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Aquatic Toxicities of Halogenated Benzoic Acids to *Tetrahymena pyriformis*

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Halogenated benzoic acids are widespread environmental pollutants resulting primarily from the production and use of xenobiotics. Two significant environmental sources of chlorinated benzoic acids are the microbial metabolism of herbicides (Maybury et al. 1996) and polychlorinated biphenyls (Abramowicz 1990; Harkness et al. 1993). Bryant and Schultz (1994) and Schultz and Dawson (1995) demonstrated that partial biodegradation or biotransformation produces byproducts that are more toxic than the parent substance. Aquatic studies while limited, have demonstrated benzoic acids to be toxic to a range of biota including bacteria (Ramos-Nino et al. 1996) as well as invertebrates and fish (Zhao et al. 1996).

The purpose of these experiments was to evaluate the aquatic toxicities of selected halogenated benzoic acids under neutralized and non-neutralized conditions, using both mortality and a population growth inhibition-toxicity assay employing the ciliate *Tetrahymena pyriformis*. Moreover, structure-toxicity models were evaluated for these data.

METHODS AND MATERIALS

Benzoic acid, ten chloro-, and four bromo-derivatives were obtained commercially (Aldrich Chemical Co., Milwaukee, Wisconsin, USA) at sufficient purity (>95%), making further purification unnecessary. Fresh stock solutions of each acid were prepared in dimethyl sulfoxide. Acute toxicity was assessed following the protocol of Schultz et al. (1994). With the aid of a dissecting microscope, toxicity, lack of ciliary movement, and/or cell lysis of the common freshwater ciliate *Tetrahymena pyriformis* was observed. Results were given in percent mortality. Serial dilutions were performed in sterile 20 mL 16 x 125-mm Pyrex test tubes containing 10 mL of a 0.1X Hank's Balanced Salt Solution or 10 mL of a 0.1X Hanks/Hepes solution for non-neutralized and neutralized benzoic acids, respectively. Stationary-phase cells were added to each tube to a concentration of 1 x 10^3 cells per mL, followed by incubation at 26 ± 1 °C for 16 hr.

Chronic toxicity was assessed following the protocol of Schultz (1997). This assay's uses were performed in foam-stoppered 250-mL Erlenmeyer flasks containing 50 mL of proteose-peptone media and examined *T. pyriformis* population growth impairment. In the neutralized experiments, each solution was

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Correspondence to: T. W. Schultz, Department of Ecology and Evolutionary Biology neutralized to pH 7 with 0.1N NaOH using an automatic titrator (Mettler-Toledo DL50 Titrator). For inoculation, log-growth-phase cells were added to each flask to a final concentration of 1.5 x 10^3 cells per mL, followed by incubation at 27 ± 1 °C for 40 hr. Population density was estimated spectrophotometrically as absorbance at 540 mn.

The 50% lethal concentration (LC_{50}) and 50% inhibitory growth concentration (IGC_{50}) were determined for each benzoic acid using Probit Analysis of Statistical Analysis System (SAS) software (SAS Institute 1989). The dependent variable was the toxic potency normalized as percentage of control. The independent variable was the toxicant concentration in mg/L.

The SAS software was also used to perform simple and multiple regression analysis for the development of structure-toxicity models. Log of the 1-octanol/water partition coefficient (K_{ow}) and the ionization constant (pK_a) either alone or in combination were used as the independent variables. The dependent variables were reported as concentration (mM) converted to the log of the inverse of the particular endpoint, LC_{50} or IGC_{50}. The models were measured for quality by the coefficient of determination (r^2). The square root of the mean square for error (s) was reported as an error of estimate of the coefficient of regression. Moreover, the ratio of model mean square or the Fisher Statistic (f) and the level of significance (Pr > f) were also noted for each variable and the model as a whole. Log K_{ow} values for each acid were secured as either a measured or computer estimated value from the CLOGP for Windows software (BIOBYTE Corp., Claremont, CA, USA); the measured value was used in preference to a calculated value. The pK_a values for each derivative were obtained as measured values from Dean (1992) or as estimated values from Hunter (1988).

RESULTS AND DISCUSSION

Acute (log (LC_{50}^{-1})) and chronic (log (IGC_{50}^{-1})) toxicity data for both non-neutralized and neutralized protocols are presented in Table 1. Molecular descriptor data, log K_{ow} and pK_a values, are also presented in Table 1. The non-neutralized results were consistently more acutely toxic than the neutralized results. It was also noted in general, that in both non-neutralized and neutralized experiments, the least acutely toxic derivatives were those with lower pK_a values (e.g., the 2-halogenated derivatives, especially the 2,6-dihalogenated benzoic acid). Under both conditions but with exceptions, halogenated benzoic acid toxic potencies increased as hydrophobicity increased.

In the comparison between the toxic potencies of chlorine and bromine-substituted benzoic acids, the brominated homologs were consistently more toxic than the chloro-containing ones. Since the pK_a values of bromo- and chloro-substituted homologs varied little, the difference in toxicity was attributed to the difference in hydrophobicity observed between the homologs.

With the exception of the neutralized acute data ($r^2 = 0.71$) none of the single descriptor models (data not shown) exhibited r^2 values > 0.50. Thus, none were of sufficient merit to be used for predictive purposes. In an effort to improve predictability by accounting for the contributions to toxicity of both the neutralized and non-neutralized molecules, multiple regression analyses were performed using both descriptors. The results are shown in Table 2. For both acute and chronic toxicity, the neutralized data modeled better than the non-neutralized data. This observation reflects the fact that the initial pH for acquiring the neutralized data was a constant (i.e., pH 7.0).

Outside of phenols, the studies of the toxicity of organic acids to aquatic organisms have been limited. An examination of the literature found few carboxylic acids, either aromatic or aliphatic, previously evaluated. However, toxicity data for a limited number of substituted benzoic acids were determined for the bacteria, daphnids, and fish (Zhao et al. 1996).

While a number of issues cloud the question of predictive toxicity of benzoic acids, none is more important than ionization. While the conversion of a HO(O=)C-group to a $^-$O(O=)C-group has little influence on size, it changes the hydrophobicity by more than four log units from a π values of -0.32 to one of -4.36 (Hansch and Leo 1979). In addition, it reduces electrophilicity from a *para*–σ value for 0.45 to 0.00 (Hansch and Leo 1979). In an aqueous system, the equilibrium can be calculated from the ionization constant, pK_a. At pH 7, the equilibrium shift is to the right, so > 99.9% of the total benzoic acid is in the dissociated form. Generally, the unionized form of weak acids is thought to be more toxic than the ionized analogue (Zhao et al. 1998). This toxicity difference is thought to be due to ease of passive uptake of the neutral form by biological membranes.

The pH is inversely related to the H+ ion concentration. Therefore, a lower pH corresponds to a higher H^+ ion concentration. Benzoic acids are weak and largely unionized in an acidic environment. Thus uptake is facilitated. In the non-neutralized experiments (i.e., lower pH), the halogenated benzoic acids were more toxic than the neutralized condition. This is because when non-neutralized, the higher H^+ ions in the toxicant-containing media force the formation of the uncharged species.

The effects of ionization on toxicity are also exhibited within a specific protocol. We consistently found that benzoic acids with halogens at the *meta*- and/or *para*-positions were more toxic than benzoic acids with halogens at the o&o-position. These relationships correspond directly to pK_a values. The pK_a is the pH at which half of the acid is dissociated. Benzoic acids halogen substituted at *meta*- and/or *para*- positions had pK_a values > 3.0, whereas benzoic acids with halogens at *ortho*-positions had pK_a values < 3.0. Therefore, at physiological pH, the *ortho*-substituted halogenated benzoic acids were ten-times more ionized than the *meta*- and/or *para*-substituted halobenzoic acids.

Table 1. Toxicities and descriptors for halogenated benzoic acids

Chemical	CAS Number [a]	Non-neutralized Log (LC_{50}^{-1})	Neutralized Log (LC_{50}^{-1})	Non-neutralized Log (IGC_{50}^{-1})	Neutralized log (IGC_{50}^{-1})	log K_{ow} [b]	pK_a [c]
Benzoic acid	65-85-0	0.91	-1.03	-0.30	-0.69	1.87	4.20
2-chloro	118-91-2	0.89	-1.36	-0.45	-0.84	1.99	2.88
3-chloro	535-80-8	1.01	-0.88	-0.13	-0.25	2.60	3.83
4-chloro	74-11-3	1.19	-0.41	0.11	-0.15	2.65	3.99
2,3-dichloro	50-45-3	0.98	-0.72	-0.41	-0.69	2.23	2.55[e]
2,4-dichloro	50-84-0	1.10	-0.42	-0.39	-0.63	2.86[d]	2.68[e]
2,5-dichloro	50-79-3	1.10	-0.90	-0.46	-0.90	2.82	2.55[e]
2,6-dichloro	50-30-6	0.80	-1.35	-0.67	-1.31	1.80	1.64[e]
3,4-dichloro	51-44-5	1.28	-0.20	0.31	0.23	3.46	3.68
3,5-dichloro	51-36-5	1.28	-0.20	0.27	0.23	3.00	3.46[e]
2,4,6-trichloro	50-43-1	1.05	-1.03	-0.41	-0.78	3.44[d]	1.40[e]
2-bromo	88-65-3	0.96	-0.95	-0.47	-0.78	2.20	2.85
4-bromo	586-76-5	1.30	-0.30	0.18	-0.07	2.80	3.99
2,5-dibromo	610-71-9	1.15	-0.85	-0.31	-0.57	3.09[d]	2.55[e]
3,5-dibromo	681-58-6	1.54	0.10	0.46	0.45	3.74[d]	3.46[e]

[a] Chemical Abstract Service registry number; [b] 1-octanol/water partition coefficient; [c] Ionization constant measured values. [d] Estimated value from CLOGP for Windows software; [e] Estimated value from Hunter (1988).

Table 2. Multiple regression models for acute (log (LC_{50}^{-1})) and chronic (log (IGC_{50}^{-1})) toxicity to *Tetrahymena*

Condition	Log K_{ow}	F	Pr > f	pK_a	f	Pr > f	Intercept	r^2	s	F	Pr > F
Acute Non neutralized	0.51	23	0.0005	0.25	11	0.0063	-2.85	0.74	0.24	17	0.0003
Acute Neutralized	0.26	60	0.0005	0.09	13	0.0036	0.11	0.86	0.08	36	0.0001
Chronic Non neutralized	0.37	33	0.0001	0.27	35	0.0001	-2.00	0.85	0.15	34	0.0001
Chronic Neutralized	0.55	43	0.0001	0.38	39	0.0001	-3.08	0.87	0.19	41	0.0001

This decrease in the unionized chemical accounts, in large part, for the decreased toxicity exhibited by the *ortho*-chlorinated benzoic acids.

The modeling toxicity of ionizable chemicals using only the log K_{ow} parameter for hydrophobicity fail to take into account the anisotropic nature of lipid bilayer biological membranes. The dissociated form of a chemicals can have a significantly greater affinity for biological membranes than the 1-octanol-water partition coefficient describes (Escher and Schwarzenbach 1996), but less of an affinity that the associated form. Thus as noted by Barron (1990) both the ionized and unionized forms of a chemical contribute to its toxicity.

The results of the present investigation indicate that the ionized and unionized forms of benzoic acids have different contributions to toxicity. Zhao et al. (1998) draws the same inference, and showed that the toxicity of benzoic acids to *Daphnia* greatly decreases as the pH increases. While the non-ionized form played a role in toxicity, the toxicity of benzoic acids to *Daphnia* could not be modeled by partition coefficients (log K_{ow}), or apparent partition coefficients (log D).

None of the relationships presented using a single descriptor, either log K_{ow}, or pK_a, is a quality model of toxicity for halobenzoic acids. However, using both log K_{ow} and pK_a as descriptors resulted in quality relationships. We feel this is not a reflection of the hydrophobic term quantitated the uptake of the associated from of the toxicants, the ionization constant quantitated the amount of toxicant present in this form. Rather, it is felt that pK_a is a surrogate for a yet to be defined uptake parameter for the dissociated form. Recent work (Vaes et al. 1998) suggested that the polar narcosis mechanism, the primary mechanism of toxicity for phenols, may be an artifact of partitioning. As shown by Vaes et al. (1997), polar pollutants have a higher affinity for membrane phospholipids than for 1-octanol. In contrast, almost no difference was observed between membrane/water partition coefficients and log K_{ow} for nonpolar pollutants. This observation is thought to explain the higher toxicity and the lower lethal body burdens of polar substances (Vaes et al. 1998).

The exposure ranges (tens to hundreds of mg /L) needed at physiological pH to elicit a toxic response in *T. pyriformis* (this study), *Daphnia magna,* or the fish *Cypinus carpiol* (Zhao et al. 1998) are far in excess of what can be expected in contaminated sediments (Flanagan and May 1993) and soils (Layton et al. 1994). However, exposure ranges (3 - 20 mg/L) needed at pH 5.7 to elicit a toxic response in the bacterium *Vibrio fischeri* (Zhao et al. 1998) are environmentally relevant.

Hydrophobicity and ionization can model acute and chronic toxic potency of halogenated benzoic acids. Both the non-ionized and ionized forms contribute to these toxicities.

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