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Toxicities of Six Phenolic Derivatives and Six Heavy Metals to the Fresh Water Luminescent Bacterium *Vibrio qinghaiensis* sp.-Q67

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Six phenolic derivatives (methyl phenol, 2-nitrophenol, 4-nitrophenol, 2-chlorophenol, 2,4-dichloro-phenol, phenol) and six heavy metals (Cu, Ni, Hg, Cd, Cr, Hg) were selected to investigate the joint toxicity of them, as well as their mixtures to *Vibrio qinghaiensis* sp.-Q67. The joint toxicity of twelve uniform design concentration ratio (UDCR) mixtures could be overestimated by the dose addition or by the independent action model within 95 % confidence intervals. All dose-response relationships could be effectively described by the two-parameters *i.e.*, Weibull or Logit function with correlation coefficient (R) greater than 0.99. Results indicate that when the total effects of mixtures were below 30 %, the mixture toxicity interactions were antagonistic actions, except for one uniform design ray of Mix-U1. With the increasing of the concentration, the effect of toxicity become opposite, for the mixtures are not in the same toxicity system. Value of β is large interval in the 3.90-10.98 range.

Keywords: Phenolic derivative, Heavy metals, *Vibrio qinghaiensis* sp.-Q67, Joint toxicity, Dose addition, Independent action.

INTRODUCTION

Phenol compounds are used in pesticides, dye and other industries and present in the environment widely which are one of the most important pollutions in water environment. With the development of the mining, metallurgy, chemical, electroplating, electronic industry, heavy metals have become one of the important pollutants of water environment. They are also the major carcinogenic pollutants in aquatic environments and highly toxic to all organisms. Toxic phenol can strongly inhibit the growth of bacteria, algae and mollusks[1]. There were 11 kinds of phenol compounds and 12 kinds of heavy metals among the 129 kinds of environmental priority pollutants specified by the US Environmental Protection Agency (US EPA)[2]. In recent years, the toxicological research of phenol compounds and heavy metals is becoming a hot spot in the environmental science[3-8].

Phenol derivatives and heavy metals found in the aquatic environments usually occur as relates to single pure substance, not to mixtures. Thus, the reliability of predictions made mixtures, not as single contaminant. However, the vast majority of available toxicity data regarding the aquatic toxicity of multi-component mixtures, derived from toxicity data on individual compound, is questionable. The type of toxicity test was studied by Backhaus *et al.*[9] Predictions of mixture toxicity require prior assumptions about the quantitative relations between the toxicity of single substances and those of mixtures. Essentially, two different concepts are available for this purpose, dose addition (DA) and independent action (IA).[9] Other early analysis methods of mixture toxicity included concepts such as toxic units (TU), additive index (AI) and mixture toxicity index (MTI). The similarity parameter λ was built on these two different basic concepts[9,10]. Joint toxicity studies on chemical mixtures have been slowly progressing over the past decades, with preliminary researches involving two-component mixtures in equi-effect concentration (most of which are EC_{50})[11]. 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 multi-component mixture predictions[9,12,13]. Toxicological evaluation of chemical mixtures is now considered to have truly entered into the field of multi-component mixtures[11,14-16].

In order to explore the toxicity change trend of various mixtures in the three-dimensional concentration space, the concentration ratio method of the one-dimensional charac-

teristics obviously does not meet the requirement. Uniform experiment is an optimized experimental design method considering experimental points uniformly distributed within the experimental range[17]. It is effective to select representative experimental points with the least possible number of experiments to reflect the uniform distribution of the mixture concentration and it has been widely used[7,8,18,19]. Uniform design table, referred to as U table, is expressed as $U_n(t)^q$ mathematically[17].

In the present study, the joint toxicities of six phenolic derivatives (methyl phenol, 2-nitrophenol, 4-nitrophenol, 2-chlorophenol, 2,4-dichloro-phenol, phenol) and six heavy metals (Cu, Ni, Hg, Cd, Cr, Hg) as well as those of their mixtures to *Vibrio qinghaiensis* sp.-Q67 were determined by using the microplate toxicity test procedure[7,8,18,19]. 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 Q67 bacteria can grow well in water and be luminous[7,18,19]. To effectively explore how joint toxicity varies with varying concentrations of specific compounds in a mixture, the compounds were mixed in uniform design concentration ratio (UDCR). To validate whether the dose addition model or the independent action model can predict the joint toxicity of a mixture of phenolic derivatives and heavy metals, it is necessary to inspect whether the dose-response curves (DRCs) predicted by the dose addition or independent action models locates within the 95 % confidence intervals of the experimental dose-response curves.

EXPERIMENTAL

All reagents used in this study were of analytical grade. The methyl phenol was purchased from Shanghai Fanyang Fine Chemical Co. Ltd. 2-Nitrophenol, 4-nitrophenol, 2-chlorophenol and $Cr(NO_3)_3{\cdot}9H_2O$ from Sinopharm Chemical Reagent Co. Ltd. 2,4-Dichloro-phenol from Reagent No.1 Factory of Shanghai Chemical Reagent Co., Ltd; $CuSO_4{\cdot}5H_2O$, $NiSO_4{\cdot}6H_2O$ and $HgCl_2$ from Shantou West Long Chemical Co., Ltd.; $CdSO_4{\cdot}8/_3H_2O$ from Guangdong Chemical Reagent Factory. Silver nitrate from Guangdong Taishan Daily Chemical Plant. Twelve uniform design concentration ratio mixtures of the test reagents 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 test organism is a novel freshwater photobacterium, *Vibrio-qinghaiensis* sp.-Q67 (Q67) that was kindly provided by East China Normal University. Details of the culture media and the culture condition have been given in our previous works[7,8,18,19]. 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 g yeast extract, 5 g tryptone, 3 g glycerin and 1000 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 the 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 rpm) at 22 ± 1 °C for 18 h[7,8,19,20].

The toxicity tests of the six phenolic derivatives and six heavy metals and their mixtures were performed by using a Veritas TM luminometer with a 96-well microplate (Turner Bio Systems Inc., USA). For each of the six phenolic derivatives and six heavy metals, 12 concentration gradients were designed based on our previous microplate toxicity test procedure[7,18]. 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 in the 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 phenolic derivatives and heavy metals 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 carried out by the same procedure as that of an individual toxicant.

The relative light units (RLUs) produced by the Q67 photobacteria exposed to various treatments (phenolic derivative and heavy metal or mixture) and controls (without any toxicant) were determined. The toxicity of toxicants to the Q67 was expressed as an inhibition ratio of the luminous intensity of the bacterium (E or x), which is calculated as follows:

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

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

It is well known that the concentration compositions in equi-effect concentrations ratio (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. The uniform design 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. Uniform design is an effective experimental design method established which can explore the concentration changes in three-dimension space with few experimental efforts. When the numbers of the mixture components (the factors in the uniform design) and the involved concentration levels (the levels of the factors) are large, it is very necessary and efficient to use the uniform design to study the combined effect of multiple-component mixture because the uniform design can reduced significantly the mixture experiment efforts.

A suitable use-table corresponding to the uniform table was selected according to the number of factors in real uniform design experiments. Because there are only 12 factors in this

TABLE-1
PARAMETERS OF THE CONCENTRATION-RESPONSE FUNCTIONS FOR THE 12 SELECTED CHEMICALS

No	Compound	Model	α	β	RMSE	R	EC_{50} (CI*) (mol/L)	pEC_{50}	pEC_{50}^{1}	pEC_{50}^{2}
L1	Methyl phenol	Weibull	6.61	2.61	0.01678	0.9984	2.1233E-03 (1.9169E-03, 2.4122E-03)	2.67	2.83	
L2	2-Nitrophenol	Weibull	6.33	1.94	0.02879	0.9968	3.5334E-04 (2.7906E-04, 4.5448E-04)	3.45	3.20	
L3	4-Nitrophenol	Weibull	7.21	2.32	0.01790	0.9986	5.4233E-04 (4.8420E-04, 6.1648E-04)	3.27		
L4	2,4-Dichloro-phenol	Weibull	6.14	1.78	0.01709	0.9986	2.2113 E-04 (1.5606E-04, 3.1569E-04)	3.66	3.66	
L5	Phenol	Weibull	5.50	2.58	0.02756	0.9970	5.3230E-03 (4.4709E-03, 6.3489E-03)	2.27		
L6	2-Chloro-phenol	Weibull	8.48	3.29	0.00078	0.9895	2.0612E-03 (1.8823E-03, 2.2961E-03)	2.69	2.81	
L7	Cr	Weibull	11.53	4.55	0.0245	0.9974	2.4287E-03 (2.2190E-03, 2.6604E-03)	2.61		2.39
L8	Cu	Weibull	20.47	7.21	0.0037	0.9666	1.2726E-03 (9.9719E-04, 1.5871E-03)	2.90		3.83
L9	Ni	Weibull	6.94	3.06	0.0371	0.9909	4.0950E-03 (3.3420E-03, 4.9872E-03)	2.39		2.76
L10	Cd	Weibull	8.69	2.26	0.0212	0.9969	9.8332E-05 (8.4193E-05, 1.1521E-04)	4.01		4.35
L11	Ag	Weibull	34.76	7.51	0.0178	0.9979	2.1023E-05 (1.8037E-05, 2.4055E-05)	4.68		
L12	Hg	Weibull	43.24	8.16	0.0247	0.9953	4.5296E-06 (4.1616E-06, 4.9165E-06)	5.34		

* CI refers to the 95 % confidence interval. The dates of pEC_{50}^{1} are from Liu *et al.*[23]; the dates of pEC_{50}^{2} are from Deng *et al.*[4]. EC_{50} unit - mol/L; α, β- the parameters of the Weibull model; RMSE-root mean square error

paper, 12 columns, 1 to 13, were selected from the U13(13^{12}) table to arrange the concentration compositions of 13 mixtures (Table-1)[21]. The mixture compositions allocated by uniform design (Table-1) can be built by replacing the corresponding serial number of levels in parentheses with six effect concentrations, ECx (x = 5,10, 15, 20, 30, 50). It should be noted that the experiment UD13 expressed by the last line in Table-1 was not performed because each pesticide in the mixture had the highest concentration levels (L13). The concentration ratios of six phenol compounds and six heavy metals in the mixtures, respectively, denoted as UD01, UD02, UD03, UD04, UD05, UD06, UD07, UD08, UD09, UD10, UD11 and UD12, were listed in Table-2. The mixture concentration design resulted in 12 mixture concentration ratios (UD01-UD12). Then, each mixture was regarded as one pseudo-toxicant whose concentration was the total concentration of all components and was further expanded to a series of concentration levels[22].

The purpose of uniform design mixture is to investigate the regularity of the mixture toxicity in the different concentration ranges. The uniform design can make the experimental points uniformly distributed in a three-dimensional space, then can examine the various possible mixtures in a larger range and more easily simulate the actual environment systems.

The selected model parameters of correlation coefficient (R), root mean square errors (RMSEx), the effect concentration (ECx) values of mixtures and predictions based on dose addition and independent action models were determined by the computer program APTox®[23]. APTox is a program used to perform the fitting of the dose repose curves, to predict the dose repose curve using the dose addition and independent action models and to design the microplate toxicity experiment.

RESULTS AND DISCUSSION

Based on the nonlinear least-squares fitting the experimental concentration-response data of the six phenolic derivatives and six heavy metals, the best dose-response curves (DRCs) were obtained. The DRCs of the 12 chemicals 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 micro-plate method and the experience rules that concentration points should be 5-6 times the DRCs model parameter values, the two-parameter model is markedly better 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 analytical formulae for the Weibull and Logit functions are given in equations 2 and 3, respectively.

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

$$x = 1/(1 + \exp(-\alpha-\beta\cdot\log(EC_x))) \quad (3)$$

The best simulated DRCs of the six phenolic derivatives and six heavy metals were obtained by using a non-linear least squares fit to the experimental data (Table-3). The results showed that the two-parameter Weibull or Logit function described the DRCs of the 12 chemicals on the photobacterium. The fitting parameters, *i.e.*, the root mean square error (RMSE), α, β and the correlation coefficient (R) are also shown in Table-3. The parameters α and β of the best fitting function were used to calculate the acute toxicity pEC_{50} and 95 % confidence interval of 12 kinds of compounds to fresh water luminescent bacteria. By inserting α and β into the equation for the inverse function of Weibull or Logit, the concentrations at each effect

TABLE-2
THE CONCENTRATION COMPOSITIONS OF 13 UD MIXTURE RAYS FROM U_{13} (13^{12})[a]

Mixture	L1	L2	L3	L4	L5	L6
UD01	EC_5(1)a	EC_5(2)	EC_{10}(3)	EC_{10}(4)	EC_{15}(5)	EC_{15}(6)
UD02	EC_5(2)	EC_{10} (4)	EC_{15}(6)	EC_{20}(8)	EC_{30}(10)	EC_{50}(12)
UD03	EC_{10}(3)	EC_{15}(6)	EC_{30}(9)	EC_{50}(12)	EC_5(2)	EC_{15}(5)
UD04	EC_{10}(4)	EC_{20}(8)	EC_{50}(12)	EC_{10}(3)	EC_{20}(7)	EC_{50}(11)
UD05	EC_{15}(5)	EC_{30}(10)	EC_5(2)	EC_{20}(7)	EC_{50}(12)	EC_{10}(4)
UD06	EC_{15}(6)	EC_{50}(12)	EC_{15}(5)	EC_{50}(11)	EC_{10}(4)	EC_{30}(10)
UD07	EC_{20}(7)	EC_5(1)	EC_{20}(8)	EC_5(2)	EC_{30}(9)	EC_{10}(3)
UD08	EC_{20}(8)	E EC_{10}(3)	EC_{50}(11)	EC_{15}(6)	EC_5(1)	EC_{30}(9)
UD09	EC_{30}(9)	EC_{15}(5)	EC_5(1)	EC_{30}(10)	EC_{15}(6)	EC_5(2)
UD10	EC30(10)	EC_{20}(7)	EC_{10} (4)	EC_5(1)	EC_{50}(11)	EC_{20}(8)
UD11	EC_{50}(11)	EC_{30}(9)	EC_{20}(7)	EC_{15}(5)	EC_{10}(3)	EC_5(1)
UD12	EC_{50}(12)	EC_{50}(11)	EC_{30}(10)	EC_{30}(9)	EC_{20}(8)	EC_{20}(7)
UD13[b]	EC_{60}(13)	EC_{60}(13)	EC_{60}(13)	EC_{60}(13)	EC_{60}(13)	EC_{60}(13)
Mixture	L7	L8	L9	L10	L11	L12
UD01	EC_{20}(7)	EC_{20}(8)	EC_{30}(9)	EC_{30}(10)	EC_{50}(11)	EC_{50}(12)
UD02	EC_5(1)	EC_{10}(3)	EC_{15}(5)	EC_{20}(7)	EC_{30}(9)	EC_{50}(11)
UD03	EC_{20}(8)	EC_{50}(11)	EC_5(1)	EC_{10}(4)	EC_{20}(7)	EC_{30}(10)
UD04	EC_5(2)	EC_{15}(6)	EC_{30}(10)	EC_5(1)	EC_{15}(5)	EC_{30}(9)
UD05	EC_{30}(9)	EC_5(1)	EC_{15}(6)	EC_{50}(11)	EC_{10}(3)	EC_{20}(8)
UD06	EC_{10}(3)	EC_{30}(9)	EC_5(2)	EC_{20}(8)	EC_5(1)	EC_{20}(7)
UD07	EC_{30}(10)	EC_{10}(4)	EC_{50}(11)	EC_{15}(5)	EC_{50}(12)	EC_{15}(6)
UD08	EC_{10}(4)	EC_{50}(12)	EC_{20}(7)	EC_5(2)	EC_{30}(10)	EC_{15}(5)
UD09	EC_{50}(11)	EC_{20}(7)	EC_{10}(3)	EC_{50}(12)	EC_{20}(8)	EC_{10}(4)
UD10	EC_{15}(5)	EC_5(2)	EC_{50}(12)	EC_{30}(9)	EC_{15}(6)	EC_{10}(3)
UD11	EC_{50}(12)	EC_{30}(10)	EC_{20}(8)	EC_{15}(6)	EC_{10}(4)	EC_5(2)
UD12	EC_{15}(6)	EC_{15}(5)	EC_{10}(4)	EC_{10}(3)	EC_5(2)	EC_5(1)
UD13[b]	EC_{60}(13)	EC_{60}(13)	EC_{60}(13)	EC_{60}(13)	EC_{60}(13)	EC_{60}(13)

a The figure in parentheses is the coding number of an original level in U_{13} (13^{12})
b The mixture experiment was in fact not performed because each component had the highest concentration level

TABLE-3
THE PERCENT CONCENTRATION RATIOS (PI %) OF THE TEST MIXTURE

Mixture	L1	L2	L3	L4	L5	L6
UD01	2.93	0.21	0.90	0.73	19.50	10.02
UD02	2.63	0.45	1.28	1.27	35.35	24.82
UD03	7.96	1.22	4.73	5.52	10.04	6.56
UD04	4.52	1.01	5.42	0.60	21.25	22.70
UD05	5.65	1.51	0.29	0.99	50.14	5.19
UD06	9.58	5.65	1.69	4.55	15.75	20.68
UD07	7.11	0.14	1.33	0.26	26.37	4.95
UD08	10.89	0.52	6.78	1.09	7.12	17.81
UD09	15.46	0.82	0.40	2.06	18.83	4.31
UD10	8.77	0.68	0.49	0.21	39.12	6.85
UD11	23.07	1.73	2.63	0.86	10.65	3.60
UD12	24.27	4.02	2.77	1.80	21.94	10.60
Mixture	L7	L8	L9	L10	L11	L12
UD01	18.31	12.14	34.21	0.72	0.28	0.06
UD02	7.79	8.60	17.16	0.41	0.21	0.05
UD03	26.47	25.05	11.79	0.32	0.29	0.07
UD04	7.12	9.02	28.08	0.09	0.14	0.04
UD05	16.32	5.37	13.43	0.96	0.11	0.03
UD06	14.90	16.75	9.71	0.54	0.15	0.05
UD07	15.55	6.42	37.43	0.22	0.19	0.03
UD08	12.83	17.77	24.80	0.11	0.23	0.04
UD09	31.44	11.72	13.41	1.32	0.19	0.03
UD10	8.54	4.19	30.63	0.39	0.10	0.2
UD11	26.22	11.33	19.48	0.27	0.13	0.02
UD12	13.21	9.31	11.77	0.19	0.11	0.02

level (*i.e.*, EC_x, the concentration that inhibits chemiluminescence by x (%) can be determined. To compare with the classical toxic index, the negative logarithm values of the EC_{50}, -logEC_{50} or pEC_{50}, for 12 chemicals are also given in Table-3. The results showed that the concentration-toxicity data of the 12 compounds can be well fitted by Weibull or Logit model function

and all the correlation coefficients (R), were greater than 0.96. The Weibull or Logit models with R values greater than 0.96 and RMSE values less than 0.037 exhibited not only good calibration ability but high stability[8].

Reliable concentration-response analyses for single substances are essential for predictions of mixture toxicities. The nonlinear fits to the regression models for the 12 compounds were depicted in Fig. 1. As can be seen from Fig. 1, the DRC of Hg was in the leftmost and its slope was also the largest, indicating the most toxicity and the fastest increase of response to concentration of Hg. The resulting concentration-response plots showed considerable differences in shape and position. However, the intersections among the curves clearly indicated that they were not parallel to each other in the strict mathematical sense. The most toxic compound was Hg (pEC_{50} = 5.34) and the least toxic compound was phenol (PAD13, pEC_{50} = 2.27). The toxicity order of the 12 compounds to Q67 was Hg > Ag > Cd > 2,4-Dichloro-phenol > 2-nitrophenol > 4-Nitrophenol > Cu > 2-chlorophenol > methyl phenol > Cr > Ni > phenol if the pEC_{50} (the negative logarithm of EC_{50}) value was considered as a toxicity index.

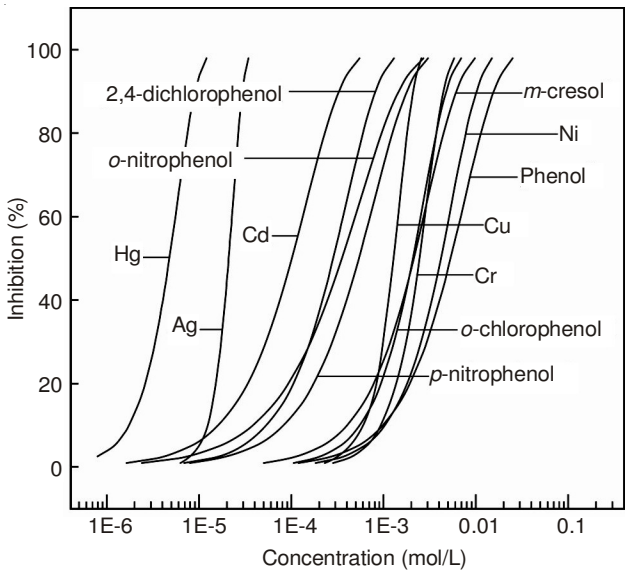

Fig. 1. Fitted dose response curves of 6 phenolic derivatives and 6 heavy metals

Compared with the pEC_{50} values of 2-chlorophenol and methyl phenol in literature[20], the relative deviation was 0.016, which shows no significant difference between these two test results. The toxicity order of the heavy metals was Cd > Cu > Ni > Cr which was in consistent with the results reported by Deng *et al.*[4]. The metal toxicity order of Hg > Cd > Cu was consistent with that of Song *et al.*[24]. The toxicity order of Cu, Ni, Hg to Q67 was Hg > Cu> Ni which was the same as to Heat Tetrahymena[5]. The DRCs of 12 individual chemicals in Fig. 1 also showed that the DRC of Hg is in the lowest concentration region and its slope is the largest, indicating the most toxicity and the fastest increase of its toxicity with the exposure concentration.

In the same way, the concentration-response (inhibition) data of the 12 mixtures on the UDCR method were determined on the veritasTM luminometer. The 12 UDCR mixtures consisted of six phenolic derivatives and six heavy metals. The results of the experimental determination of the mixture toxicities as well as the predictions made by the reference concepts, dose addition and independent action[14] are depicted in Fig. 2. Concentration-response functions for mixtures relate the total concentration of the mixture constituent c_{mix} to the inhibition of photobacterial luminosity. For the toxicities of the 12 different mixtures, the Weibull or Logit two-parameter models were used to obtain the fitting parameters RSME, α, β and correlation coefficient R (Table-4). The effect concentration EC_{50} and 95 % confidence intervals of mixture calculated by the best-fit function are also listed in the Table-4.

The Weibull or Logit models of the mixtures with R values greater than 0.99 and RMSE values less than 0.027 exhibited good calibration ability and high stability. For the set of 12 fixed concentration ratio mixtures, the $pEC_{50,mix}$ values ranged from 2.50 for UD10 to 2.85 for UD03. These values were within the range of $pEC_{50,i}$ for the 12 individual chemicals (2.27-5.34), *i.e.*, the values of the mixtures fall into the span between the most toxic and the least toxic individual chemicals, indicating the absence of strong synergistic or antagonistic interactions within the mixture[15].

According to dose addition and independent action models[14], the combined toxicities of the 12 mixtures were predicted to explore the toxicity interaction between various phenol and aniline derivatives in the mixtures (Table-5). Based on the optimal non-linear model of the 12 single chemicals (Weibull or Logit models), the effect concentrations $EC_{mix,DA}$ and $EC_{mix,IA}$ of the 20 points ranging from 1 % to 99 % in the conditions of the dose addition and independent action models

TABLE-4
DOSE-RESPONSE MODELS AND EFFECT CONCENTRATION VALUES OF THE 12 MIXTURES

Mixture	Model	α	β	RMSE	R	R^2	EC_{50} (CI) (mol/L)	pEC_{50}
UD01	Weibull	12.47	4.79	0.01328	0.9977	0.9954	2.0900E-03 (1.9357E-03, 2.2637E-03)	2.68
UD02	Weibull	11.24	4.44	0.00731	0.9993	0.9985	2.4318E-03 (2.1852E-03, 2.7165E-03)	2.61
UD03	Weibull	22.60	8.05	0.01768	0.9985	0.9970	1.4029E-03 (1.2454E-03, 1.5659E-03)	2.85
UD04	Weibull	17.61	6.86	0.00899	0.9995	0.9990	2.3962E-03 (2.2188E-03, 2.5811E-03)	2.62
UD05	Weibull	19.60	7.94	0.02711	0.9954	0.9907	3.0572E-03 (2.7968E-03, 3.3374E-03)	2.51
UD06	Weibull	12.06	4.44	0.02016	0.9966	0.9933	1.5894E-03 (1.4743E-03, 1.7113E-03)	2.79
UD07	Logit	28.53	10.98	0.02218	0.9970	0.9940	2.5214E-03 (2.3318E-03, 2.7398E-03)	2.59
UD08	Weibull	11.83	4.50	0.02008	0.9959	0.9918	1.9483E-03 (1.7268E-03, 2.1965E-03)	2.71
UD09	Weibull	13.14	5.09	0.02182	0.9958	0.9916	2.2320E-03 (2.0700E-03, 2.4132E-03)	2.65
UD10	Weibull	11.05	4.55	0.01215	0.9988	0.9975	3.1280E-03 (2.8615E-03, 3.4154E-03)	2.50
UD11	Logit	20.49	7.74	0.01587	0.9982	0.9965	2.2528E-03 (1.9894E-03, 2.5814E-03)	2.64
UD12	Weibull	10.13	3.90	0.02260	0.9958	0.9917	2.0351E-03 (1.6988E-03, 2.4148E-03)	2.69

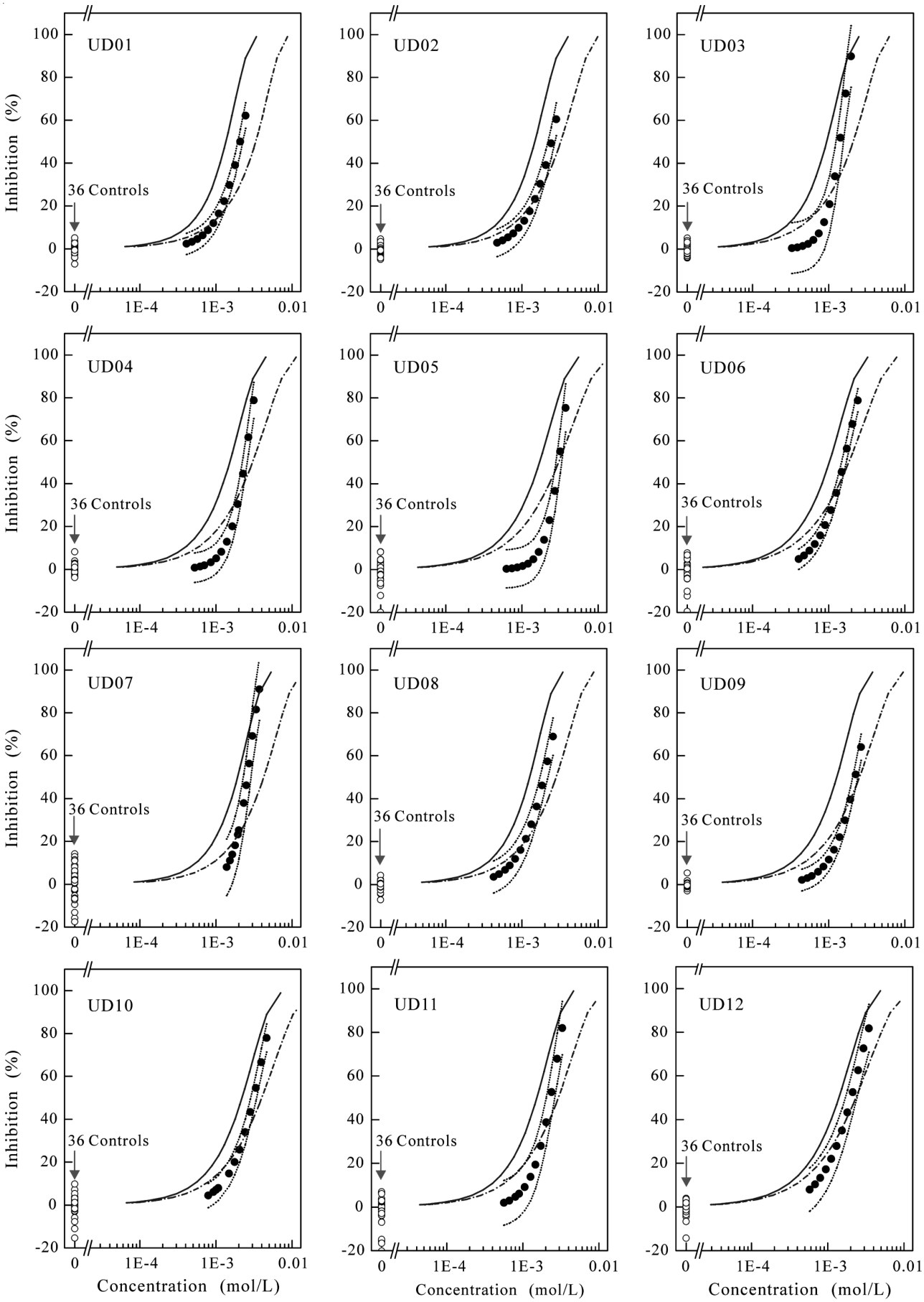

Fig. 2. The concentration-inhibition ratio (%) relationship of the 12 mixtures. (• Observed; -predicted by DA; --- predicted by IA; o Controls; ···· 95 % confidence interval of the experimental DRC)

TABLE-5
STATISTICS FOR TOXICITY PREDICTIONS BY THE DOSE ADDITION AND INDEPENDENT ACTION MODELS OF THE 12 MIXTURES

Mixture	$RMSE_{DA}$	R_{DA}	R^2_{DA}	$pEC_{50,mix\ DA}$	$RMSE_{IA}$	R_{IA}	R^2_{IA}	$pEC_{50,mix\ IA}$
UD01	7.937E-04	0.9997	0.9994	2.87	1.304E-03	0.9988	0.9977	2.51
UD02	1.043E-03	0.9998	0.9996	2.84	9.877E-04	0.9971	0.9941	2.55
UD03	5.043E-04	0.9922	0.9845	3.05	1.009E-03	0.9778	0.9561	2.77
UD04	8.390E-04	0.9939	0.9878	2.82	1.481E-03	0.9798	0.9600	2.56
UD05	1.303E-03	0.9861	0.9723	2.78	1.463E-03	0.9632	0.9277	2.58
UD06	5.897E-04	0.9979	0.9958	3.01	6.789E-04	0.9841	0.9684	2.83
UD07	6.951E-04	0.9941	0.9883	2.71	2.462E-03	0.9855	0.9713	2.40
UD08	7.359E-04	0.9998	0.9996	2.91	1.075E-03	0.9971	0.9941	2.60
UD09	9.520E-04	0.9981	0.9962	2.90	8.526E-04	0.9875	0.9752	2.66
UD10	8.941E-04	0.9988	0.9976	2.66	1.494E-03	0.9234	0.8527	2.42
UD11	7.576E-04	0.9960	0.9920	2.82	1.319E-03	0.9841	0.9685	2.58
UD12	6.566E-04	0.9990	0.9980	2.87	8.214E-04	0.9887	0.9776	2.70

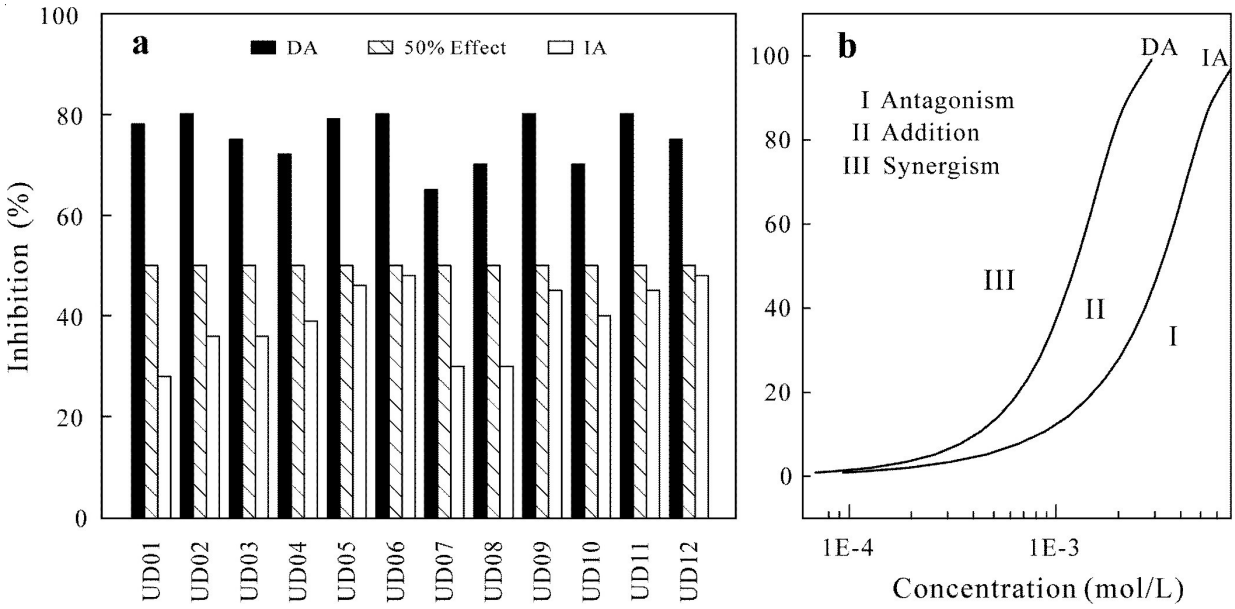

Fig. 3. The toxic percentage response of the 12 mixtures

were calculated using APTox[23] software. The plots of the effects predicted by the dose addition and independent action models compared with experimental with the 95 % confidence intervals can be seen in Fig. 2. The results indicated that the dose addition overestimated the effects of mixtures in EC_{50}.

When the total effects of mixtures were below 30 %, the experimental points were in I area (Fig. 3) and the mixture toxicity interactions were antagonistic actions, except for one uniform design ray of Mix-U1. With the increasing of the total effect, the experimental effect of numerical point fall into II area (between dose addition and independent action) and effect is additive action.The result of low toxic effect (< 30 %) is antagonistic action, the same as the result of Deng *et al.*[4]. With the increasing of the concentration, the effect of toxicity become opposite, for the mixtures are not in the same toxicity system. For mixtures from different system, toxicity results are not the same as that of Song *et al.*[24]. values of β is large interval in the 3.90-10.98 in Table-2, but β is smaller interval in the 2.86-4.30 in Song's research. The toxicity results of a mixture of different system whether related with the interval of β value need to be further studied.

Conclusion

For the uniform design mixtures which is no chemical reaction of phenol compounds with heavy metals of a wide range concentration. When the effect of mixtures was low (< 30 %), its effect is antagonistic action. With the concentration increasing (> 30 %), the toxicity would be additive effect. except for one uniform design ray of UD01, because β is large interval in the 1.78-8.16 of the individual DRCs. In fact, the relationships between the models for combined action depend on the distribution functions, the corresponding slope parameters and on the mixture concentrations administered[25]. From the results, it is clear that the mixture concentrations ratio is a very important factor.

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