6	A1	A2
EECR-1	EC_{10}	EC_{10}	EC_{10}	EC_{10}	EC_{10}	EC_{10}	EC_{10}	EC_{10}
EECR-2	EC_{50}	EC_{50}	EC_{50}	EC_{50}	EC_{50}	EC_{50}	EC_{50}	EC_{50}
UDCR-1	EC_8	EC_{10}	EC_{15}	EC_{20}	EC_{25}	EC_{30}	EC_{40}	EC_{50}
UDCR-2	EC_{10}	EC_{20}	EC_{25}	EC_{30}	EC_{15}	EC_{50}	EC_8	EC_{40}
UDCR-3	EC_{15}	EC_{25}	EC_8	EC_{10}	EC_{40}	EC_{20}	EC_{50}	EC_{30}
UDCR-4	EC_{20}	EC_{30}	EC_{10}	EC_{50}	EC_8	EC_{40}	EC_{15}	EC_{25}
UDCR-5	EC_{25}	EC_{15}	EC_{40}	EC_8	EC_{50}	EC_{10}	EC_{30}	EC_{20}
UDCR-6	EC_{30}	EC_{50}	EC_{20}	EC_{40}	EC_{10}	EC_8	EC_{25}	EC_{15}
UDCR-7	EC_{40}	EC_8	EC_{50}	EC_{15}	EC_{30}	EC_{25}	EC_{20}	EC_{10}
UDCR-8	EC_{50}	EC_{40}	EC_{30}	EC_{25}	EC_{20}	EC_{15}	EC_{10}	EC_8

Table 3 The optimal DRC models for PADs mixtures

Mixture	Model	α	β	$RMSE_x$	R	R^2	$pEC_{50,mix}$
EECR-1	Weibull	5.86	2.19	0.01541	0.9985	0.9970	2.84
EECR-2	Weibull	7.11	2.58	0.02150	0.9976	0.9951	2.90
UDCR-1	Weibull	5.11	1.84	0.01231	0.9990	0.9980	2.98
UDCR-2	Weibull	6.00	2.03	0.01417	0.9988	0.9975	3.14
UDCR-3	Weibull	6.10	2.35	0.01717	0.9982	0.9965	2.75
UDCR-4	Weibull	6.52	2.38	0.02534	0.9959	0.9918	2.89
UDCR-5	Weibull	6.32	2.29	0.01068	0.9994	0.9989	2.92
UDCR-6	Weibull	5.75	2.12	0.01505	0.9985	0.9970	2.89
UDCR-7	Weibull	5.49	1.89	0.02838	0.9961	0.9922	3.10
UDCR-8	Weibull	6.27	2.19	0.01996	0.9977	0.9959	3.03

Table 4 Statistics for toxicity predictions by DA and IA models

Mixture	$RMSE_{DA}$	$pEC_{50,mix\ DA}$	R_{DA}	$RMSE_{IA}$	$pEC_{50,mix\ IA}$	R_{IA}
EECR-1	4.82×10^{-4}	2.94	0.9953	1.06×10^{-3}	3.19	0.9901
EECR-2	1.21×10^{-3}	2.96	0.9826	4.84×10^{-4}	3.21	0.9823
UDCR-1	3.71×10^{-4}	2.93	1.0000	8.78×10^{-4}	3.14	1.0000
UDCR-2	6.36×10^{-4}	3.11	0.9976	3.29×10^{-4}	3.33	0.9976
UDCR-3	1.26×10^{-3}	2.80	0.9918	6.66×10^{-4}	3.01	0.9913
UDCR-4	1.00×10^{-3}	2.95	0.9900	4.85×10^{-4}	3.17	0.9896
UDCR-5	7.45×10^{-4}	2.98	0.9930	5.28×10^{-4}	3.19	0.9927
UDCR-6	1.02×10^{-3}	2.87	0.9970	4.90×10^{-4}	3.08	0.9968
UDCR-7	1.41×10^{-4}	3.12	0.9997	7.48×10^{-4}	3.34	0.9996
UDCR-8	7.99×10^{-4}	3.04	0.9944	3.58×10^{-4}	3.26	0.9944

For the toxicities of the 10 different mixtures, the Weibull two-parameter model was again used to obtain the fitting parameters RSME, α, β, and correlation coefficient R (Table 3). The Weibull models of the mixtures with R values greater than 0.99 and RMSE values less than 0.029 exhibited good calibration ability and high stability. For the set of 10 fixed concentration ratio mixtures, the $pEC_{50,mix}$ values ranged from 2.75 to 3.14 mol/L. These values are within the range of $pEC_{50,i}$ for the eight individual PADs (2.52–3.66), i.e., the values of the mixtures fall into the span between the most toxic and the least toxic individual PAD, indicating the absence of strong synergistic or antagonistic interactions within the mixture (Arrhenius et al. 2004).

To compare with the classical method, two mixtures were constructed by using an equivalent-effect concentration ratio (EECR) method (Liu et al. 2009). According to DA and IA models (Faust et al. 2003), the combined toxicities of EECR mixtures of EC_{10} and EC_{50} (EECR-1 and EECR-2) were predicted to explore the toxicity interaction between various phenol and aniline derivatives in the mixtures (Table 4). Based on the optimal non-linear model of the eight single benzene derivatives (Weibull model), the effect concentrations $EC_{mix,\ DA}$ and $EC_{mix,\ IA}$ of the 20 points ranging from 1% to 99% in the conditions of the DA and IA models were calcualated using APTox software. The plots of the effects predicted by DA and IA compared with experimental can be seen in Fig. 1. This shows that the toxicities of the two EECR mixtures, EC_{10} and EC_{50}, can be well predicted by the DA and IA models. The fitted curves all appear very similar, with the IA model predicting inhibitory effects at very lower concentrations in all cases, but the IA model tends to overestimate the toxicity of the mixtures at higher concentrations (Fig. 1). The DRCs predicted by the DA model are located within the 95% confidence intervals of the experimental DRCs in all cases. Although the DRCs predicted by the IA model are located within the 95% confidence intervals of the experimental DRCs at very lower concentrations, the DRCs predicted by the IA model are located outside of the 95% confidence intervals of the experimental DRCs at higher concentrations (Fig. 1), which may be a result of a slight antagonistic effect (Huang et al. 2011). These findings are in congruence with the pharmacological assumptions that the mixture toxicity of strictly similar-acting substances will be accurately predicted by DA (Arrhenius et al. 2004;

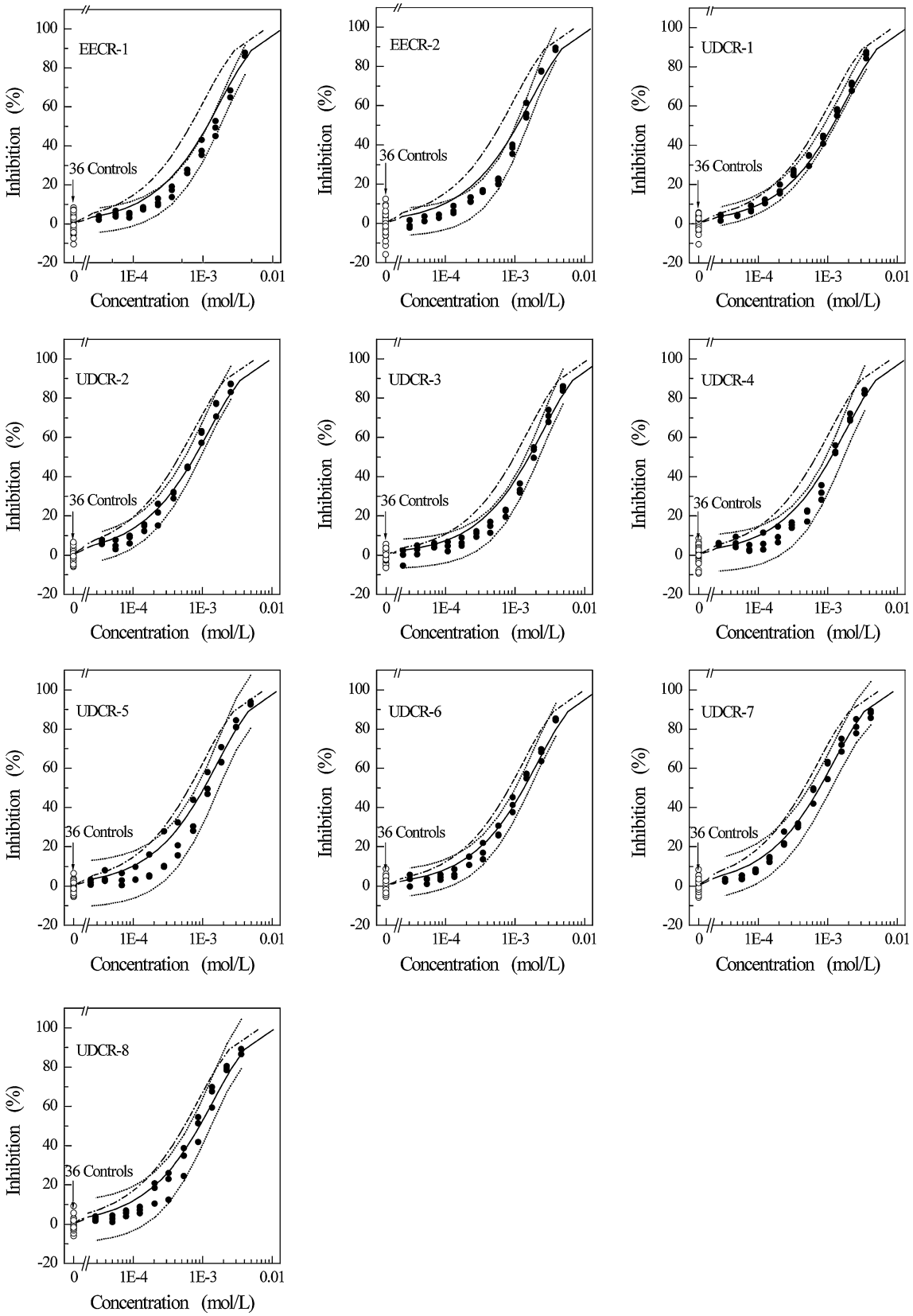

Fig. 1 The concentration – inhibition ratio (%) relationship of 10 PADs mixtures (• Experimental data; — predicted by DA; -·- predicted by IA; o Controls; ···· 95% confidence intervals of the experimental DRC)

Backhaus et al. 2004). The results correspond well with an earlier study with phenylurea mixtures in which identical predictions by DA and IA approaches indicated that the toxicity of similar-acting substances could be accurately predicted by the concept of DA (Backhaus et al. 2004). Given the inherent variability of natural epipsammon and periphyton communities, the mixture toxicities of the 12 strictly congeneric phenylureas with a similar toxicological mechanism of action were well predicted by both concepts when applied in complex biological systems. However, DA described the observed mixture toxicity better with respect to position and shape of the concentration–response curves (Arrhenius et al. 2004). The DA model may be used as a slightly conservative, but broadly applicable model with a relatively small likelihood of underestimating effects due to interactions (Belden et al. 2007).

The combined toxicities of eight UDCR mixtures, UDCR-1, UDCR-2, UDCR-3, UDCR-4, UDCR-5, UDCR-6, UDCR-7 and UDCR-8, were predicted (Table 4). To explore the toxicity interaction between various PADs in the mixtures, we integrated the *t*-DRCs predicted by the DA model and the IA model together with the experimental points observed and their 95% confidence intervals (CIs), and displayed them in Fig. 1.

The equivalent-effect concentration ratios, EC_{10} and EC_{50}, can only reflect a mixture concentration of a certain space of a direction. Since the linear or non-linear relationship existed among each EC_x, even if various mixtures of the equivalent-effect concentration ratios were measured, they only reflect several similar directions of space concentrations and can not explain the overall situation. On the contrary, the UDCR mixtures can be designed to study the law of the toxicity at the different space concentrations of the derivatives. The combined toxicities of the UDCR mixtures can be predicted by the DA model, and the IA model overestimated the toxicities at higher concentrations (Table 4 and Fig. 1). For the 10 different mixtures, the correlation coefficients (R_{DA}) for the toxicity predictions by the DA models were equal to or higher than the correlation coefficients (R_{IA}) for the toxicity predictions by the IA models (Table 4). The $pEC_{50,mix\ DA}$ values predicted by the DA model were in good agreement with the $pEC_{50,mix}$ values estimated by the Weibull two-parameter model, but the $pEC_{50,mix\ IA}$ values predicted by the IA model were obviously higher than the $pEC_{50,mix}$ values estimated by the Weibull model (Tables 3 and 4).

Phenol, aniline, and their derivatives are polar narcotics and exhibit a reasonably wide range of toxicity (Lu et al. 2008). The toxicity of these chemicals to many organisms in aquatic ecosystems (*Scenedesmus obliquus*, *Tetrahymena pyriformis*, *Chlorella pyrenoidosa* and *Vibrio fischeri*, *etc*.) has been extensively investigated (Könemann and Musch 1981; Cronin and Schultz 1996; Ramos et al. 1999; Lu et al. 2008). Their toxicity increased with hydrophobicity, good hydrogen bonding donor capacity, and low hydrogen bonding acceptor capacity (Ramos et al. 1999). The joint toxicity of each of the binary mixtures of aniline and each of its derivatives to *photobacterium phosphoreum* is mainly simple addition of the toxicities of the two compounds (Yuan et al. 2004). The toxicity of substituted phenols and anilines to the alga (*Scenedesmus obliquus*) could be well predicted by quantitative structure–activity relationship (QSAR) models. In addition, a model derived from the structural parameters of single components in binary mixtures was used successfully to predict toxicity of mixtures (Wang et al. 2008). The results of this study indicated that the toxic effects of the mixtures of phenol and aniline derivatives exceed that of the most active component alone. The low effect concentrations of individual PADs contribute to the overall toxicity, and they show that the concept of concentration addition provides a reliable tool for the predictive hazard assessment of multi-component benzene derivatives mixtures, irrespective of the effect level and the concentration ratios of the PADs in the mixtures. Therefore, risk assessments of PADs in aquatic systems may no longer be restricted to single pure toxicants. Risk assessments of PAD mixtures may be possible using the DA approach.

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