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Toxicity and quantitative structure–activity relationships of benzoic acids to *Pseudokirchneriella subcapitata*

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ABSTRACT

The present study presents the toxicity data of benzoic acid and its derivatives on *Pseudokirchneriella subcapitata*, in terms of EC50 and NOEC values. Median effective concentrations (EC50) range from 0.55 to 270.7 mg/L (based on final yield) and 1.93 to 726.3 mg/L (based on algal growth rate). No-observed-effect concentration (NOEC) is within the range of <0.0057–179.9 mg/L. From both the NOEC and EC50 values, it was found that, 2,4,6-trihydroxybenzoic acid, 4-chlorobenzoic acid, 3-bromobenzoic acid, 4-bromobenzoic acid, 2,6-dihydroxybenzoic acid, and 2,3,4-trihydroxybenzoic acid possess much higher risks to the aquatic organisms as compared to the other benzoic acids. These data are useful for risk assessment and protection of the aquatic environments, because such information is not available in the existing toxicological databases. The toxicity of halogenated benzoic acids was found to be directly related to the compound's hydrophobicity (the logarithm of the 1-octanol/water partition coefficient, log*Kow*). On the other hand, the number of hydroxyl groups (N_{OH}) had a determinant influence to the toxicity of hydroxybenzoic acids. Quantitative structure–activity relationships were established to correlate the observed toxicity with log*Kow* and N_{OH} values. These statistical correlations are highly significant with the predictive power Q^2 ranging from 0.896 to 0.955. Furthermore, in terms of the species sensitivity, the luminescent bacteria (Microtox) and the alga *P. subcapitata* appeared to be more susceptible to benzoic acids than the water flea and ciliate.

1. Introduction

Benzoic acid is a common additive for preserving foods, fats, fruit juices, alkaloid solutions, and curing tobacco. A major source of benzoic acid and its derivatives released into the aquatic environments are from the effluents of coal refining, paper and pulp mills, and in agricultural runoff [1]. In addition, benzoic acids are the major key metabolites for biodergration of alkyl benzenes or PAH via aerobic, anaerobic, or oxidation pathways [2,3]. Moreover, several benzoic acid derivatives with hydroxyl or halogen substitutes are known to be a group of secondary plant metabolites as well as aerobic microbial degradation products of lignin, an important plant cell wall polymer [4].

The effects of benzoic acids on bacteria, ciliate, daphnids, and fish have been reported by previous researchers [5–8]. Muccini et al. [7] and Zhao et al. [5,6] demonstrated that ionization is an important factor governing the toxicity of benzoic acids and the unionized form of these weak acids is thought to be generally more toxic than the ionized analogue. For the different isomers of benzoic acids, Muccini et al. [7] concluded that benzoic acids with halogens at the meta- and para-positions were more toxic than those with ortho-substitutions. They explained the difference by the general concept that the ortho-halogenated benzoic acids having lower pKa values are more ionized, and therefore less toxic than meta- and/or para-substituted ones. Kamaya et al. [8] indicated that ortho-hydroxylated benzoic acids displayed higher toxicity than the meta- and/or para-hydroxylated ones, pointing out that the hydroxybenzoate derivatives behaved differently from the halobenzoates. On the other hand, toxicity data describing the effects of benzoic acids on algae are very rare. Kamaya et al. [9] studied the effects of some hydoxybenzoates to the freshwater green alga *Pseudokirchneriella subcapitata* and concluded that 2-hydroxybenzoic acid (2-HBA) is relatively more toxic than 4-HBA and 3-HBA. In addition, 4-HBA may stimulate the algal growth at lower concentrations ranging from 0.1 to 1.0 mmol/L.

Quantitative structure–activity relationships (QSARs) attempt to statistically relate the toxicity of a group of compounds to their physio-chemical structure. Kamaya et al. [8] found that the toxicity of benzoic acids can be described by the logarithm of n-octanol/water partition coefficient (log*Kow*) and the number of hydroxyl groups (N_{OH}) of benzoic acid replaced with hydroxyl. Zhao et al. [6] also used pKa, E_{LUMO}, and log*Kow* to construct QSARs for

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prediction of the toxicity of halobenzoic acids to *Daphnia magna*, *V. fischeri*, and fish.

The alga *P. subcapitata* (formerly known as *Selenastrum capricornutum*) is a common biological indicator studied most extensively by ecotoxicologists. However, the effects of benzoic acids on phytoplankton have rarely been studied. In addition, the low toxic effects of benzoic acids on algae are seldom found in literature. The objective of the present study was to estimate the toxicity (in terms of EC50 and NOEC) of benzoic acids on *P. subcapitata* based on two response endpoints, i.e., final yield and algal growth rate. Furthermore, quantitative structure–activity relationships were derived for the prediction of toxicity of various benzoic acids.

2. Materials and methods

The test technique applied in the present study is a closed-system algal toxicity test, which was originally designed for testing both volatile organic compounds and metallic toxicants [10]. However, the author's previous work also indicated that the test technique works well for both high and low volatile organic compounds [11]. Detailed information regarding the test method and the concept of experimental design can be found in the author's previous work [10]. A brief description for toxicity testing and data analyses is given below.

Algal inoculum was withdrawn from the chemostat operated under a steady state, and transferred into 300 mL BOD bottles, together with dilution water (with growth medium) and toxicants. The BOD bottles were filled completely, leaving no headspace. A water seal was provided to ensure a closed test environment. The bottles were then placed on an orbital shaker operated at 100 rpm. Temperature and light intensity were kept at 24 ± 1 °C and 65 μEm-2s-1 (±10%), respectively. US EPA [12] bottle medium, with no EDTA content, was used for toxicity testing. Two response endpoints were used to evaluate the toxicity of the toxicants; the final yield and algal growth rate based on cell density counts. The median effective concentration (EC50) was defined as the toxicant concentration, which reduced the response to half of that obtained by the control. The initial inoculated cell density was 15,000 cells/mL and the duration of the test was 48 h. The population density of the algae was determined using an electronic particle counter (Culter Electronics, Luton, UK). The initial pH for toxicity testing was set at 6.5. All chemicals used were of reagent grade and were tested at least twice, i.e., range finding test and definitive test. For the definitive test, one control and 6 (or 7) different treatments were performed in triplicate. Stock solution was freshly prepared, and its concentration was analyzed using a HPLC analyzer before commencing the experiment.

Probit analysis was applied to determine the concentration–response relationship and the median effective concentration (EC50). One-tail Dunnett's procedure was applied for the estimation of NOEC and LOEC values at 5% level of significance. The studentized range (*SI*) can be calculated according to Eq. (1) as shown below.

$$SI = \frac{Xc - Xi}{Sw\sqrt{(1/nc) + (1/ni)}} \quad (1)$$

where *Xc* and *Xi* are mean observations from controls and treatments, respectively. *Sw* is the square root of the within-group variance and, *nc* and *ni* are the numbers of replicates for the control and treatment. A specific treatment is considered to be significantly different from the controls if the corresponding *SI* value is greater than the critical value (*T*). Obviously, *T* serves as a cut-off point for the Dunnett's test. We may hence calculate the cut-off value (in term of % reduction) by transforming Eq. (1) into the following expression:

$$\%\text{Reduction} = \frac{Xc - Xi}{Xc} \times 100 = \frac{T}{Xc} \times Sw\sqrt{\frac{1}{nc} + \frac{1}{ni}} \times 100 \quad (2)$$

Correlation analyses were performed using MINITAB (Ver 14.2; MINITAB, State College, PA, USA) to establish QSARs. Leave-one-out cross-validation was carried out to test the significance of each QSAR. The statistical quality was judged by the square of the correlation coefficient (r^2), the Fisher criterion (*F*), the root mean square error (*S*), and the cross-validated correlation coefficient (Q^2).

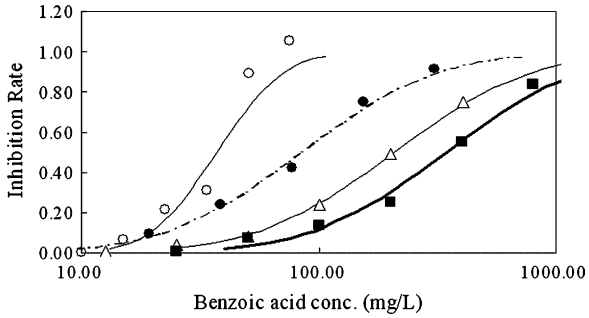

Fig. 1. Effects of benzoic acid at four different initial pH conditions on algal growth rate (○ non-neutralized; ● pH = 6.5; Δ pH = 7; ■ pH = 7.5).

3. Results and discussion

Fig. 1 displays the concentration–response relationships for benzoic acid tested under four different initial pH conditions (6.5, 7.0, 7.5, and non-neutralized), based on the endpoint of the algal growth rate. EC50 values for the aforementioned test conditions were 83.3, 207.5, 342.8, and 37.1 mg/L, respectively. It is obvious that the most severe condition occurred when the test was conducted without pH adjustment. However, for the non-neutralized test, the initial pH level was significantly depressed to below 6.0 when the benzoic acid concentration exceeded 22.5 mg/L. The responses observed under such a condition are partially due to acidity, as the algal growth will be considerably inhibited when pH is below 6.0. Considering that pH level in most aquatic environments is between 6.0 and 9.0, the initial condition of pH equal to 6.5 was chosen for testing the remaining benzoic acids. This means that the assessment is based on a conservative consideration, as in some instances, the pH value in the natural aquatic environment may be higher than 6.5. The reason for increasing toxic effect at decreasing pH is the degree of ionization for a weak acid [7,13]; Although both the ionized and unionized forms of benzoic acids contribute to the toxicity, the unionized fraction contributes significantly to the toxicity

Table 1
Algal responses to benzoic acid.

Conc mg/L	Final cells cells/ml	Growth rate μ	Inihibit rate Final yield	Inihibit rate Growth rate
Control	2.811 E+05	1.470*	0	0
307.84	1.927 E+04*	0.130*	0.980	0.910
153.92	3.112 E+04*	0.370*	0.940	0.750
76.96	8.197 E+04*	0.840*	0.750	0.420
38.48	1.401 E+05*	1.120*	0.530	0.240
19.24	2.159 E+05*	1.330*	0.250	0.090
9.62	2.458 E+05*	1.400	0.130	0.050
4.81	2.779 E+05	1.460	0.010	0.004
EC50			36.39	83.29

* Significantly different from the controls at $p = 0.05$ using the one-tail Dunnett's test.