

# Quantitative Structure–Activity Relationships for Predicting the Joint Toxicity of Substituted Anilines and Phenols to Algae

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Aniline, phenol, and their derivatives are widely used industrial chemicals that consequently have a high potential for environmental pollution. The toxicity of these chemicals has been investigated extensively using fish, *Tetrahymena pyriformis* and *Vibrio fischeri* (Cronin and Schultz, 1996; Könemann and Musch, 1981; Lu et al., 2003). However, in aquatic ecosystems, there often are many coexistent chemicals, and information on the joint toxicity of a mixture of aniline and phenol derivatives is scarce.

Quantitative structure–activity relationships (QSARs) are powerful tools for predicting the toxicologic effect of chemicals. In the past two decades, QSARs for the toxicity of single compounds have been developed fleetly. The joint toxicity of organic chemicals can be assessed objectively by QSAR studies, and the toxicologic effect of coexistent pollutants on aquatic organisms can be predicted.

In an early study analyzing the joint toxicity of binary mixtures, Placket and Hewlett (1967) identified four types of actions: simply additive, more than additive (synergism), less than additive (partial addition), and no interaction (independent). These actions have been quantitatively classified using the Toxic Unit (TU), the Additivity Index (AI), the Similarity Parameter ( $\lambda$ ), and the Mixture Toxicity Index (MTI).

Recently, joint toxicity prediction based on QSARs has been attempted. Xu and Nirmalakhandan (1998) applied

QSAR models derived from single chemical toxicity assays to predict the joint toxicity of mixtures of organic chemicals to microorganisms. Yuan et al. (2002) determined the joint toxicity of binary mixtures of 2,4-dinitrobenzene, 8 nitrobenzenes, and anilines to *Vibrio fischeri* according to an equiconcentration ratio (1:1) and developed a QSAR for joint toxicity using  $E_{\text{LUMO}}$  as the structural descriptor. Huang et al. (2003) measured the joint toxicity of phenol derivatives to tadpoles (*Rana japonica*) and predicted mixture toxicity using  $\log P$ . This study aimed to determine experimentally the toxicity of single substituted anilines and phenols and related binary mixtures to the algae *Scenedesmus obliquus*, and to assess, model, and predict joint toxicity using molecular structural descriptors.

## Materials and Methods

*Scenedesmus obliquus* was supplied by Wuhan Institute of Aquatic Organisms, Chinese Academy of Science. The toxicity test method was referred to the algae inhibition test (OECD, 1981). The culture was maintained in a liquid medium, and the formula of culture medium was referred to Lu et al. (2001). For each compound studied, seven concentration steps in a logarithmic gradient ranging from no effect to 100% growth inhibition concentration were planned after range-finding experiments. The algae in the logarithmic growing period were inoculated into 250-mL Erlenmeyer flasks, which amounted to 60 mL of the culture media, the compound, and the algae. The culture media without test compounds served as the control solution. The initial algae cell concentration in the test culture was approximately  $3 \times 10^4$  cells/mL. The culture was incubated at  $20 \pm 1^\circ\text{C}$ . A 12-h light and 12-h dark photoperiod

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was programmed, and the average illumination intensity was about 4,000 lux produced by a white fluorescent lamp. All experiments were performed with two replicates of each treatment run simultaneously.

Growth was monitored using the electron microscope ( $\times 400$ ). Data were handled according to the following formulas:  $\mu = \ln(N_t/N_0)/(t - t_0)$ , where  $\mu$  is the average specific growth rate,  $N_0$  is the initial cell concentration,  $N_t$  is the cell concentration after culturing for 48 h, and  $t - t_0$  is the experimental period (48 h in this study); and  $I = [\mu(b) - \mu(\text{tox})]/\mu(b) \times 100\%$ , where  $I$  is the inhibition rate,  $\mu(b)$  is the average specific growth rate of the control, and  $\mu(\text{tox})$  is the average specific growth rate of added toxic compound. Logarithms of the inverse median effective inhibition concentration after 48 h of exposure, expressed as  $\log 1/EC_{50}$  (mol/L), were gained 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 the mixture, binary mixtures consisting of 2,4-dichloroaniline and another anilines or phenols were conducted at an equitoxic ratio (1:1) based on observed  $EC_{50}$  values. All toxicity testing procedures of mixtures were performed as with the single chemicals. The joint toxicity of mixtures was described as follows based on the work of Preston et al. (2000):

$$EC_{50\text{mix}} = (C_A + C_B) / (C_A/EC_{50A} + C_B/EC_{50B}). \quad (1)$$

In this equation,  $EC_{50\text{mix}}$  is the median effective inhibition concentration of binary mixtures;  $EC_{50A}$  and  $EC_{50B}$  are the median effective inhibition concentrations of components A and B; and  $C_A$  and  $C_B$  are the concentrations of components A and B in binary mixtures.

The logarithm of the n-octanol/water partition coefficient ( $\log P$ ) was obtained from ClogP for Windows software (version 3.55; Biobyte Company, Claremont, CA, USA). The measured values of  $\log P$  were selected for QSAR study. The energy of the lowest unoccupied molecular orbital ( $E_{\text{LUMO}}$ ) and the energy of the highest occupied molecular orbital ( $E_{\text{HOMO}}$ ) were obtained from the ChemOffice 2004 program using the quantum chemical method MOPAC (<http://www.cambridgesoft.com>). The parameter values of the chemicals studied are listed in Table 1. The linear regression analyses were carried out using the SPSS statistical package (version 9.0; SPSS Company, Chicago, IL, USA).

## Results and Discussion

The observed 48-h  $\log 1/EC_{50}$  of 21 substituted anilines and phenols and the  $\log 1/EC_{50\text{mix}}$  of 20 binary mixtures

are given in Table 1. The 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  $\alpha$ -naphthol. For the purpose of comparison, the 5-min bioluminescent bacterium Shk1  $EC_{50}$  values of phenolic compounds (Ren et al., 2003) and the 15-min *Chlorella vulgaris*  $EC_{50}$  values of anilines (Netzeva et al., 2004) also are shown. When the toxicity of four anilines on *S. obliquus* was compared with those from *C. vulgaris*, all the chemicals tested exhibited higher toxicity on *S. obliquus* than on *C. vulgaris*. The comparison of *S. obliquus* and Shk1  $EC_{50}$  data shows that chlorophenols and nitrophenol were discovered to be more toxic to Shk1, but for phenol itself and Naphthols, the situation was the converse.

It is well known that the nonspecific toxicity of chemicals can be described by two kinds of action: nonpolar narcosis (type 1 narcosis) and polar narcosis (type 2 narcosis). Nonpolar narcotic chemicals are considered baseline toxicants. Their toxicity is proportional to their concentrations at the site of action and 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 octanol/water partition coefficient ( $\log P$ ) dependent model (Schultz et al., 1989).

The relationship of the single toxicity of 21 substituted anilines and phenols with  $\log P$  was analyzed, and Equation 2 was obtained. However, the  $\log P$ -dependent model explains only 57.1% of the variance.

$$\log 1/EC_{50} = 0.663(0.132) \log P + 1.913(0.300) \quad (2)$$

$$n = 21, \quad r^2 = 0.571, \quad r^2_{\text{adj}} = 0.548, \quad SE = 0.471, \quad F = 25.25, \quad p < 0.001.$$

In this equation,  $n$  is the number of observations;  $r^2$  is the square of the correlation coefficient;  $r^2_{\text{adj}}$  is the adjusted  $r^2$  value;  $SE$  is the standard error;  $F$  is the mean square ratio; and  $p$  is the probability greater than the  $F$  value.

To improve the prediction for the toxicity of polar narcotic anilines and phenols studied in this report, the frontier orbital energy gap  $\Delta E$  was introduced and defined as  $\Delta E = E_{\text{LUMO}} - E_{\text{HOMO}}$ , where  $\Delta E$  is a critical parameter determining the molecular admittance. It can be said that the larger the  $\Delta E$  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:

**Table 1** Toxicity data and structural parameters

Compounds	Log1/EC <sub>50</sub>		log1/EC <sub>50mix</sub>		logP	logP <sub>mix</sub>	E <sub>HOMO</sub>	E <sub>LUMO</sub>	ΔE	ΔE <sub>mix</sub>
	Obs.	Pre <sup>a</sup>	Obs.	Pre <sup>b</sup>						
2,4-Dichloroaniline	3.40	3.63			2.78		−8.96	−0.09	8.87	
2-Chloroaniline	2.81	3.10		3.01	1.90	2.08	−8.85	0.20	9.05	9.01
3-Chloroaniline	2.90	3.04	2.69 <sup>c</sup>	3.05	1.88	2.09	−8.93	0.19	9.11	9.05
4-Chloroaniline	2.91	3.08		3.09	1.83	2.06	−8.81	0.22	9.03	9.00
4-Bromoaniline	3.13	3.30	2.67 <sup>c</sup>	3.24	2.26	2.44	−8.87	0.13	9.00	8.95
Diphenylamine	4.49	3.84		3.67	3.50	2.83	−8.56	0.45	9.01	8.88
Aniline	2.60	2.79	1.66 <sup>c</sup>	2.84	0.90	1.16	−8.28	0.60	8.88	8.88
2,4,6-Trichloroaniline	4.55	4.36	4.11 <sup>c</sup>	3.67	3.52	2.83	−8.71	−0.33	8.38	8.84
α-Naphthylamine	4.29	4.32		3.65	2.25	2.72	−8.08	−0.36	7.72	8.74
N-Methylaniline	2.79	2.95		3.01	1.66	1.89	−8.53	0.57	9.10	9.05
2,4-Dichlorophenol	3.42	3.60	3.74 <sup>d</sup>	3.41	3.06	2.92	−9.45	−0.38	9.07	8.97
2-Chlorophenol	2.84	2.98	2.93 <sup>d</sup>	3.03	2.15	2.28	−9.40	−0.07	9.33	9.23
3-Chlorophenol	2.92	3.09		3.10	2.50	2.57	−9.43	−0.04	9.39	9.26
4-Chlorophenol	3.14	3.19	3.81 <sup>d</sup>	3.25	2.39	2.53	−9.28	−0.08	9.20	9.08
Phenol	2.61	2.51	2.29 <sup>d</sup>	2.85	1.46	1.65	−9.25	0.29	9.54	9.46
α-Naphthol	4.56	4.54	3.43 <sup>d</sup>	3.67	2.85	2.78	−8.31	−0.54	7.77	8.80
β-Naphthol	4.53	4.47	4.08 <sup>d</sup>	3.67	2.70	2.77	−8.31	−0.53	7.78	8.79
3-Nitrophenol	3.15	3.27		3.26	2.00	2.28	−10.15	−1.26	8.89	8.88
4-Nitrophenol	3.33	3.10	3.51 <sup>d</sup>	3.36	1.91	2.31	−10.30	−1.24	9.06	8.97
Hydroquinone	2.79	2.40	.	3.00	0.59	1.02	−9.07	0.12	9.19	9.13
Resorcinol	2.76	2.35	2.76 <sup>d</sup>	2.97	0.80	1.17	−9.24	0.12	9.36	9.28

<sup>a</sup> Predicted log1/EC<sub>50</sub> calculated by Equation 3<sup>b</sup> Predicted log1/EC<sub>50mix</sub> calculated by Equation 6<sup>c</sup> 15 min log1/EC<sub>50</sub> for *Chlorella vulgaris* from Netzeva et al. (2004)<sup>d</sup> 5 min log1/EC<sub>50</sub> for bioluminescent bacterium Shk1 from Ren et al. (2003)

$$\log 1/EC_{50} = 0.445(080) \log P - 0.801(0.121) \Delta E + 9.501(1.154) \quad (3)$$

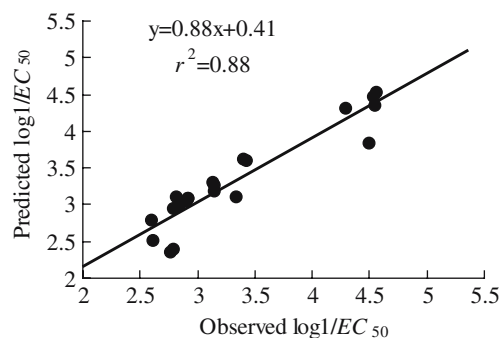
$n = 21$ ,  $r^2 = 0.876$ ,  $r^2_{\text{adj}} = 0.862$ ,  $SE = 0.261$ ,  $F = 63.33$ ,  $p < 0.001$ .

Equation 3 was used to predict toxicity, and the predicted values are presented in Table 1. The plot of observed log1/EC<sub>50</sub> to algae versus calculated values by the model (Equation 3) is shown in Fig. 1.

On the basis of an independence assumption, which implies 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 the n-octanol/water partition coefficient for mixtures (logP<sub>mix</sub>):

$$\log P_{\text{mix}} = (C_A \times \log P_A + C_B \times \log P_B + \dots) / (C_A + C_B + \dots). \quad (4)$$

In this equation, logP<sub>mix</sub> is the n-octanol/water partition coefficient of binary mixtures; logP<sub>A</sub> and logP<sub>B</sub> are the

**Fig. 1** Calculated log1/EC<sub>50</sub> from Equation 3 versus observed log1/EC<sub>50</sub> of single compounds to algae

n-octanol/water partition coefficient of components A and B; and C<sub>A</sub> and C<sub>B</sub> are the concentration of components A and B in binary mixtures. An obvious correlation between the joint toxicity to tadpoles and logP<sub>mix</sub> for 23 mixtures of substituted phenols was obtained, and the logP<sub>mix</sub>-dependent model could explain 88% of variance (Huang et al., 2003).

In our study,  $\log P_{\text{mix}}$  of 20 binary mixtures was calculated on the basis of Equation 4 (Table 1). In addition, we proposed that the orbital energy of a mixture also is a simple sum of individual components and calculated the frontier orbital energy gap of mixtures  $\Delta E_{\text{mix}}$ . A regression method similar to single chemical analyses was used to model joint toxicity ( $\log 1/EC_{50\text{mix}}$ ). The  $\log P_{\text{mix}}$ -dependent model can explain only 66.3% of variance. By adding  $\Delta E_{\text{mix}}$ , a two-descriptor QSAR model was obtained as follows:

$$\log 1/EC_{50\text{mix}} = 0.297(0.062) \log P_{\text{mix}} - 0.679(0.195) \Delta E_{\text{mix}} + 8.699(1.834) \quad (5)$$

$n = 20$ ,  $r^2 = 0.803$ ,  $r^2_{\text{adj}} = 0.780$ ,  $SE = 0.137$ ,  $F = 34.7$ ,  $p < 0.001$ .

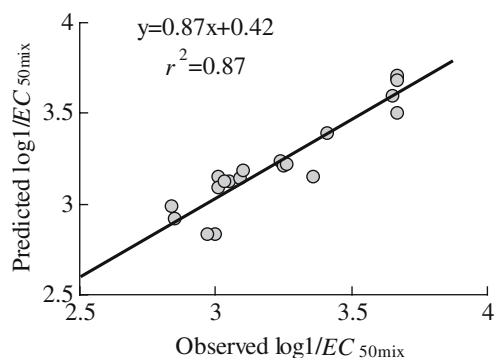
From a predictive standpoint, Equation 5 was not satisfactory. Because the binary mixtures were composed of 2,4-dichloroaniline and other substituted anilines and phenols, respectively, according to an equitoxic ratio (1:1) in this study, there was an obvious relationship between the  $\log P$  of single compounds and the  $\log P_{\text{mix}}$  of binary mixtures ( $r^2 = 0.90$ ). The toxicity of mixtures then may be related to the structural parameters of single components (Yuan et al., 2002). Through multivariable linear regression analyses, an improved QSAR between  $\log 1/EC_{50\text{mix}}$  and  $\log P$  and  $\Delta E$  was developed as follows:

$$\log 1/EC_{50\text{mix}} = 0.214(0.034) \log P - 0.280(0.051) \Delta E + 5.282(0.489) \quad (6)$$

$n = 20$ ,  $r^2 = 0.873$ ,  $r^2_{\text{adj}} = 0.858$ ,  $SE = 0.110$ ,  $F = 58.6$ ,  $p < 0.001$ .

Equation 6 explains most of the variance (87.3%), with maximum  $F$  values (58.6) and the minimum standard error of the estimate (0.110), with neither statistical nor obvious visual outliers observed. This model was used to predict the joint toxicity, and the predicted values are presented in Table 1. The plot of observed  $\log 1/EC_{50\text{mix}}$  to algae versus calculated values by Equation 6 is shown in Fig. 2. The quality of model fit and prediction of Equations 3 and 6 were similar to comparable  $r^2$ , slope, and intercept in Figs. 1 and 2. It is shown that the model derived from the structural parameters of single components can be used successfully to predict joint toxicity.

The obtained models showed that both the single and joint toxicities of aniline, phenol, and their derivatives to algae are related mainly to hydrophobicity and electronic properties. Because  $\log P$  is a hydrophobicity parameter, the higher the  $\log P$ , the stronger the hydrophobicity and the easier the compounds are bioconcentrated in an organism. The substituted anilines and phenols investigated in this



**Fig. 2** Calculated  $\log 1/EC_{50\text{mix}}$  from Eq.6 versus observed  $\log 1/EC_{50\text{mix}}$  of mixtures

study are polar narcotics. Such compounds exhibit effects similar to those of nonpolar narcotics, but at potency levels greater than estimated by their hydrophobicity. The addition of the frontier orbital energy gap  $\Delta E$  can enormously improve the prediction of  $\log P$ -dependent models. Toxicity increases with greater negative  $\Delta E$  (i.e., the smaller the value of  $\Delta E$ , the easier the electron transfers from HOMO orbital to LUMO orbital and the stronger the toxicity). This result is consistent with that reported by Yan et al. (2006).

In conclusion, the 48-h toxicity of anilines and phenols and their binary mixtures to the algae was determined. Not only for single toxicity, but also for joint toxicity, successful two-descriptor QSAR models, accounting for hydrophobicity and frontier orbital energy gap, were developed. However, this study investigated only the toxicity of binary mixtures containing 2,4-dichloroaniline, and the model derived from the structural parameters of single components can be used to predict joint toxicity. The prediction of the joint toxicity of multiple-component mixtures should be studied continuously.

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