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Quantitative structure-activity relationships for joint toxicity of substituted phenols and anilines to *Scenedesmus obliquus*

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Abstract

There are often many chemicals coexisting in aquatic ecosystems, and few information on the joint toxicity of a mixture of organic pollutants is available at present. The 48-h toxicity of substituted phenols and anilines and their binary mixtures to *Scenedesmus obliquus* was determined by the algae inhibition test. The median effective inhibition concentration EC_{50} values for single compounds and EC_{50mix} values for coexistent compounds were obtained. The *n*-octanol/water partition coefficient ($\log P_{mix}$) and the frontier orbital energy gap (ΔE_{mix}) for mixtures were calculated. The following two-descriptor quantitative structure-activity relationships (QSARs) models were developed to predict single toxicity and joint toxicity respectively: $\log(1/EC_{50}) = 0.445\log P - 0.801\Delta E + 9.501$ (r^2 = 0.876) and $\log(1/EC_{50mix}) = 0.338\log P_{mix} - 0.492\Delta E_{mix} + 6.928$ (r^2 = 0.831). The two equations were found to fit well. In addition, the model derived from the structural parameters of single components in binary mixtures $\log(1/EC_{50mix}) = 0.222\log P - 0.277\Delta E + 5.250$ (r^2 = 0.879) can be used successfully to predict the toxicity of a mixture.

Key words: joint toxicity; QSARs; frontier orbital energy gap; 2,4-dichlorophenol

Introduction

Phenol, aniline and their derivatives are widely used industrial chemicals, and consequently have a high potential to become environmental pollutants. In which, 2,4-dichlorophenol is one of prior monitoring pollutants regulated by the Environmental Protection Agency of China. Toxicity of these chemicals has been extensively investigated using fish, *Tetrahymena pyriformis* and *Vibrio fischeri* (Könemann and Musch, 1981; Cronin and Schultz, 1996; Lu *et al*., 2003). However, in aquatic ecosystems there are often many chemicals coexistent, and few information on joint toxicity of a mixture of aniline and phenol derivatives is available. Quantitative structure-activity relationships (QSARs) are powerful tools in predicting the toxicological effects of chemicals. Multiple QSARs for the toxicity of single chemicals have been developed in the past two decades. QSAR studies on the joint toxicity of organic chemicals can assess objectively and predict the toxicological effect of coexisting pollutants on aquatic organisms. In an early study, Placket and Hewlett (1967) identified four types of actions in analyzing the joint toxicity of binary mixtures, i.e., simply additive, more than additive/synergism, less than-additive/partial addition and no interaction/independent. The above actions have been quantitatively classified using the toxic unit (TU); additivity index (AI); the similarity parameter (λ); and mixture toxicity index (MTI). Recently, joint toxicity prediction based on QSARs has been attempted. Xu and Nirmalakhandan (1998) applied QSARs models derived from single chemical toxicity assays to predict joint toxicity of mixtures of organic chemicals to microorganisms. Yuan *et al*. (2002) determined the joint toxicity of binary mixtures of 2,4-dinitrobenzne and 8 nitrobenzenes or anilines to *V. fischeri* according to an equiconcentration ratio (1:1) and developed a QSAR for joint toxicity using molecular orbital energy as a structural descriptor. Huang *et al*. (2003) measured the joint toxicity of phenol derivatives to tadpoles (*Rana japonica*) and predicted mixture toxicity using *n*-octanol/water partition coefficient.

Since algae are the primary producers in many aquatic ecosystems, their susceptibility to contaminants has been the subject of many reports. The purpose of this study was to experimentally determine the toxicity of substituted phenols and anilines and their binary mixtures to the algae (*Scenedesmus obliquus*), and assess, model and predict joint toxicity using molecular structural descriptors.

1 Materials and methods

1.1 Chemicals

Substituted phenols and anilines were purchased from Shanghai Chemical Reagent Co., China (analytical reagent grade). Other compounds used for preparation of liquid

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medium were of chemically pure quality. The test chemical was dissolved in 1 ml of acetone and added distilled water to 500 ml. The ultrasonic cleaner was utilized to accelerate dissolution for those undissolved compounds.

1.2 Bioassay

S. obliquus was supplied by Wuhan Institute of Hydrobiology, Chinese Academy of Sciences. The algae inhibition test was employed to determine toxicity (OECD, 1981). The culture was maintained in a liquid medium, prepared as described in Lu *et al*. (2001). After initial range finding experiments, each compound was tested in a logarithmic concentration series (6–8 concentrations). Algae in the logarithmic growing period were inoculated into 250-ml Erlenmeyer flasks, adjusting the final volume of the culture media, compound and alga to 60 ml. Culture media without test compounds served as the control solution. The initial algal cell concentration in the test culture was approximately 3×10^4 cells/ml. The culture was incubated at 20±1°C. A 12-h light and 12-h dark photoperiod was programmed and the average illumination intensity was about 4000 lx produced by a white fluorescent lamp. All experiments were performed with two replicates run simultaneously.

Growth was monitored by the electron microcope (400 magnification). Data were handled according to the following equations:

$$\mu = \ln(N_t/N_0)/(t - t_0) \quad (1)$$

where, μ is the algal average growth rate, N_0 is the initial cell concentration, N_t is the cell concentration after being cultured for 48 h, and (t–t_0) is the experimental period (48 h in the current study).

$$I = (\mu_b - \mu_{tox)}/\mu_b \times 100\% \quad (2)$$

where, I is the inhibition rate, μ_b is the algal average growth rate of the control, and μ_{tox} is the algal average growth rate under exposure of toxicants. Logarithms of the inverse median effective inhibition concentration after 48-h exposure, expressed as log ($1/EC_{50}$) (mol/L), were determined through one variable linear regression analysis of the negative logarithm of compound concentrations and the inhibition rates as the relative toxic potency for each single chemical.

To test the toxicity of each mixture, binary mixtures consisted of 2,4-dichlorophenol and another phenols or anilines were conducted at an equal-toxic ratio (1:1) based on observed EC_{50} values. All toxicity testing procedures for mixtures were the same as those for single chemicals. The joint toxicity of mixtures was described as Eq.(3) based on the work of Preston *et al*. (2000).

$$EC_{50mix} = (C_A + C_B)/(C_A/EC_{50A} + C_B/EC_{50B}) \quad (3)$$

In this equation, EC_{50mix} is the median effective inhibition concentration of a mixture, EC_{50A} and EC_{50B} are the median effective inhibition concentration of component A and B, and C_A and C_B are the concentration of component A and B in binary mixtures.

1.3 Statistical analyses

QSAR models were developed through multiple linear regression analyses using the SPSS statistical package (ver. 11.0, SPSS Company, Chicago, IL, USA). Model quality was characterized by the number of observations (n), the square of correlation coefficient (r^2), the adjusted square of correlation coefficient (r^2_{adj}), the standard error (SE), the mean square of residual for estimated parameter (MR), the mean square ratio (F), the significance level (Pr).

The logarithm of each n-octanol/water partition coefficient (log$P$) was obtained from log$P$ for Windows software (ver. 3.55, Biobyte Company, Claremont, CA, USA). The energy of the lowest unoccupied molecular orbital (E_{LUMO}) and the energy of the highest occupied molecular orbital (E_{HOMO}) were obtained from the ChemOffice 2004 program using the quantum chemical method MOPAC (http://www.cambridgesoft.com). The parameter values of the studied chemicals are listed in Table 1.

2 Results and discussion

The observed 48-h log($1/EC_{50}$) of 21 substituted phenols and anilines and the log($1/EC_{50mix}$) of 20 binary mixtures are given in Table 2. Compounds exhibited a reasonably wide range of algal toxicity and log($1/EC_{50}$) values ranged from 2.60 for aniline to 4.56 for α-naphthol. For the purpose of comparison, the 5-min bioluminescent bacterium EC_{50} values of phenolic compounds (Ren and Frymier, 2003) and the 15 min *Chlorella vulgaris* EC_{50} values of anilines (Netzeva *et al*., 2004) are also shown. When comparing the toxicity of 4 anilines on *S. obliquus* with those from *C. vulgaris*, all the chemicals tested exhibit higher toxicity on *S. obliquus* than on *C. vulgaris*. The comparison of *S. obliquus* and bioluminescent bacterium EC_{50} data shows that chlorophenols and nitrophenol were more toxic to bioluminescent bacterium than to *S. obliquus*, but for phenol itself and naphthols, the situation was inverse.

It is well known that non-specific toxicity of chemicals can be described by two kinds of actions: non-polar narcosis (type I narcosis) and polar narcosis (type II narcosis). Non-polar narcotic chemicals are considered baseline toxicants. Their toxicity is proportional to their concentrations at the site of action and is caused solely by membrane perturbation (Schultz *et al*., 1986). Polar narcotic chemicals, typified by most phenols and anilines, exhibit toxic potency higher than that estimated by their hydrophobicity due to the existence of polar substituents in the molecules (Kamlet *et al*., 1986). The addition of an electronic parameter can improve the prediction of a log$P$ dependent model (Schultz *et al*., 1989).

The relationship of the individual toxicity of 21 substituted phenols and anilines with log$P$ was analyzed and Eq.(4) was obtained. However, the log$P$-dependent model explains only 57.1% of variance.

$$\log(1/EC_{50}) = (0.663 \pm 0.132)\log P + (1.913 \pm 0.300) \quad (4)$$
$$n = 21,\ r^2 = 0.571,\ r^2_{adj} = 0.548,\ SE = 0.471,\ MR = 0.222,\ F = 25.25,\ Pr > F = 0.000$$

Table 1 Structural parameters of phenols and anilines

Compound	log$P$	logP_{mix}	E_{HOMO} (eV)	E_{LUMO} (eV)	ΔE (eV)	ΔE_{mix} (eV)
2,4-Dichlorophenol	3.06		–9.45	–0.38	9.07	
2-Chlorophenol	2.15	2.34	–9.40	–0.07	9.33	9.26
3-Chlorophenol	2.50	2.61	–9.43	–0.04	9.39	9.31
4-Chlorophenol	2.39	2.62	–9.28	–0.08	9.20	9.12
Phenol	1.46	1.68	–9.25	0.29	9.54	9.46
α-Naphthol	2.85	3.05	–8.31	–0.54	7.77	8.89
β-Naphthol	2.70	3.03	–8.31	–0.53	7.78	8.88
3-Nitrophenol	2.00	2.37	–10.15	–1.26	8.89	8.92
4-Nitrophenol	1.91	2.42	–10.30	–1.24	9.06	9.02
Hydroquinone	0.59	1.06	–9.07	0.12	9.19	9.14
Resorcinol	0.80	1.20	–9.24	0.12	9.36	9.30
2,4-Dichloroaniline	2.78	2.92	–8.96	–0.09	8.87	8.97
2-Chloroaniline	1.90	2.13	–8.85	0.20	9.05	9.03
3-Chloroaniline	1.88	2.15	–8.93	0.19	9.12	9.08
4-Chloroaniline	1.83	1.99	–8.81	0.22	9.03	9.02
4-Bromoaniline	2.26	2.53	–8.87	0.13	9.00	8.98
Diphenylamine	3.50	3.09	–8.56	0.45	9.01	8.97
Aniline	0.90	1.19	–8.28	0.60	8.88	8.89
2,4,6-Trichloroaniline	3.52	3.09	–8.71	–0.33	8.38	8.93
α-Naphthylamine	2.25	2.97	–8.08	–0.36	7.72	8.82
N-Methylaniline	1.66	1.94	–8.53	0.57	9.10	9.07

Table 2 Predicted toxicity and the residuals of compounds

Compound	log(1/EC_{50}) (mol/L)				log(1/EC_{50mix}) (mol/L)				
	Obs.[a]	Pre.[b]	Res.[c]	Obs.	Obs.[d]	Pre.[e]	Res.[f]	Pre.[g]	Res.[h]
2,4-Dichlorophenol	3.42	3.60	–0.18	3.74*					
2-Chlorophenol	2.84	2.98	–0.14	2.93 *	3.03	3.16	–0.13	3.14	–0.11
3-Chlorophenol	2.92	3.09	–0.17		3.19	3.23	–0.04	3.20	–0.01
4-Chlorophenol	3.14	3.20	–0.06	3.81*	3.26	3.32	–0.06	3.23	0.03
Phenol	2.61	2.51	0.10	2.29 *	2.85	2.84	0.01	2.93	–0.08
α-Naphthol	4.56	4.55	0.01	3.43 *	3.69	3.58	0.11	3.73	–0.04
β-Naphthol	4.53	4.47	0.06	4.08*	3.69	3.58	0.11	3.70	–0.01
3-Nitrophenol	3.15	3.27	–0.12		3.27	3.34	–0.07	3.23	0.04
4-Nitrophenol	3.33	3.09	0.24	3.51*	3.37	3.30	0.07	3.16	0.21
Hydroquinone	2.79	2.40	0.39		3.00	2.79	0.21	2.84	0.16
Resorcinol	2.76	2.36	0.40	2.76 *	2.98	2.75	0.23	2.83	0.15
2,4-Dichloroaniline	3.40	3.63	–0.23		3.41	3.50	–0.09	3.41	0.00
2-Chloroaniline	2.81	3.10	–0.29		3.02	3.20	–0.18	3.17	–0.15
3-Chloroaniline	2.90	3.03	–0.13	2.69#	3.08	3.18	–0.10	3.14	–0.06
4-Chloroaniline	2.91	3.08	–0.17		3.07	3.16	–0.09	3.16	–0.09
4-Bromoaniline	3.13	3.30	–0.17	2.67#	3.25	3.36	–0.11	3.26	–0.01
Diphenylamine	4.49	3.84	0.65		3.69	3.56	0.13	3.53	0.16
Aniline	2.60	2.79	–0.19	1.66 #	2.85	2.95	–0.10	2.99	–0.14
2,4,6-Trichloroaniline	4.55	4.36	0.19	4.11 #	3.69	3.58	0.11	3.71	–0.02
α-Naphthylamine	4.29	4.32	–0.03		3.67	3.59	0.08	3.61	0.06
N-Methylaniline	2.79	2.95	–0.16		3.02	3.12	–0.10	3.10	–0.08

[a] Observed data; [b] values calculated by Eq.(5); [c] residuals calculated by Eq.(5); [d] observed data; [e] predicted values calculated by Eq.(7); [f] residuals calculated by Eq.(7); [g] predicted values calculated by Eq.(9); [h] residuals calculated by Eq.(9); *5 min log(1/EC_{50}) for bioluminescent bacterium; #15 min log(1/EC_{50}) for *C. vulgaris*.

The standard errors for parameters were given in the parentheses in Eq.(4).

To improve the prediction for the toxicity of polar narcotic phenols and anilines studied in this paper, the frontier orbital energy gap ΔE was introduced and defined as $\Delta E = E_{LUMO} - E_{HOMO}$. ΔE is a critical parameter determining the molecular admittance. It can be said that the larger the ΔE value, the more stable the molecule and, thus, the harder the rearrangement of its electron density under the presence of an external charge or external electric field (Mielczarek, 2005). The following two-descriptor model was obtained from multivariable regression analyses:

$$\begin{aligned}\log(1/EC_{50}) &= (0.445 \pm 0.080)\log P - (0.801 \\ &\quad \pm 0.121)\Delta E + (9.501 \pm 1.154) \\ n &= 21,\ r^2 = 0.876,\ r^2_{adj} = 0.862,\ SE = 0.261, \\ MR &= 0.067,\ F = 63.33,\ Pr > F = 0.000\end{aligned} \quad (5)$$

Eq.(5) was used to predict toxicity, and the predicted values and residuals are presented in Table 2. The plot of observed log(1/EC_{50}) to algae versus calculated values by the model (Eq.(5)) is shown in Fig.1. Eq.(5) was found to fit very well and the correlation coefficient of observed toxicity and predicted toxicity is 0.936.

Based on an independence assumption, which implies

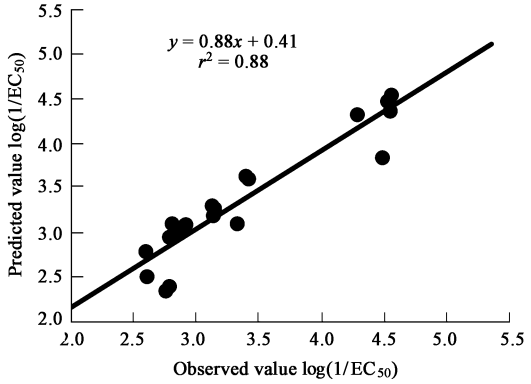

Fig. 1 Calculated $\log(1/EC_{50})$ from Eq.(5) verse observed $\log(1/EC_{50})$ of single compounds.

that partitioning of a mixture is simply the summed partitioning of individual chemicals and ignores the interactions between components, Huang *et al.* (2003) proposed an equation to predict *n*-octanol/water partition coefficient for mixtures ($\log P_{mix}$) (Eq.(6)).

$$\log P_{mix} = (C_A \times \log P_A + C_B \times \log P_B + \cdots)/(C_A + C_B + \cdots) \quad (6)$$

where, $\log P_{mix}$ is *n*-octanol/water partition coefficient of the binary mixtures, $\log P_A$ and $\log P_B$ are *n*-octanol/water partition coefficients of component A and B, and C_A and C_B are the concentrations of component A and B in binary mixtures. An obvious correlation between the joint toxicity to tadpoles and $\log P_{mix}$ for 23 mixtures of substituted phenols was obtained and the $\log P_{mix}$-dependent model could explain 88% of variance (Huang *et al.*, 2003).

In our study, the $\log P_{mix}$ of 20 binary mixtures were calculated based on Eq.(6) (Table 1). In addition, we proposed the orbital energy of a mixture is also a simple sum of the individual components and calculated the frontier orbital energy gap of mixtures ΔE_{mix}. A regression method similar to single chemical analyses was used to model joint toxicity ($\log(1/EC_{50mix})$). A two-descriptor QSAR model was obtained as follows in Eq.(7).

$$\log(1/EC_{50mix}) = (0.338 \pm 0.051)\log P_{mix} - (0.492 \pm 0.198)\Delta E_{mix} + (6.928 \pm 1.856)$$
$$n = 20,\ r^2 = 0.831,\ r^2_{adj} = 0.811,\ SE = 0.129,\ MR = 0.017,\ F = 41.8,\ Pr>F = 0.000 \quad (7)$$

Eq.(7) was characterized by a high coefficient (0.831) and small error of estimate (0.129). Thus it appears that joint toxicity is well correlated with the $\log P_{mix}$ and ΔE_{mix} of the mixtures. Eq.(7) was used to predict joint toxicity, and the predicted values and residuals are presented in Table 2. A comparison of the predicted values from the model (Eq.(7)) with the observed toxicity shows that they were very close (Fig.2a).

To further test the robust and predictive capability of Eq.(7), 20 mixtures were randomly divided into two sets. Among these, 5 treatments (4-chlorophenol, β-naphthol, resorcinol, 4-chloroaniline and α-naphthylamine) were included in the test set and the remaining data in the training set. A regression analysis for the training data was performed and resulted in Eq.(8). The toxicity of the test set mixtures was predicted using Eq.(8). The relationship between the predicted and observed $\log(1/EC_{50mix})$ showed a good consistency with r^2=0.860. Since 5 mixtures were randomly excluded from the procedure and the correlation between the observed toxicity and the predicted toxicity by this model was satisfactory, the QSAR-based model therefore can be used to predict joint toxicity of aromatic anilines and phenols to algae.

$$\log(1/EC_{50mix}) = (0.356 \pm 0.056)\log P_{mix} - (0.508 \pm 0.214)\Delta E_{mix} + (7.014 \pm 1.984)$$
$$n = 15,\ r^2 = 0.837,\ r^2_{adj} = 0.810,\ SE = 0.127,\ MR = 0.016,\ F = 30.79,\ Pr>F = 0.000 \quad (8)$$

Since the binary mixtures were composed of 2,4-dichlorophenol and other substituted phenols and anilines respectively according to equal-toxic ratio (1:1) in this study, there was an obvious relationship between $\log P$ of single compounds and $\log P_{mix}$ of binary mixtures (r^2 = 0.91), then the toxicity of mixtures may be related to the structural parameters of single components (Yuan *et al.*, 2002). Through multivariable linear regression analyses, the following QSAR model between log ($1/EC_{50mix}$) and $\log P$ and ΔE was developed:

$$\log(1/EC_{50mix}) = (0.222 \pm 0.035)\log P - (0.277 \pm 0.052)\Delta E + (5.250 \pm 0.497)$$
$$n = 20,\ r^2 = 0.879,\ r^2_{adj} = 0.864,\ SE = 0.109,\ MR = 0.012,\ F = 61.5,\ Pr>F = 0.000 \quad (9)$$

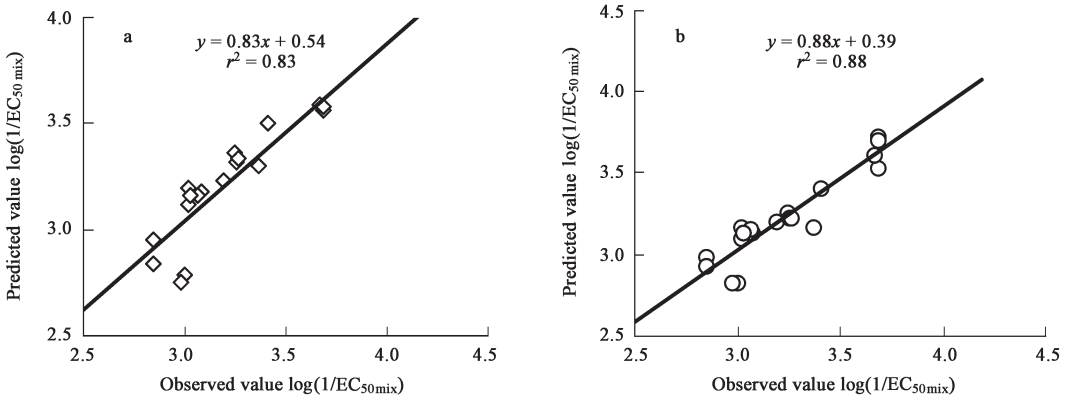

Fig. 2 Calculated $\log(1/EC_{50mix})$ from Eq.(7) (a) and Eq.(9) (b) verse observed $\log(1/EC_{50mix})$ of mixtures.

Eq.(9) explains most of the variance (87.9%), with maximum F values (61.5) and minimum standard error of estimate (0.109) and with neither statistical nor obvious visual outliers observed. The model 9 was used to predict joint toxicity, the predicted values and residuals are presented in Table 2, and the plot of observed $\log(1/EC_{50mix})$ to algae versus calculated values is shown in Fig.2b.

The quality of model fit and prediction of Eq.(5) and Eq.(9) was similar with comparable r^2, slope and intercept in Figs.1 and 2b. It is shown that the model derived from the structural parameters of single component can be used successfully to predict the joint toxicity of binary mixtures contained 2,4-dichlorophenol.

The obtained models revealed that both individual toxicity and joint toxicity of phenol, aniline and their derivatives to algae were related mainly to their hydrophobicity and electronic properties. log$P$ is a hydrophobicity parameter, the higher the log$P$, the stronger the hydrophobicity and the easier the compounds were bioconcentrated in an organism. The substituted phenols and anilines studied in this paper are polar narcotics. Such compounds exhibit toxicity similar to non-polar narcotics, but at potency levels greater than estimated by their hydrophobicity. The addition of the frontier orbital energy gap ΔE can enormously improve the prediction of log$P$-dependent models. Toxicity increases with greater negative ΔE. That is, the smaller the value of ΔE, the easier the electron transfers from HOMO orbital to LUMO orbital and the stronger the toxicity. This result is consistent with Yan *et al*. (2006).

3 Conclusions

Acute toxicity of substituted phenols and anilines and their binary mixtures to the algae was determined. The median effective inhibition concentration EC_{50} values for single compound and EC_{50mix} values for coexistent compounds were obtained. Not only for individual toxicity but also for joint toxicity, successful two-descriptor QSAR models, accounting for octanol/water partition coefficient and frontier orbital energy gap, were developed. The substituted phenols and anilines studied in this paper are polar narcotics, and their toxicity to algae is related mainly to electronic properties and hydrophobicity. However, this paper only investigated toxicity of binary mixtures containing 2,4-dichlorophenol, and the model derived from the structural parameters of individual components can be used to predict joint toxicity. The prediction for joint toxicity of multiple-component mixtures should be studied in the further.

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