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QSAR for Prediction of Joint Toxicity of Substituted Phenols to Tadpoles (*Rana japonica*)

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Contaminated ecosystems, whether aquatic or terrestrial, are often polluted with several chemicals rather than a single pollutant. As a result of time and financial constraints, toxicity testing has generally been restricted to studying the effects of single pollutants on a target organism after a specific exposure time and under controlled environmental conditions (Preston et al. 2000). Few detailed studies of joint toxicity of mixtures and their prediction have been conducted.

Toxicity based quantitative structure-activity relationships (QSARs) provide useful tools of predicting toxicological effect of single chemicals (McKinney et al. 2000). Konemann (1981), Hermens and Leeuwangh (1985) had reported some pioneering studying using QSAR models and the concepts of toxic units (TU), mixture toxicity index (MTI), and additive index (AI) to analyze joint toxicity in mixtures. Recently Nirmalakhandan et al. (1994), Xu and Nirmalakhandan (1998) extended two QSAR models of toxicity of single chemicals to the field of mixtures. They provided an impetus to utilize the large number of single chemical QSAR models reported in the literature in predicting toxicity of mixtures in aquatic ecotoxicological fields. Prakash et al. (1996) developed a QSAR-based approach using the similarity parameter λ to predict the concentrations of individual chemicals. Lin et al. (2002), based on the Empore^{TM} disk/water partition coefficient of a mixture, presented a QSAR model to predict the toxicity of mixed halogenated benzenes to *Vibrio fischeri*. However, a QSAR model based on calculated physicochemical descriptors in predicting the toxicity of mixtures has not been attempted.

Phenol and its derivatives are widely used as industrial chemicals. They have many large-scale commercial uses, such as intermediates for dyes and organic synthesis processes, and as disinfectants and antiseptics. Thus they have a high potential for environmental pollution. Toxicity of phenol and its derivatives has been extensively investigated using a protozoan *Tetrahymena pyriformis*, amphibian tadpoles (*Rana japonica*) and fathead minnow *Pimephales promelas* (Wang et al. 2002). However, information on joint toxicity of a mixture of phenol derivatives is rather scarce, particularly in relation to amphibians. The purpose of this study was to experimentally measure the toxicity of single phenol derivatives and the related mixtures to tadpoles (*Rana japonica*), and analyze, model and predict mixture toxicity using molecular physicochemical descriptors.

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MATERIALS AND METHODS

Rana japonica tadpoles were employed for toxicity testing because they are very common in China and these green frog tadpoles proved to be more sensitive to hazards than other tadpoles (Berrill et al. 1995; Wang et al. 2001). Naturally fertilized egg masses of tadpoles were collected from a single pond in Nanjing, where the tadpoles were exclusively bred. All eggs had been laid no more than 2 days prior to collection. Eggs and the resulting tadpoles were cultured in aerated water in a glass tank at room temperature. A 12h light and 12h dark photoperiod was programmed. Tadpoles were fed a mixture of bread crumb and cooked rice three times per week and water was renewed every other day. Before experiments started, the tadpoles were acclimated under the same conditions for a week. Feeding was halted 24h prior to testing. Healthy individuals of similar size and weight were employed for the tests. The average body length and body weight were 3.0 ± 0.1cm, and 0.12 ± 0.01g, respectively.

All exposures took place at 25±1°C water temperature in a controlled environmental chamber. Aerated chloride-free water was used for preparing the chemical stock solutions and test dilutions. All chemicals tested were of sufficient purity (purity 98%). A six-step concentration series in geometric grade ranging from no effect to 100% lethal concentration was prepared after range finding experiments. 10 tadpoles were employed for each test and placed in a 500ml glass beaker containing 300ml test solution or aerated chloride-free water. The aerated chloride-free water without test compounds served as the control solution, and the solutions were renewed every 6h to achieve semi-static acute exposure. All experiments were performed with three replicates of each treatment run simultaneously. Mortality was recorded and the concentration needed to obtain 50% mortality for each chemical was calculated based on nominal concentrations. The pH values of the test solution at the LC_{50} for all chemicals were measured by a pH-Meter (PHS-3C). Logarithms of the inverse median lethal concentrations after 24-h exposure, expressed as log $1/LC_{50}$ (mol/l), were calculated as the relative toxic potency for each single pure chemical.

To test the toxicity of the mixture, we employed the sum of toxic units (M), which was defined by the following equation:

$$M = C_1 / LC_{1\,50} + C_2 / LC_{2\,50} + \cdots C_i / LC_{i\,50} + \cdots \qquad 1$$

In this equation, C_i is the concentration of the individual chemical presented in a mixture, LC_{50} is the median lethal concentration of the substance, and subscripts 1, 2, and i are the components of the mixture, respectively. Binary mixtures were conducted at three ratios: 1:1, 1:3, 3:1(identical fraction of LC_{50}) based on observed LC_{50} values. Multiple-component mixtures were tested at an equitoxic ratio (1:1). Toxicity of mixtures with different M (say, 2.0, 1.6, 1.2, 0.8, 0.4, 0.2) was tested. All toxicity testing procedures of mixtures were performed as with the single chemicals. The toxicity of mixtures was described as follows (Preston et al. 2000) Eq. 2. In this equation, LC_{50} is the median lethal concentration, C is the concentration of a chemical, and subscripts A, B, etc., and mix are the

components and mixture, respectively.

$$LC_{50mix}=(C_A + C_B + \cdots) /(C_A/LC_{50A} + C_B/LC_{50B} + \cdots) \quad 2$$

Logarithms of the 1-octanol/water partition coefficient (Log K_{ow}), which classically expresses the hydrophobic character as a result of various and complex interactions among many factors and plays a dominant role in the transport of the molecule, were computer estimated or retrieved as measured values from *SRC-WSKOW* for Microsoft Windows (version 1.26). The acid dissociation constants (*pKa*) were cited from the literature (Wang et al. 2001).

The multiple regression analysis procedure of STATISTICA for Windows software (version 5.0) was employed for QSAR analysis. The toxic potency, log $1/LC_{50}$, was employed as the dependent variable, and calculated Log K_{owmix} as the independent variables. Model quality was characterized by the number of observations (n), the square of correlation coefficient (r^2), the standard error of estimate (S.E.), the Fisher criterion (F), the significance level (P).

RESULTS AND DISCUSSION

A summary of toxicity of single substituted phenols to tadpoles and the calculated log K_{ow} value are shown in Table 1. The pH of the solutions of single substituted phenol at median lethal concentration to tadpoles, as well as the corresponding *pKa* of the chemical, are also presented in Table 1.

Based on an independece 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, a K_{MD}-based QSAR model was successfully developed to quantify the baseline toxicity of environmental pollutants (Lin et al. 2001). In our study, we proposed an equation to predict 1-octanol-water partition coefficient for mixtures (Log K_{owmix}) (Eq. 3). Where C is concentration of a substance, subscripts A, B, etc., and mix represent the components and the mixtures, respectively.

$$\text{Log } K_{owmix} = (C_A \times \text{Log } K_{owA} + C_B \times \text{Log } K_{owB} + \cdots) / (C_A + C_B + \cdots) \quad 3$$

The log K_{owmix} calculated by Eq. 3 is listed in Table 2. The toxicity of mixtures determined by the experiments and calculated from Eq. 2, together with toxicity ratios of components in the mixtures, are also given in Table 2. No mortality occurred in the controls of any treatments. The comparison of pH and *pKa* for each compound indicated that the pH of the solution with 50% mortality was much lower than corresponding *pKa*, so dissociation hardly occurred and these chemicals were present mainly in neutral molecules under test conditions.

Several mechanisms of toxic action may result from substituted phenols. However, phenol itself and the most simple alkyl, alkoxyl, mono- and di-halogen substitutedphenols are thought to produce polar narcosis (Cronin and Schultz, 1996; Wang et al. 2001). Generally speaking, the toxicity of compounds acting by polar narcosis is modeled well by log K_{ow} alone. The relationship of toxicity of 12

Table 1. Acute toxicity to the tadpoles (*Rana japonica*) and physiochemical parameters of single substituted phenols

ID[1]	Chemical name	CAS No.[2]	pH[3]	*pKa*[4]	log1/LC_{50}[5]	log K_{ow}[6]
A	4-Tert-Butylphenol	98-54-4	6.26	10.30	4.033	3.42
B	2-Methylphenol	95-48-7	6.57	10.23	2.837	1.94
C	4-Methylphenol	106-44-5	6.35	10.19	3.057	2.06
D	4-Methoxyphenol	150-76-5	6.62	10.21	2.624	1.59
E	4-Fluorophenol	371-41-5	6.37	9.79	2.693	1.77
F	4-Chlorophenol	106-48-9	6.41	9.38	3.421	2.39
G	4-Bromophenol	106-41-2	6.52	9.79	3.664	2.59
I	Bisphenol E	108-46-3	6.78	9.44	3.914	3.19
J	Bisphenol A	80-05-7	6.69	9.44	4.201	3.64
K	1-Naphthol	90-15-3	6.95	9.30	3.807	2.85
L	2-Naphthol	135-19-3	7.01	9.57	3.886	2.70
M	2-Methoxyphenol	1990-5-1	6.35	10.19	2.654	1.34

[1] ID =A name assigned to test chemicals
[2] CAS No.= Chemical Abstract Services registry number
[3] pH = pH of the solution at log 1/LC_{50} to tadpoles of each substituted phenol
[4] *pKa* = negative logarithmic form of the acid dissociation constant
[5] log 1/LC_{50} = logarithms of inverse median lethal concentration
[6] log K_{ow} = logarithm of the 1-octanol/water partition coefficient

studied phenols with log K_{ow} was analyzed and the model (Eq. 4) was obtained. Equation 4 explains most of the variance (93.7%), with maximum F values (149) and minimum standard error of estimate (0.156) and with neither statistical nor obvious visual outliers observed.

$$\text{Log } 1/LC_{50} = 0.778 \log K_{ow} + 1.488 \qquad 4$$
$$n=12,\ r^2= 0.937,\ \text{S.E.}= 0.156,\ F= 149,\ p< 0.000001$$

Predictions of mixture toxicity require sound assumptions about the quantitative relationship between the toxicity of single substances and those of mixtures. Basically, two different concepts are available for that purpose, concentration addition and independent action (Boedeker et al. 1992). Concentration addition is based on the idea of a similar action of mixture components. In the case that chemicals meet this requirement of similar action, concentration addition is generally accepted as a reasonable expectation for their joint toxicity (Calamari and Vighi 1992). In this study, all tested compounds are polar narcotics, that is, these chemicals meet the requirement of similar action. It is therefore assumed that, similarly to single substituted phenols, the toxicity of mixtures may be related to the total hydrophobicity of the mixtures. A regression method similar to single chemical analyses was used. The relationship between log1/LC_{50mix} and log K_{owmix} for 23 mixtures was developed as follows Eq. 5. A comparison of the predicted values from the model (Eq. 5) with the experimental toxicity shows that they are very close (Table 2). The plot of observed log 1/LC_{50mix} (mol/l) to tadpoles versus calculated value by the model (Eq. 5) is shown in Figure 1.

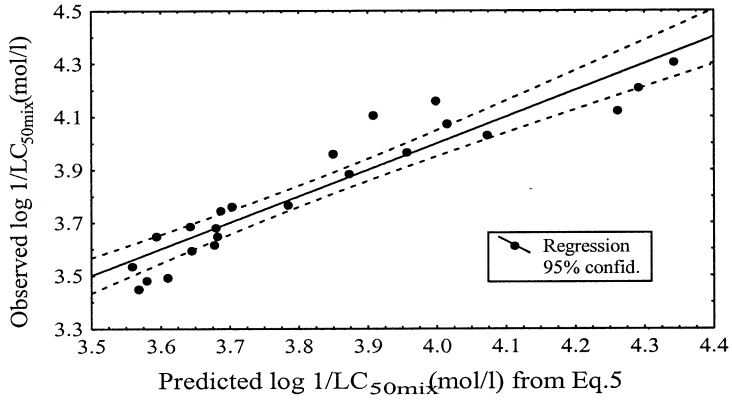

Figure 1. Calculated log $1/LC_{50mix}$ (mol/l) from Eq.5 verse observed log $1/LC_{50\ mix}$ (mol/l) of the mixtures to tadpoles (*Rana japonica)*

$$\text{Log } 1/LC_{50mix} = 0.876 \log K_{owmix} + 1.445 \qquad 5$$
$$n=23,\ r^2=0.88,\ \text{S.E.}=0.089,\ F=160,\ p<0.000001$$

The model (Eq. 5) was characterized by a high correlation coefficient (0.88) and small error of estimate (0.089). Thus it appears that the $\log 1/LC_{50mix}$ is well correlated with logarithms of the 1-octanol-water partition coefficient ($\log K_{owmix}$) of the mixtures. It does not matter whether binary or multiple components mixtures or the variant ratios (1:1, 3:1, 1:3, identical fraction of LC_{50}) in mixtures were used, the quality of the model was not affected.

As an additional test of the predictive capability of Eq.5, a prediction was performed in which five treatments (1#, 7#, 9#, 16#, 23#) were excluded from the data set to provide for a test, the remaining data set served as the training set. A regression analysis for the training data was performed and resulted in Eq. 6. The toxicity of the test set compounds was predicated using Eq. 6. The relationship between the predicted $\log 1/LC_{50mix}$ by Eq. 6 and the observed ones shows a good consistency, with $r^2=0.946$, S.E.=0.0493, and F=52.8 at a level of significance $P<0.0054$. Since 5 mixtures were randomly excluded from the mixtures and the agreement between the observed $\log 1/LC_{50mix}$ and the predicted $\log 1/LC_{50mix}$ by the model is generally satisfactory, the QSAR-based model therefore can be used to predicted toxicity of the phenol mixtures to tadpoles.

$$\text{Log } 1/LC_{50mix} = 0.861 \log K_{owmix} + 1.467 \qquad 6$$
$$n=18,\ r^2=0.899,\ \text{S.E.}=0.088,\ F=124,\ p<0.000001$$

We have not considered chronic toxicity. It should be kept in mind that chemicals for which acute toxic action is simple similar, do not necessarily have simple similar chronic actions. However, the chronic toxicity of chemicals is a complex function of their biological handling. We have not attempted to correlate log K_{owmix} with their chronic toxicities. Furthermore, it is unlikely that such a correlation would be predictive of the long-term risk from exposure to organic mixtures.

Table 2. Mixture constituents and results of joint toxicity experiment.

No.	Components of mixture	xLC_{50}:y LC_{50}	log $1/LC_{50mix}$ [1]		log Kow_{mix} [2]
			Observ.	Calcu.	
1#	A:F	1.0:1.0	3.649	3.592	2.451
2#	A:I	1.0:1.0	4.209	4.291	3.249
3#	A:L	1.0:1.0	4.109	3.906	2.809
4#	F:I	1.0:1.0	3.596	3.643	2.509
5#	F:L	1.0:1.0	3.495	3.608	2.469
6#	I:L	1.0:1.0	3.966	3.957	2.867
7#	A:F:I	1.0:1.0:1.0	3.748	3.687	2.559
8#	A:F:L	1.0:1.0:1.0	3.688	3.641	2.507
9#	A:I:L	1.0:1.0:1.0	4.075	4.013	2.931
10#	F:I:L	1.0:1.0:1.0	3.650	3.681	2.552
11#	A:F:I:L	1.0:1.0:1.0:1.0	3.764	3.703	2.577
12#	A:F	3.0:1.0	3.681	3.679	2.550
13#	A:F	1.0:3.0	3.538	3.557	2.411
14#	A:I	3.0:1.0	4.305	4.342	3.307
15#	A:I	1.0:3.0	4.121	4.261	3.214
16#	A:L	3.0:1.0	4.161	3.998	2.914
17#	A:L	1.0:3.0	3.962	3.848	2.743
18#	F:I	3.0:1.0	3.482	3.578	2.435
19#	F:I	1.0:3.0	3.769	3.783	2.669
20#	F:L	3.0:1.0	3.453	3.568	2.423
21#	F:L	1.0:3.0	3.618	3.677	2.547
22#	I:L	3.0:1.0	4.033	4.072	2.999
23#	I:L	1.0:3.0	3.887	3.871	2.769

[1] the negative logarithm of median lethal concentrations (mol/l) to tadpoles, Obs = observed values, Calcu.. = Calculated by Eq. (5)

[2] log K_{owmix} is log K_{ow} value of the mixture, calculated by Eq. (3).

Finally, this paper only investigated toxicity of mixtures containing of polar narcotic phenols, a QSAR model based on the total hydrophobicity of a mixture was developed, but toxicity of mixtures containing different action toxicants were not studied. We need to continue our research to develop joint toxicity of complex mixtures by introducing other physic-chemical parameters to describe these specific toxic effects in mixtures.

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