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Joint Toxicity Evaluation and QSAR Modeling of Aromatic Amines and Phenols to Bacteria

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Abstract Acute toxicity of aromatic amines and phenols and their mixtures to bacteria in natural waters was determined and the median inhibition concentration IC_{50} values for single compounds and IC_{50mix} values for binary and multiple mixtures were obtained. Based on the quantitative structure–activity relationship model for single chemical toxicity, a two-descriptor model accounting for n-octanol/water partition coefficient ($\log P_{mix}$) and the energy of the lowest unoccupied molecular orbital ($E_{LUMOmix}$) was developed for the toxicity of a mixture: $\log(1/IC_{50mix}) = 0.326 \log P_{mix} - 0.660 E_{LUMOmix} + 3.323$ ($n = 32$, $R^2 = 0.834$). This model can be used successfully to predict the toxicity of a mixture, whether binary mixtures or multiple mixtures of three or four chemicals are used as predictors.

Keywords Joint toxicity · QSARs · Aromatic amines · Bacteria

The aromatic amines are commonly used in the chemical manufacture of dyes, rubber and textiles and can also originate from gasoline and coal combustion (Palmiotto et al. 2001). Of which, 3,4-dichloroaniline (DCA) is an environmental contaminant, precursor for synthesis and a degradation product of several herbicides (e.g. diuron, linuron and propanil) (Crossland 1990). It has been reported that chlorinated anilines are more difficult to be biodegraded than chlorinated phenols in the natural waters, and have a high environmental persistence (Lu et al. 2002).

Aquatic organisms are typically not exposed to single substances but rather simultaneously to multiple mixtures of chemicals. Interest in assessing the toxicity of complex chemical mixtures has increased substantially within the past decade. Quantitative structure–activity relationships (QSARs) are powerful tools in predicting the toxicological effects of chemicals (Bradbury 1994). Multiple QSARs for the toxicity of single chemicals have been developed in the past two decades. Recently, QSAR has also been utilized in the study of joint toxicity of mixtures, and some mixture physiochemical parameters derived from experiment or calculation were used to predict mixture toxicity (Yuan et al. 2002; Huang et al. 2003; Lu et al. 2007).

The Yangtze River Delta is one of the most densely urbanized areas in China, with a series of big, medium and small cities and towns along the river. In recent years, water pollution in the lower reaches of the Yangtze River has become more serious, and many polychlorinated aromatics have been detected in the water and/or sediment of Nanjing section (Jiang et al. 2000). The aim of the present work was to experimentally determine the toxicity of aromatic amines and phenols and their mixtures to the river bacteria, develop QSAR models, and assess and predict joint toxicity using molecular structural descriptors.

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Materials and Methods

Compounds used in this study were obtained from different sources. 3,4-Dichloroaniline, 2,4-dichloroaniline, 2,5-dichloroaniline, 2,4,6-trichloroaniline, 2,4-dichlorophenol, 2,4,6-trichlorophenol and 2,4-dinitroaniline were purchased from Acros Organics (Geel, Belgium) and the stated purities were 99%. Aniline, monochloroanilines, 4-bromoamine and N-methylaniline (>99%) were obtained from Shanghai Tingxin Chemical Reagent Co., China (Shanghai, China); 3-nitrophenol and 4-nitrophenol (>99%) from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) and 95% ethanol from Shanghai Jiuyi Chemical Reagent Co., China (Shanghai, China). Other compounds used for preparation of culture medium were of chemically pure quality.

A water sample was gathered from the Nanjing section in the Yangtze River (Jiangsu Province, China). The sample was obtained at a depth of 0.5 m and 50 m away from the bank. There were no large industry enterprises or new pollutant sources in the vicinity of this section of the river. The pollutants from the upper reaches of the river have been admixed equably, and the concentration of most halogenated aromatic hydrocarbons is at the ng/L level (Jiang et al. 2000). The temperature of the water sample was 19°C, the pH was 6.5 and the dissolved oxygen (DO) was 7.6 mg/L. The water sample was stored at 4°C when not being used. Bacterial counts were 2.15×10^5 CFU/ml determined by standard plate count techniques (Wang 1988).

The bacterial growth inhibition test was employed to determine toxicity (Alsop et al. 1980). The culture was maintained in liquid medium containing beef extract, 3 g; peptone, 10 g; NaCl, 5 g; distilled water, 1 L. The pH of the culture medium was adjusted to 7.2–7.4 and the medium was sterilized for 20 min at 121°C. After initial range finding experiments, each compound was tested in a logarithmic concentration series (5–7 concentrations). Stock standard solution of every compound was prepared by dissolving each compound in 50 ml of 95% ethanol in concentrations that are required to obtain the highest exposure concentration in water. The solutions were stored in brown glass bottles and kept at 4°C. Working solutions were prepared by a serial dilution of the stock solutions using 95% of ethanol. One millilitre of working solution of test chemical and 1 ml of river water were added to 48 ml of culture medium in 250 ml conical flasks, bringing the final volume to 50 ml. One millilitre of 95% ethanol solution and 1 ml of sterilized river water added to 48 ml of culture medium served as a blank control, and 1 ml of 95% ethanol solution and 1 ml of river water added to 48 ml of culture medium served as a seed control. For each concentration and control, experiments were performed in triplicate. All samples were incubated for 24 h at 20 ± 1°C.

The turbidities were measured using a spectrophotometer (UV-1201) at 530 nm against a blank control. The inhibition rates of bacterial growth are calculated as follow:

$$\text{Inhibition rate}\,(\%) = \left(1 - \frac{\text{Absorbanceoftestbottle}}{\text{Absorbance of seed control}}\right) \times 100\% \quad (1)$$

Logarithms of the inverse median inhibition concentration after 24 h exposure, expressed as log(1/IC_{50}) (mol/L), were calculated using concentration-response curves of the negative logarithm of compound concentrations and the inhibition rates as the relative toxic potency for each chemical (see Fig. 1). To test the toxicity of each mixture, toxic units (TU) and their sums were calculated based on the following equation:

$$M = \Sigma TU_i = \Sigma C_i / IC_{50i} \quad (2)$$

In Eq. 2, M is the sum of toxic units of mixtures, C_i is the concentration of an individual chemical present in a mixture, and IC_{50i} is the median inhibition concentration of individual component. Binary and multiple-component mixtures were tested at an equitoxic ratio (identical fractions of their respective IC_{50}s). The toxicity of mixtures with different M values were tested. All toxicity testing procedures for mixtures were the same as those for single chemicals. The concentration-response curve for the mixture of 2-chloroaniline, 3,4-dichloroaniline and 2,4,6-trichloroaniline is shown in Fig. 2.

The joint toxicity of mixtures was calculated as follows in Eq. 3.

$$IC_{50mix} = \Sigma(TU_{50i} \times IC_{50i}) \quad (3)$$

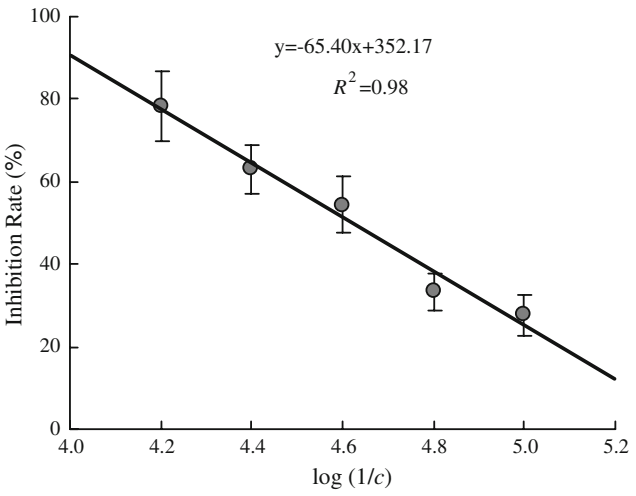

Fig. 1 Concentration-response curve for 2,4-dinitroaniline. *Bars* indicate standard error of the mean

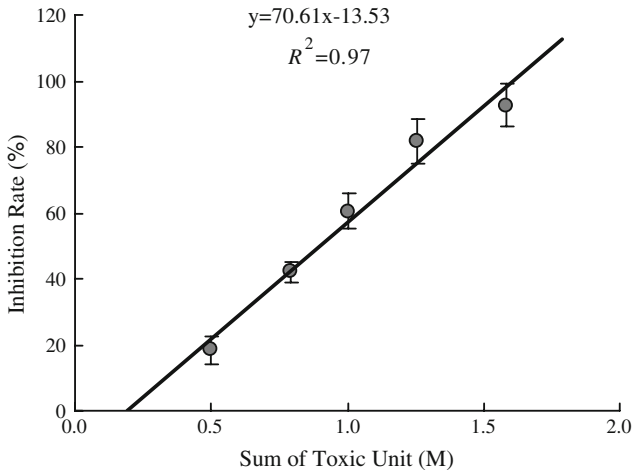

Fig. 2 Concentration-response curve for mixture of 2-CA, 3,4-DCA and 2,4,6-TCA. *Bars* indicate standard error of the mean

In this equation, IC_{50mix} is the median inhibition concentration of a mixture to the bacteria, TU_{50i} is the toxic unit of component i in mixtures at 50% inhibition rate of bacterial growth and IC_{50i} is the median inhibition concentration of the individual substances.

The logarithm of each n-octanol/water partition coefficient (log *P*) was obtained from Clog *P* for Window software (ver. 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_{LUMO}) was obtained from the ChemOffice 2004 program using the quantum chemical method MOPAC (http://www.cambridgesoft.com). The parameter values of studied chemicals are listed in Table 1. The linear regression analyzes were carried out using the MINITAB statistical software (version 15, MINITAB Inc.). Model quality was characterized by the number of observations (n), the square of correlation coefficient (R^2), the cross-validated correlation coefficient (Q^2), the standard error of the estimate (*s*), and the Fisher criterion (*F*).

Results and Discussion

The observed 24 h $\log(1/IC_{50})$ of 11 aromatic amines and 4 substituted phenols and the log $(1/IC_{50mix})$ of 32 mixtures are given in Tables 1 and 2, respectively. The values of $\log(1/IC_{50})$ ranged from 3.36 for N-methylaniline to 4.74 for 2,4,6-trichloroaniline. For chloroanilines and chlorophenols, toxicity is directly related to the number of chlorine atoms contained by the compound. Also, the nitro substituent seems to be more toxic than chlorine substituent.

Table 1 Acute toxicity to the river bacteria and structural parameters of the individual chemicals

Compounds	Abbr[a]	Toxicity (mol/L)				log *P*	E_{LUMO} (eV)
		Bacteria[b]	Alga[c]	Fish[d]	Pre.[e]		
Aniline	AN	3.66	1.66	2.84	3.39	0.90	0.60
2-Chloroaniline	2-CA	3.68		4.35	3.83	1.90	0.20
3-Chloroaniline	3-CA	3.83	2.69		3.83	1.88	0.19
4-Chloroaniline	4-CA	3.90		3.62	3.81	1.83	0.21
4-Bromoamine	4-BA	3.88	2.67		3.96	2.26	0.13
2,4-Dichloroaniline	2,4-DCA	4.11			4.20	2.78	−0.09
3,4-Dichloroaniline	3,4-DCA	4.41		4.36	4.22	2.69	−0.23
2,5-Dichloroaniline	2,5-DCA	4.30			4.21	2.75	−0.16
2,4,6-Trichloroaniline	2,4,6-TCA	4.74	4.11		4.50	3.52	−0.33
2,4-Dinitroaniline	2,4-DNA	4.65	2.64	4.07	4.54	1.84	−1.81
N-Methylaniline	N-MA	3.36			3.63	1.66	0.57
3-Nitrophenol	3-NP	4.26			4.39	2.00	−1.26
4-Nitrophenol	4-NP	4.28		3.38	4.35	1.91	−1.24
2,4-Dichlorophenol	2,4-DCP	4.22		4.32	4.38	3.06	−0.38
2,4,6-Trichlorophenol	2,4,6-TCP	4.62		4.33	4.67	3.69	−0.65

[a] Abbr is an abbreviation of compound name

[b] Observed log $(1/IC_{50})$ for the river bacteria

[c] Log $(1/EC_{50})$ for *C. vulgaris* from Netzeva et al. (2004)

[d] Log $(1/LC_{50})$ for the fathead minnow from Netzeva et al. (2005)

[e] Predicted log $(1/IC_{50})$ calculated by Eq. 6

Table 2 Joint toxicity to the river bacteria and structural parameters of the mixtures

No.	Components of mixture	log (1/IC_{50mix})			log P_{mix}	$E_{LUMOmix}$ (eV)	MTI	Joint effects
		Obs[a]	Pre.[b]	Res.[c]				
1	3,4-DCA + 2,5-DCA	4.38	4.34	0.04	2.72	−0.19	1.10	Simple addition
2	3,4-DCA + N-MA	3.49	3.56	−0.07	1.74	0.50	0.55	Partial addition
3	3,4-DCA + 2-CA	3.85	3.86	−0.01	1.92	0.13	0.80	Partial addition
4	3,4-DCA + 3-CA	3.92	3.93	−0.01	2.05	0.10	0.64	Partial addition
5	3,4-DCA + 4-CA	3.94	3.91	0.03	2.03	0.11	0.51	Partial addition
6	3,4-DCA + 4-BA	3.98	4.06	−0.08	2.36	0.05	0.71	Partial addition
7	3,4-DCA + 3-NP	4.55	4.62	−0.07	2.29	−0.83	1.73	Potentiation
8	3,4-DCA + 4-NP	4.66	4.60	0.06	2.24	−0.82	2.09	Potentiation
9	3,4-DCA + 2,4,6-TCA	4.69	4.46	0.23	2.95	−0.26	1.49	Potentiation
10	3,4-DCA + 2,4,6-TCP	4.62	4.56	0.06	3.00	−0.39	1.38	Potentiation
11	3,4-DCA + 2,4-DCP	4.62	4.48	0.14	2.91	−0.32	2.06	Potentiation
12	3,4-DCA + 2,4-DCA	4.19	4.31	−0.12	2.75	−0.14	0.84	Partial addition
13	3,4-DCA + 2,4-DNA	4.45	4.63	−0.18	2.38	−0.81	0.79	Partial addition
14	2-CA + 3,4-DCA + 2,4,6-TCA	4.10	3.95	0.15	2.13	0.10	1.10	Simple addition
15	2-CA + 2,4-DCA + 2,4,6-TCA	3.82	3.99	−0.17	2.22	0.09	0.42	Partial addition
16	3-CA + 2,4-DCA + 2,4,6-TCA	3.85	4.03	−0.18	2.29	0.06	0.51	Partial addition
17	3-CA +2,5-DCA + 2,4,6-TCA	4.08	4.00	0.08	2.22	0.07	−0.18	Antagonism
18	3-CA + 3,4-DCA + 2,4,6-TCA	3.89	3.99	−0.10	2.18	0.06	0.43	Partial addition
19	4-CA + 3,4-DCA + 2,4,6-TCA	4.10	4.03	0.07	2.18	0.01	0.78	Partial addition
20	4-CA + 2,5-DCA + 2,4,6-TCA	3.82	4.01	−0.19	2.23	0.06	0.23	Partial addition
21	4-CA + 2,4-DCA + 2,4,6-TCA	4.08	4.03	0.05	2.30	0.06	0.89	Partial addition
22	4-CA + 2,4-DNA + 2,4,6-TCA	4.06	4.09	−0.03	2.02	−0.17	0.58	Partial addition
23	3-CA + 2,4-DNA + 2,4,6-TCA	4.11	4.05	0.06	2.03	−0.10	0.81	Partial addition
24	2-CA + 2,4-DNA + 2,4,6-TCA	4.25	3.99	0.26	2.01	−0.02	1.36	Potentiation
25	4-BA + 2,4-DNA + 2,4,6-TCA	4.46	4.20	0.26	2.34	−0.17	1.46	Potentiation
26	AN + 2,4-DNA + 2,4,6-TCA	3.34	3.37	−0.03	1.16	0.50	0.20	Partial addition
27	4-BA + 2,4-DCA + 2,4,6-TCA	3.90	4.14	−0.24	2.54	0.02	0.54	Partial addition
28	4-BA + 2,5-DCA + 2,4,6-TCA	4.00	4.13	−0.13	2.50	0.02	0.62	Partial addition
29	4-BA + 3,4-DCA + 2,4,6-TCA	4.15	4.12	0.03	2.47	0.01	0.89	Partial addition
30	AN + 3,4-DCA + 2,4,6-TCA	3.65	3.48	0.17	1.33	0.42	0.19	Partial addition
31	4-CA + 2,4-DCA + 2,4,6-TCA + 2,4,6-TCP	4.20	4.13	0.07	2.44	−0.02	0.99	Simple addition
32	4-CA + 2,4-DNA + 2,4,6-TCA + 2,4,6-TCP	4.03	4.18	−0.15	2.23	−0.19	0.51	Partial addition

[a] Observed log (1/IC_{50mix}) for the river bacteria

[b] Predicted log (1/IC_{50mix}) calculated by Eq. 8

[c] Residual = observed log (1/IC_{50mix}) − predicted log (1/IC_{50mix})

For the purpose of comparison, the 15 min *Chlorella vulgaris* EC_{50} values of anilines (Netzeva et al. 2004) and the 96 h fathead minnow LC_{50} values of anilines and phenols (Netzeva et al. 2005) are also shown in Table 1. When the toxicity of five anilines on bacteria was compared with those from *C. vulgaris*, all the chemicals tested exhibited higher toxicity on bacteria than on *C. vulgaris*. The comparison of bacterial and fish toxicity data shows that all the chemicals except for 2-chloroaniline and 2,4-dichlorophenol were discovered to be more toxic to bacteria. Hence, it seems that the river bacteria assay is more sensitive than the alga or fish assay.

Joint toxicity was evaluated with the mixture toxicity index (MTI) as proposed by Könemann (1981). MTI was defined as follows: $MTI = 1 - \log M / \log M_0$, where $M = \Sigma TU_i$ and $M_0 = M/TU_{max}$ (TU_{max} is the largest TU value in the mixture). For the mixtures at an equitoxic ratio, the above formula can be simplified as follows: $MTI = 1 - \log M / \log N$, where N is the number of components in the mixture. Simple addition is characterized by

MTI = 1. MTI < 0 represents antagonism, 0 < MTI < 1 partial addition, MTI > 1 potentiation and MTI = 0 indicates independent. MTI values for the mixtures studied were calculated and the joint effects were evaluated (see Table 2). The results showed that most mixtures exhibited partial addition effects, the binary mixtures of 3,4-DCA and nitro- or chloro- substituted phenols showed potentiation effects, three mixtures (3,4-DCA + 2,5-DCA, 2-CA + 3,4-DCA + 2,4,6-TCA and 4-CA + 2,4-DCA +2,4,6-TCA + 2,4,6-TCP) were close to simple addition effects, and the mixture toxicity of 3-CA, 2,5-DCA and 2,4,6-TCA was antagonism.

Verhaar et al. (1992) recognised four modes of action associated with different structural classes: class I – inert chemicals, class II – relatively inert chemicals, class III – reactive chemicals, and class IV – specifically acting chemicals. Class I is typically associated with non-polar narcosis. Class II is associated with polar narcosis. Class III summarizes different types of reactive chemicals, which in principle are difficult to model together but the net result of reactivity in most cases is enhanced toxicity. 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 compilation if data sets of more diverse chemicals, acting by different mechanisms of toxic action, requires additional terms for successful QSAR modeling (Netzeva et al. 2004). Lu et al. (2001) measured 48 h toxicity of 40 substituted benzenes (nitro-, chloronitro-, dinitro-, anilines and phenols) to the alga *S. obliquus* and developed QSAR model based on log P and E_{LUMO}: $\log(1/EC_{50}) = 0.272\log P - 0.659E_{LUMO} + 2.54$ (n = 40, $R^2 = 0.79$). Netzeva et al. (2004) determined the toxicity of 14 anilines to the alga *Chlorella vulgaris* and developed a successful two-descriptor QSAR model accounting for hydrophobicity and electrophilicity: $\log 1/EC_{50} = 0.740\log P - 0.406E_{LUMO} - 1.852$ (n = 14, $R^2 = 0.939$). Chen et al. (2007) developed QSARs for anilines, supposedly acting by polar narcosis. As an endpoint, they measured both dissolved oxygen production (DO) and algal growth rate EC_{50} to *C. subcapitata*. The log P models for the two endpoints were improved by inclusion of E_{LUMO}: log (1/EC_{50})oxygen demand $= 0.59\log P - 1.33E_{LUMO} - 0.24$ (n = 19, $R^2 = 0.92$) and log (1/EC_{50})growth rate $= 0.24\log P - 1.97E_{LUMO} + 0.81$ (n = 18, $R^2 = 0.88$).

In this study, the relationship of the individual toxicity of 11 aromatic amines and 4 substituted phenols with log P was analyzed and Eq. 4 was obtained.

$$\log(1/IC_{50}) = 0.365(0.108)\log P + 3.283(0.261)$$
$$n = 15, R^2 = 0.470, Q^2 = 0.339, s = 0.303, F = 11.54 \quad (4)$$

In this equation, the numbers in parentheses are the standard errors on the coefficients. It was found that 2,4-dinitroaniline had a large residual. Its exclusion improved the statistical fit of Eq. 4. The model without the outlier is present in Eq. 5. However, the log P-dependent model explains only 66.3% of variance.

$$\log(1/IC_{50}) = 0.410(0.085)\log P + 3.126(0.208)$$
$$n = 14, R^2 = 0.663, Q^2 = 0.555, s = 0.235, F = 23.59 \quad (5)$$

The above results showed that hydrophobicity alone is not enough to describe accurately the toxicity of aromatic amines and phenols. In order to improve the prediction for the toxicity of polar narcotics and reactive chemicals (such as 2,4,6-TCP, 4-NP and 2,4-DNA) studied in this paper, the reactivity term E_{LUMO} was included in Eq. (6):

$$\log(1/IC_{50}) = 0.296(0.062)\log P - 0.361(0.067)E_{LUMO} + 3.340(0.148)$$
$$n = 15\ R^2 = 0.844\ Q^2 = 0.717\ s = 0.172\ = 32.41 \quad (6)$$

Equation 6 was characterized by a high coefficient (0.844) and small error of estimate (0.172). There were no statistical outliers to this relationship. Both descriptors are statistically significant ($p < 0.001$) and have a coefficient to error ratio (t-criterion) equal to 4.77 and −5.36 for log P and E_{LUMO}, respectively.

Based on an independence assumption (Chiou et al. 1984), 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 n-octanol/water partition coefficient (log P_{mix}) for mixtures as follows:

$$\log P_{mix} = (C_a \times \log P_a + C_b \times \log P_b + \ldots C_i \times \log P_i)/(C_a + C_b + \ldots + C_i) \quad (7)$$

In equation, log P_{mix} is the logarithm of n-octanol/water partition coefficient of the mixtures, log P_a, log P_b and log P_i are the logarithm of n-octanol/water partition coefficients of component a, b and i, and C_a, C_b and C_i are the concentrations of component a, b and i in mixtures.

In our study, the log P_{mix} of 32 mixtures were calculated based on Eq. 7. In addition, we proposed the orbital energy of a mixture is also a simple sum of the individual components and calculated the energy of the lowest unoccupied molecular orbital of mixtures $E_{LUMOmix}$ (see Table 2). A regression method similar to single chemical analyses was

used to model joint toxicity ($\log(1/IC_{50mix})$). A two-descriptor QSAR model was obtained as follows in Eq. 8.

$$\log(1/IC_{50mix}) = 0.326(0.076)\log P_{mix} - 0.660(0.095)E_{LUMOmix} + 3.323(0.169)$$
$$n = 32,\ R^2 = 0.834,\ Q^2 = 0.798, s = 0.139,\ F = 73.01 \quad (8)$$

Equation 8 explains most of the variance (83.4%), with maximum F values (73.01) and minimum standard error of estimate (0.139) and with neither statistical nor obvious visual outliers observed. Furthermore, this QSAR model is also significant for prediction as Q^2 equals to 0.798. Figure 3 depicts the correlation between log ($1/IC_{50mix}$) values and descriptors ($\log P_{mix}$ and $E_{LUMOmix}$) as described by Eq. 8. It appears that joint toxicity is well correlated with the $\log P_{mix}$ and $E_{LUMOmix}$ of the mixtures. In addition, both parameters exerted significant influences on the observed toxicity. Equation 8 was used to predict joint toxicity, and the predicted log ($1/IC_{50mix}$) values to the bacteria and the residuals are presented in Table 2.

The obtained models revealed that both individual toxicity and joint toxicity of aromatic amines and phenols to the river bacteria are related mainly to their electronic properties and hydrophobicity. Aniline and partial substituted anilines such as N-MA and chloroanilines studied in this paper are polar narcotics. Such compounds exhibit effects similar to non-polar narcotics, but at potency levels greater than estimated by their hydrophobicity. Whilst NPs, 2,4,6-TCP and 2,4-DNA are reactive compounds. The addition of the energy of the lowest unoccupied molecular orbital (E_{LUMO}) can enormously improve the prediction of log P-dependent models. E_{LUMO} reflects the overall ability

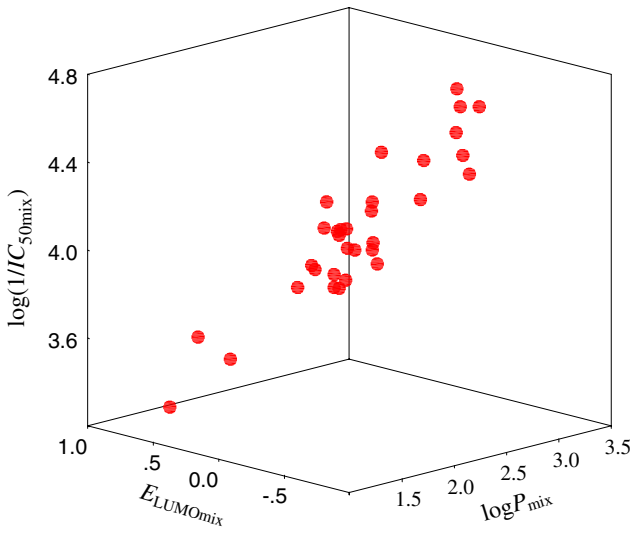

Fig. 3 The correlation between log ($1/IC_{50mix}$), log P_{mix}, and $E_{LUMOmix}$ values

of the molecule to accept an electron pair from a potential nucleophile, the negative values of the slopes for the QSAR models suggest that toxicity increases with an increase in the electrophilicity of the chemicals. The positive coefficients for log P in QSAR models indicate that toxicity increases with an increase of chemical's hydrophobicity.

In conclusion, two QSAR models based on hydrophobicity and frontier orbital energy were developed and used to predict the joint toxicity of mixtures containing toxicants with different mechanisms of action. However, this paper only investigated toxicity of mixtures at an equitoxic ratio, and the prediction for joint toxicity of mixtures in variant toxic ratios should be studied in the further.

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