Research Article

Prediction and Evaluation of the Mixture Toxicity of Twelve Phenols and Ten Anilines to the Freshwater Photobacterium *Vibrio qinghaiensis* sp.-Q67

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Twelve substituted phenols and ten substituted anilines were chosen to investigate if the dose addition and independent action models can be used to evaluate the mixture toxicity of phenolic and aniline derivatives (PADs). Their photobacterium toxicity to the freshwater luminescent bacterium *Vibrio qinghaiensis* sp.-Q67 showed that the two-parameter Weibull or Logit function could be effectively applied to describe the dose-response relationships. The joint toxicity of three equivalent-effect concentration ratio (EECR) mixtures and twelve uniform design concentration ratio (UDCR) mixtures could be well evaluated using the dose addition (DA) or the independent action (IA) model within 95% confidence intervals.

1. Introduction

Phenol and aniline derivatives (PADs) are important chemical materials and intermediates in synthesis of other compounds, such as dyes, pharmaceuticals, polymers, and synthetic resins. However, they are also the major carcinogenic pollutants in aquatic environments and extremely toxic to organisms [1, 2]. Many of PADs are priority pollutants in the water [3].

Phenol and aniline derivatives usually occur as mixture contaminants in the aquatic environments [4]. However, most of the toxicity data obtained are information about pure and individual chemicals, not related to mixtures. Therefore, the reliability is questionable to evaluate the multicomponent mixture toxicity from the toxicity data about single chemicals. Several mixtures in different toxicity tests were studied by some researchers to get better results in the predictive evaluation [5].

In order to evaluate the combined toxicity, it is required firstly to assume the relationships between the toxicity of individual chemicals and their mixtures quantitatively. Essentially, two different basic models are available for this purpose, that is, the independent action (IA) and the dose addition

(DA) [5]. Other early analysis methods of mixture toxicity included concepts such as toxic units, mixture toxicity index, and additive index. On these two different basic models, the similarity parameter λ was built [4, 5]. Joint toxicity studies on chemical mixtures have been slowly progressing over the past decades, with preliminary researches involving two-component mixtures in equieffect concentration (most of which are EC_{50}) [4, 6]. Since the year 2000, the nonlinear simulation of the dose-effect curve and the recurrent points have been used to estimate the low effects concentration, and the dose addition and independent action for multicomponent mixture predictions [5, 7, 8]. The mixture toxicity of nonpolar narcotic chemicals could be predicted by two QSAR approaches, that is, the forward stepwise multilinear regression (MLR) and the radial basis function neural networks (RBFNNs) from molecular descriptors which are calculated and defined as composite descriptors according to the fractional concentrations of the mixture components [9]. In order to enable the prioritization of ionic liquids with a favourable cytotoxicity profile, a Classification and Regression Tree (CART) classifier has been recently proposed due to its simplicity, reliability, good predictive capability, and transparent chemical interpretation based on structural

TABLE 1: Some physical-chemical properties of 22 PADs.

Number	Name ^a	CAS RN	Purity (%)	MW	Manufacturer
PAD01	3,5-Dihydroxytoluene	504-15-4	98	124.14	Sinopharm Chemical Reagent Co., Ltd, China
PAD02	2,3-Dimethylphenol	526-75-0	98	127.17	Fluka AG, Chemische Fabrik
PAD03	<i>p</i> -Chlorophenol	106-48-9	99	128.56	Sinopharm Chemical Reagent Co., Ltd, China
PAD04	<i>o</i> -Chlorophenol	95-57-8	98	128.56	Sinopharm Chemical Reagent Co., Ltd, China
PAD05	2,4-Dichlorophenol	120-83-2	98	163.00	Shanghai No. 1 Reagent Factory, China
PAD06	Phenol	108-95-2	99.9	94.11	Guangdong Shantou Xilong Chemical
PAD07	<i>m</i> -Cresol	108-39-4	98	108.13	Shanghai Fanyang Fine-chemical Co., Ltd, China
PAD08	<i>o</i> -Cresol	95-48-7	98	108.13	Sinopharm Shanghai Chemical Reagent Co., Ltd, China
PAD09	<i>p</i> -Cresol	106-44-5	98	108.13	Sinopharm Shanghai Chemical Reagent Co., Ltd, China
PAD10	<i>m</i> -Nitrophenol	554-84-7	98	139.11	Shanghai No. 1 Reagent Factory, China
PAD11	<i>p</i> -Nitrophenol	100-02-7	99.5	139.11	Sinopharm Chemical Reagent Co., Ltd, China
PAD12	<i>o</i> -Nitrophenol	88-75-5	98	139.11	Sinopharm Chemical Reagent Co., Ltd, China
PAD13	Aniline	62-53-3	99.5	93.13	Guangdong Shantou Xilong Chemical
PAD14	<i>m</i> -Methylaniline	108-44-1	98.5	107.16	Sinopharm Chemical Reagent Co., Ltd, China
PAD15	<i>p</i> -Methylaniline	106-49-0	99	107.16	Shanghai Hengxin Chemical Reagent Factory
PAD16	<i>o</i> -Methylaniline	95-53-4	99%	107.16	Shanghai Hengxin Chemical Reagent Factory
PAD17	<i>p</i> -Nitroaniline	100-01-6	99.5	138.13	Sinopharm Chemical Reagent Co., Ltd, China
PAD18	<i>o</i> -Nitroaniline	88-74-4	98.5	138.13	Shanghai No. 3 Reagent Factory, China
PAD19	<i>m</i> -Nitroaniline	99-09-2	98.5	138.13	Shanghai No. 1 Reagent Factory, China
PAD20	<i>o</i> -Chloroaniline	95-51-2	99	127.57	Sinopharm Chemical Reagent Co., Ltd, China
PAD21	<i>m</i> -Chloroaniline	108-42-9	99	127.57	Sinopharm Chemical Reagent Co., Ltd, China
PAD22	<i>p</i> -Chloroaniline	106-47-8	98	127.57	Sinopharm Chemical Reagent Co., Ltd, China

^a Common name; CAS RN: Chemical Abstracts Services Registry Number; MW: molecular weight.

molecular fragments [10, 11]. Toxicological evaluation of chemical mixtures is now considered to have truly entered into the field of multicomponent mixtures [4, 6, 12–15].

In this work, the joint toxicities of 22 species of phenol and aniline derivatives (PADs) and the toxicities of their mixtures to the freshwater photoluminescent bacterium *Vibrio qinghaiensis* sp.-Q67 (Q67) were measured through a microplate toxicity test procedure [16–19]. To seek how the mixture toxicity changes with changing concentrations of individual chemicals in the mixture effectively, the compounds were mixed in equieffect concentrations ratio (EECR) or in uniform design concentration ratio (UDCR).

2. Materials and Methods

2.1. Materials. The PADs are common in water environment and all have a high solubility. Some physical-chemical properties of 22 tested PADs are listed in Table 1. Three EECR mixtures and twelve UDCR mixtures of the test agents were made by the stock solutions, which were prepared with pure agents in distilled water, kept in critically cleaned glass containers, and stored at 4°C.

The freshwater photoluminescent bacterium *Vibrio qinghaiensis* sp.-Q67 (Q67) was used as the test organism that was kindly provided by East China Normal University, China. The culture condition and media have been described in detail in some of our earlier works [16–19]. To prepare the culture medium, the following chemicals were dissolved

into 1000 mL distilled water: 5.0 g tryptone, 5.0 g yeast extract, 3.0 g glycerin, 35.8 mg $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 13.6 mg KH_2PO_4 , 33.0 mg CaCl_2 , 1.54 g NaCl , 0.61 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.34 g NaHCO_3 , 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The solution was adjusted to pH 8.8–9.0 and turned into a solid culture medium after adding 2% agar. 50 mL culture medium was put into a 100 mL conical flask, which was then obturated by using a brown paper and sterilized at 121°C for 20 min through high pressure steam. For each experiment, the photoluminescent bacteria Q67 were inoculated from a stock culture medium into a fresh culture medium, which was then maintained and cultured for 24 h at $22 \pm 1^\circ\text{C}$. Finally, the bacteria Q67 were further cultured in a liquid medium at $22 \pm 1^\circ\text{C}$ by shaking (120 r/min) for 18 h [16, 18–20].

2.2. Methods. A Veritas luminometer with a 96-well microplate was bought from the Turner BioSystems Inc., USA, and used to examine the toxicity of PADs and their mixtures. Regarding our earlier microplate toxicity test procedure, twelve concentration gradients were planned for each of the twelve phenols and ten anilines [16, 17]. 100 μL Milli-Q water was injected into twelve wells of the first row of the microplate, which were regarded as twelve controls (without any toxicant). Twelve different toxicant solutions were injected into twelve wells of the second row of the microplate and then filled with Milli-Q water to the total volume of 100 μL . To obtain a chemiluminescence inhibition (response) from 1% to the maximum, the twelve toxicant solutions were

TABLE 2: The effect concentration of 22 PADs in 15 mixtures (%).

Mixture	PAD01	PAD02	PAD03	PAD04	PAD05	PAD06	PAD07	PAD08	PAD09	PAD10	PAD11
EE22-01	5.070	1.540	0.164	0.816	0.119	7.150	1.240	3.080	0.752	3.010	0.545
EE22-10	5.240	2.070	0.482	2.190	0.310	8.800	2.550	3.090	1.650	2.500	0.991
EE22-50	4.420	2.170	0.959	3.950	0.570	8.600	3.770	2.560	2.670	1.800	1.330
UD22-01	0.858	0.389	0.0864	0.514	0.0957	3.461	1.106	1.516	1.027	1.636	0.792
UD22-02	1.491	0.795	0.237	1.409	0.324	9.338	4.053	4.841	4.878	5.750	5.447
UD22-03	1.066	0.633	0.240	1.629	0.367	11.159	7.764	0.404	0.340	0.899	0.584
UD22-04	2.003	1.290	0.586	4.900	1.495	1.686	1.014	2.253	2.246	3.826	4.364
UD22-05	1.803	1.399	0.716	7.904	0.040	3.745	2.200	3.957	7.883	0.772	0.591
UD22-06	2.187	1.755	1.274	0.231	0.132	8.370	6.940	0.693	0.874	2.335	3.042
UD22-07	1.555	1.442	1.615	0.436	0.201	11.225	0.354	1.382	2.184	0.302	0.324
UD22-08	2.876	3.104	0.0578	1.529	0.749	1.972	2.137	4.914	0.382	1.943	2.681
UD22-09	3.847	4.376	0.153	3.067	2.236	5.552	6.107	1.031	1.929	6.198	0.550
UD22-10	3.141	4.384	0.185	4.127	0.073	7.993	0.267	1.977	6.065	1.101	1.729
UD22-11	3.754	6.751	0.383	10.156	0.191	20.020	1.263	5.249	0.710	3.288	0.372
UD22-12	3.897	0.292	0.537	0.309	0.398	2.125	3.355	0.933	2.695	0.895	1.710
Mixture	PAD12	PAD13	PAD14	PAD15	PAD16	PAD17	PAD18	PAD9	PAD20	PAD21	PAD22
EE22-01	0.227	23.710	3.690	2.500	28.890	0.226	0.103	2.300	16.440	4.610	1.800
EE22-10	0.750	22.430	7.040	5.220	16.430	0.345	0.295	2.980	7.660	4.270	2.680
EE22-50	1.620	22.300	9.730	7.790	11.250	0.538	0.567	3.050	3.990	3.310	3.060
UD22-01	0.789	19.435	8.179	7.088	17.527	0.619	0.728	5.871	9.874	8.997	9.437
UD22-02	0.083	7.864	2.987	3.009	12.431	0.366	0.446	4.540	9.805	9.086	10.863
UD22-03	0.701	21.886	13.823	19.032	3.054	0.088	0.116	1.796	4.628	4.328	5.461
UD22-04	0.125	11.288	6.563	7.864	25.988	2.074	0.069	1.659	6.086	5.408	7.210
UD22-05	1.000	39.558	0.811	1.854	10.891	0.512	1.389	0.692	3.628	3.450	5.206
UD22-06	0.147	14.832	9.674	21.063	5.625	0.264	0.574	9.827	2.478	3.166	4.514
UD22-07	0.810	45.852	1.769	3.625	18.247	0.063	0.172	3.261	1.020	1.645	2.516
UD22-08	0.197	19.296	23.592	1.853	14.752	1.500	0.120	4.410	6.201	2.143	3.591
UD22-09	1.874	6.132	7.203	21.175	8.530	0.568	0.037	2.665	11.148	1.939	3.681
UD22-10	0.197	21.319	1.044	4.166	30.788	0.190	0.832	1.051	6.075	1.016	2.279
UD22-11	1.801	8.060	11.055	1.363	15.889	0.237	0.394	0.583	5.476	0.756	2.258
UD22-12	0.257	32.948	2.967	13.247	7.900	1.056	0.180	7.875	4.520	9.890	2.013

prepared after a proper dilution factor. Similarly, some other toxicant solutions were prepared and injected into twelve wells of the third or fourth row of the microplate. The fifth row of the microplate was used as a test duplication of the second row. Finally, each well of the microplate was filled with 100 μ L bacterial Q67 suspension. All microplate tests were triplicated. Considering the sum of all component concentrations as the total concentration of a PADs mixture, the microplate toxicity test with the PADs mixture was conducted in the same manner as the procedure of a single PAD toxicant.

In each test, the photoluminescent bacteria Q67 were exposed to different treatments and controls, and the relative light unit (RLU) was measured. An inhibition ratio of the luminous intensity of the bacterium (E or x) was applied to express the PADs toxicity to the photoluminescent bacteria and can be calculated by the following equation:

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

where I is a mean RLUs value of a treatment and I_o a mean RLUs value of all untreated controls.

2.3. Mixture Design. The test mixtures were prepared by both the EECR and UDCR methods. According to the measured results for the 22 PAD toxic pollutants, the best nonlinear simulation was performed and the given effect x (%) was expressed as a concentration EC_x . For the three EECR mixtures, the EC_1 , EC_{10} , and EC_{50} percentages of the total mixture concentration (c_{mix}) were designated as the concentration ratios of each PAD in the mixtures, that is, EE22-01, EE22-10, and EE22-50, respectively. The concentrations percentages of different PAD compounds in the mixtures were showed in Table 2.

To avoid the significant range limitation of the concentration percentages in EECR mixtures, 12 UDCR mixtures (denoted as UD22-01, UD22-02, UD22-03, UD22-04, UD22-05, UD22-06, UD22-07, UD22-08, UD22-09, UD22-10, UD22-11, and UD22-12) were designed using the former

11 lines in the uniform design table (U-table), $U_{13}(13^{22})$ (Table 2). The uniform design (UD) experiment is planned in terms of the uniform design table (U-table). Like orthogonal design, UD offers lots of experimental tables to the users for convenient utilization. U-tables of form $U_n(n^s)$, where the subscript n refers to the number of the mixture experiments and the superscript s to the number of pesticides (factors) in the experiment (mixture), are purposely chosen to mimic the tables of orthogonal designs, $L_n(q^s)$, except that the number of levels equals the number of experiments ($q = n$) [16]. In light of $U_{13}(13^{22})$ given in Table 2, twelve 22-component mixtures and a control group were designed. For each mixture design, proper concentrations were selected based on the results of some preexperiments and no uniform dilution factors were applied. As an effective experimental design method, the uniform design (UD) can save time and effort in investigating the changes of concentration in three-dimension space. When the factors in the uniform design (the numbers of mixture components) and the levels of the factors (the concentration levels) are large, it is very necessary and efficient to use the uniform design (UD) to investigate the joint effect of a multiple-component mixture because the UD can reduce significantly the mixture experiment efforts.

By using the computer program APTox, the dose-response curves (DRCs) were predicted based on both of the DA and IA models within a confidence interval of 95%; the root mean square error (RMSE_x) and the correlation coefficient (R) of the models and the mixture EC_x values were also calculated [16, 20]. Mathematically, the DA model is expressed as

$$\sum_{i=1}^n \frac{c_i}{EC_{x,i}} = 1, \quad (2)$$

where n is the number of mixture components, $EC_{x,i}$ the concentration of the i th component that provokes $x\%$ effect when applied singly, and c_i the concentration of the i th component in the mixture [16, 20]. The IA model can be formulated mathematically as

$$E(c_{\text{mix}}) = 1 - \prod_{i=1}^n (1 - E(c_i)), \quad (3)$$

where c_{mix} and $E(c_{\text{mix}})$ are the total concentration and total effect of the mixture, respectively, and $E(c_i)$ is the effect of the i th component with the concentration of c_i in the mixture [16, 20].

3. Results and Discussion

3.1. Photobacterial Toxicity of Single PADs. Based on the nonlinear least-squares fitting the experimental concentration-response data of the 22 species of PADs, the best dose-response curves (DRCs) were obtained. The DRCs of the 22 PADs to the photobacterium could be well described by the two-parameter function Weibull or Logit. According to the experimental results of the 12 concentration points designed with the microplate method and the experience rules that concentration points should be 5-6 times the parameter

values of the DRCs model, the two-parameter model is markedly more effective than the three-parameter model in fitting the experimental data. Therefore, all DRCs simulations in the present paper have applied only two parameters with the Weibull and Logit model in the data analysis.

The Weibull and Logit functions can be expressed in (2) and (3), respectively,

$$x = 1 - \exp(-\exp(\alpha + \beta \cdot \log(EC_x))), \quad (4)$$

$$x = \frac{1}{(1 + \exp(-\alpha - \beta \cdot \log(EC_x)))}. \quad (5)$$

From the nonlinear least-squares fitting to the experimental data, the best simulated DRCs of the 22 PADs were obtained (Table 3). The results indicated that the dose-response curves (DRCs) of the 22 PADs on the luminescent bacterium Q67 could be described by the two-parameter Weibull or Logit models. The RMSE, R , α and β parameters of the fitting models are also calculated and given in Table 3. By substituting the α and β values into the Weibull or Logit functions, the values of the concentration (EC_x) that inhibited the chemiluminescence by $x(\%)$ at each effect level were calculated. In Table 3, the pEC_{50} , that is, the negative logarithm of the EC_{50} ($-\log EC_{50}$), for 22 PADs are also given in comparison with classical toxic indices. Both of the Weibull and Logit functions could well describe the concentration-toxicity data of 22 PADs with $RMSE < 0.037$ and $R > 0.99$, which indicated the high stability and good calibration ability of the fitting of the two models [19].

A believable concentration-response examination of individual chemicals is indispensable to evaluate mixture toxicities. As a typical example for the concentration-inhibition ratio relationship of the single substance, the experimental and fitting results for *o*-methylaniline (PAD16) are illustrated in Figure 1(a). The photobacterial toxicity relationship for PAD16 was a nonsymmetrical and sigmoidal curve, which was sharper in the high inhibition section than in the low inhibition section, which was a general character of the 22 PADs tested. Additionally, Figure 1(a) reveals the relation between EC_1 and EC_{50} in the low inhibition section. For the EC_x estimations, $x = 1\%$ was chosen as a lower limit of the effect level, since the obtained test data commonly tolerated the interpolative approximation of inhibitions down to this level.

The nonlinear fitting with the regression model was depicted in Figure 1(b). The obtained concentration-response curves showed significant discrepancy in position and shape. In the strict mathematical term, however, the intersections among the curves obviously indicated that the curves did not occur in parallel with each other. It was very obvious, as the ratio between EC_1 and EC_{50} was used as a gradient indicator in the low effect sections of the dose-response curves. The ratio was diverse for the substance examined and varied from 0.00357 of *p*-chlorophenol (PAD03) to 0.04138 of *o*-chloroaniline (PAD20). In Table 3, for the extreme case PAD03, the EC_{50} was more than 280 times greater than the EC_1 , but the EC_{50} was only 24 times higher than the EC_1 for PAD20. The toxic dose range of PAD03 was the widest among the 22 compounds. The most toxic

TABLE 3: Parameters of the concentration-response functions for the individual PADs.

Number	Model	RMSE _x	R	α	β	EC ₅₀ (mol/L)	pEC ₅₀	EC ₁ (mol/L)	EC ₁ /EC ₅₀	EC ₅₀ /EC ₁
PAD01	Weibull	0.03646	0.9933	7.06	2.70	1.72E-03	2.77	4.01E-05	0.02330	42.8
PAD02	Weibull	0.02356	0.9972	6.74	2.31	8.48E-04	3.08	1.24E-05	0.01460	68.4
PAD03	Weibull	0.00897	0.9996	5.57	1.73	3.70E-04	3.43	1.32E-06	0.00357	280.3
PAD04	Weibull	0.03120	0.9907	4.63	1.78	1.55E-03	2.81	6.43E-06	0.00415	241.0
PAD05	Weibull	0.01709	0.9987	6.14	1.78	2.21E-04	3.66	9.24E-07	0.00418	239.2
PAD06	Weibull	0.01331	0.9992	5.55	2.39	3.35E-03	2.47	5.71E-05	0.01700	58.7
PAD07	Weibull	0.01605	0.9983	5.161	1.95	1.47E-03	2.83	9.79E-06	0.00666	150.2
PAD08	Weibull	0.01490	0.9991	7.56	2.64	9.90E-04	3.00	2.50E-05	0.02530	39.6
PAD09	Weibull	0.01913	0.9985	5.28	1.89	1.03E-03	2.99	5.88E-06	0.00570	175.2
PAD10	Weibull	0.02714	0.9972	8.76	2.89	6.95E-04	3.16	2.38E-05	0.03420	29.2
PAD11	Weibull	0.03131	0.9951	6.45	2.08	5.26E-04	3.29	4.73E-06	0.00899	111.2
PAD12	Weibull	0.02897	0.9940	4.98	1.67	5.71E-04	3.20	4.86E-06	0.00851	117.5
PAD13	Weibull	0.02358	0.9961	4.55	2.41	9.11E-03	2.04	1.61E-04	0.01767	56.6
PAD14	Weibull	0.01533	0.9964	4.48	2.00	3.77E-03	2.42	2.88E-05	0.00764	130.9
PAD15	Weibull	0.00926	0.9993	4.45	1.91	3.01E-03	2.52	1.83E-05	0.00608	164.5
PAD16	Weibull	0.01349	0.9993	6.67	2.98	4.35E-03	2.36	1.65E-04	0.03793	26.4
PAD17	Logit	0.02819	0.9954	8.21	2.23	2.08E-04	3.68	1.78E-06	0.00856	116.9
PAD18	Weibull	0.01894	0.9971	6.14	1.78	2.21E-04	3.65	9.32E-07	0.00422	237.1
PAD19	Weibull	0.01781	0.9978	6.48	2.34	1.18E-03	2.93	1.80E-05	0.01525	65.6
PAD20	Weibull	0.00119	0.9910	8.14	3.06	1.66E-03	2.78	6.87E-05	0.04138	24.2
PAD21	Weibull	0.01379	0.9992	7.56	2.74	1.28E-03	2.89	3.69E-05	0.03094	34.7
PAD22	Weibull	0.01237	0.9994	6.071	2.20	1.19E-03	2.93	1.43E-05	0.01202	83.2

FIGURE 1: The concentration-inhibition ratio relationship of the single substance. (a) *o*-Methylaniline; (b) 22 PADs (●: observed; ○: controlled; —: fitted by Weibull model).

PAD was *p*-nitroaniline (PAD17, pEC₅₀ = 3.68) and the least toxic PAD was aniline (PAD13, pEC₅₀ = 2.04). The toxicity order of the 22 PADs was *p*-nitroaniline (PAD17, pEC₅₀ = 3.68) > 2,4-dichlorophenol (PAD05, 3.66) > *o*-nitroaniline (PAD18, 3.65) > *p*-chlorophenol (PAD03, 3.43) >

p-nitrophenol (PAD11, 3.29) > *o*-nitrophenol (PAD12, 3.20) > *m*-nitrophenol (PAD10, 3.16) > 2,3-dimethylphenol (PAD02, 3.08) > *p*-chloroaniline (PAD22, 2.93) > *o*-cresol (PAD08, 3.00) > *p*-cresol (PAD09, 2.99) > *m*-nitroaniline (PAD19, 2.93) > *m*-chloroaniline (PAD21, 2.89) > *m*-cresol (PAD07,

TABLE 4: The t-DRC model as well as statistics for toxicity predictions by DA and IA models of 15 PAD mixtures.

Mixture	Model	α	β	RMSE _x	R	pEC _{50,mix}	RMSE _{DA}	RMSE _{IA}	R _{DA}	R _{IA}
EE22-01	Weibull	5.74	2.20	0.02380	0.9976	2.78	5.798E-04	3.246E-04	0.9947	0.9960
EE22-10	Weibull	6.18	2.38	0.00917	0.9996	2.75	9.930E-05	1.255E-04	0.9999	0.9999
EE22-50	Weibull	5.98	2.23	0.00091	0.9916	2.84	6.707E-04	1.516E-04	1.0000	0.9998
UD22-01	Weibull	5.03	1.97	0.02364	0.9968	2.74	1.112E-03	1.159E-03	0.9951	0.9965
UD22-02	Logit	11.21	3.91	0.01696	0.9988	2.87	2.491E-03	2.590E-03	0.9549	0.9486
UD22-03	Weibull	5.73	2.31	0.03317	0.9943	2.64	5.856E-04	3.100E-04	0.9987	0.9993
UD22-04	Weibull	6.59	2.52	0.02744	0.9962	2.76	5.157E-04	3.758E-04	0.9984	0.9976
UD22-05	Weibull	5.96	2.31	0.00983	0.9995	2.74	6.681E-04	2.482E-04	0.9985	0.9988
UD22-06	Weibull	5.76	2.20	0.01695	0.9985	2.78	2.536E-04	2.624E-04	1.0000	0.9998
UD22-07	Weibull	4.99	2.02	0.01067	0.9994	2.65	3.919E-04	4.128E-04	0.9983	0.9989
UD22-08	Logit	8.32	2.93	0.02280	0.9972	2.84	8.979E-03	9.426E-03	0.9288	0.9184
UD22-09	Logit	10.70	3.64	0.01825	0.9989	2.94	2.177E-03	2.668E-03	0.9584	0.9454
UD22-10	Weibull	7.09	2.74	0.02152	0.9980	2.72	1.190E-03	9.284E-04	0.9939	0.9950
UD22-11	Weibull	6.19	2.30	0.01711	0.9986	2.85	8.249E-04	5.027E-04	0.9997	0.9999
UD22-12	Weibull	6.04	2.30	0.01124	0.9994	2.79	8.381E-04	2.847E-04	0.9993	0.9998

FIGURE 2: The concentration-inhibition ratio relationship of three PADs mixtures constructed by the EECR method (●: observed; ○: controlled; —: predicted by DA; - - -: predicted by IA).

2.83) > *o*-chlorophenol (PAD04, 2.81) > *o*-chloroaniline (PAD20, 2.78) > 3,5-dihydroxytoluene (PAD01, 2.77) > *p*-methylaniline (PAD15, 2.52) > phenol (PAD06, 2.47) > *m*-methylaniline (PAD14, 2.42) > *o*-methylaniline (PAD16, 2.36) > aniline (PAD13, 2.04).

3.2. Photobacterial Toxicity of PADs Mixtures. The concentration-response relationships of three EECR mixtures and twelve UDCR mixtures were determined on the Veritas luminometer in the same procedure as described above. The three EECR mixtures (EE22-01, EE22-10, and EE22-50) and 12 UDCR mixtures (UD22-01~UD22-12) consisted of all 22 PADs. The experimental data of the mixture toxicities and the predictions of the DA and IA models are illustrated in Figures 2 and 3. The dependence of the inhibition of photobacterial luminosity on the total concentration of the mixture

constituent (c_{mix}) can be expressed by the concentration-response equations.

The fitting parameters of the Weibull or Logit models, α , β , RSME, and R , were calculated for the toxicity prediction of the 15 PADs mixtures (Table 4). The Weibull or Logit functions could well fit the concentration-toxicity data of the 15 PADs mixtures with $\text{RMSE} < 0.033$ and $R > 0.99$, which indicated the high stability and good calibration ability of the fitting of the two models. The $\text{pEC}_{50,\text{mix}}$ values of the 15 PADs mixtures varied from 2.64 for UD22-03 to 2.94 for UD22-09, which were within the $\text{pEC}_{50,i}$ range from the least toxic PAD (2.04 for PAD13) to the most toxic PAD (3.68 for PAD17), indicating the absence of strong synergistic or antagonistic interactions within the mixture [13].

A mixture concentration can be reflected only within a certain space of a direction for the equieffect concentrations

FIGURE 3: The concentration-inhibition ratio relationship of twelve PADs mixtures constructed by the UDCR method (●: observed; ○: controlled; —: predicted by DA; - - -: predicted by IA).

ratio (EECR) method; that is, it cannot describe the whole situation and can only represent some alike directions of space concentrations because of the existing relationship among the effect concentrations (EC_x), even though different EECR mixtures were measured. Instead, the PAD mixtures can be designed by the uniform design concentration ratio (UDCR) method to investigate the toxicity rule at the various space concentrations of PADs [16, 19]. To study the toxicity interaction among different PADs in the mixtures, the joint toxicities of all 15 PADs mixtures were also evaluated according to the DA and IA models (Table 4). Based upon the optimal nonlinear Weibull or Logit models for the 22 individual PADs, the 20 points varying between 1% and 99% for the inhibition concentrations of the DA model ($EC_{mix,DA}$) and the inhibition concentrations of the IA model ($EC_{mix,IA}$) were determined by the APTox computer program.

In Figures 2 and 3, the comparisons of the experimental data with the results calculated using the DA and IA models are illustrated. The DA and IA models could well predict the toxicity effects of all 15 PADs mixtures within 95% confidences of the experimental DRCs. The calculated DRC model parameters (β) indicated that the difference between the predicted results of these two models was insignificant in the present experiment.

All of the predicted curves are very similar to each other, but the inhibition effects evaluated by DA model were slightly lower than those predicted by IA model in all cases except for the EE22-01 mixture (Figures 2 and 3), which might be related to a very small synergistic or antagonistic effect [15, 19]. With regard to the existing quantitative structure-activity relationship (QSAR) method for the prediction of mixture toxicity, the independent action (IA) and the dose addition (DA) are two ideal and prominent reference models of additive behavior for the analysis of joint activity, but the two approaches do not consider the interactions of constituents in a mixture, which can result in complex and substantial changes in the apparent properties of its components, causing synergistic or antagonistic effects [9]. The result of the present work corresponded with the pharmacological presumption that the combined toxicities of the rigorously similar-acting chemicals could be well evaluated using the DA model [6, 13]. This finding was well in accordance with the result of a previous experimental study on phenylurea mixtures; that is, the identical evaluations of the DA and IA models showed that the DA model can be used to predict the toxicities of the similar-acting chemicals exactly [6]. Assuming the inherent variance of natural periphyton and epipsammon communities, the joint inhibitions of these 22 rigorously congeneric phenylureas having an analogous toxicological action mechanism could be well evaluated by the DA and IA models. It was found that the $RMSE_{DA}$ and $RMSE_{IA}$, the R_{DA} and R_{IA} were almost identical for both the DA and IA models (Table 4). As shown in Figures 2 and 3, both concepts created nearly the same concentration-response curves in all cases, the obtained data points of three EECR and twelve UDCR mixtures scattered around the solid lines of the DA model and the dotted lines of the IA model. Thus, the effects of 15 PADs mixtures could be well predicted using both DA and IA models. For these 15 various PADs mixtures, the R_{IA} values for

the toxic effect calculations using IA model were lower than or identical to the R_{DA} values for the toxic effect calculations using DA model (Table 4).

The polar and narcotic PADs show a fairly broad range of toxicities for numerous aquatic organisms that have been comprehensively studied [21–24]. Their toxicity was related to the property of being water-repellent, weak H bonding acceptor ability, and strong H bonding donor ability [23]. The mixture toxicities of two aniline compounds to *Photobacterium phosphoreum* could be principally described by the simple summing of the two-component toxicities [25]. The quantitative structure-activity relationship could be well used to express the PADs toxicities to *Scenedesmus obliquus*. Additionally, the toxicity of mixtures could be successfully predicted by the ideal based on structural factors of individual chemicals in two-component mixtures [26]. The data of the present study indicated that the toxicity inhibitions of the PADs mixtures were greater than that of the most toxic PAD compound. The DA model can be well utilized to evaluate the toxic effects of multicomponent mixtures of benzene derivatives, regardless of the inhibition levels and the PADs concentration ratios [19]. The PADs toxic evaluation in water systems is no more limited to individual pure chemicals. Both of the DA and IA approaches may be used to evaluate the toxic effects of PADs mixtures.

4. Conclusions

Assessment research of the individual toxicity of the 22 PADs confirmed that these compounds elicited the common effect on the inhibition luminescence of the photobacterium Q67 at concentrations below those that were lethal to the organisms. Based on the nonlinear least-squares fitting the experimental dose-response data of the 22 species of PADs, the best dose-response curves (DRCs) obtained for the 22 PADs to the photobacterium can be accurately expressed using the two-parameter Weibull or Logit model. The toxic inhibitions of multicomponent PADs mixtures can be well evaluated using the DA and IA models based on the concentration-response curves of individual components. Both of these models were proved to be suitable methods for the toxic evaluation of PADs mixtures in the aquatic ecosystem, when the models for the evaluation of multicomponent PADs mixtures were used with the EECR or UDCR mixture design.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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