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Regression Comparisons of Aquatic Toxicity of Benzene Derivatives: *Tetrahymena pyriformis* and *Rana japonica*

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The foundation of aquatic toxicity testing lies in *in vivo* mortality assays utilizing fish as test organisms. Databases for several fish species including the fathead minnow (*Pimephales promelas*), guppy (*Poecilia reticulata*) and zebrafish (*Brachydanio rerio*) have been developed. Recently, Wang et al. (2001) proposed using tadpoles of the frog *Rana japonica* as a test organism.

Because of the expected high costs of programs such as REACH, the Registration, Evaluation and Authorization of CHemicals, (Anon 2001) the development and use of microscale surrogate test systems is being examined as a means to fill data gaps. Compared to *in vivo* testing, these surrogates are more rapid and efficient; by and large they are based on ecologically important organisms. Included among the surrogate systems are assays using ciliated protozoa (Gilron and Lynn 1998). One such system, population growth impairment of the ciliate *Tetrahymena pyriformis* (i.e., TETRATOX), has been standardized (Schultz 1997). As *in vitro* aquatic toxicity test organisms, ciliates such as *Tetrahymena* afford several advantages including a short generation time (Schultz 1997). Interspecies comparisons (Schultz et al. 1998; Sinks and Schultz 2001; Seward et al. 2002) support the proposition that TETRATOX is an effective assay, which may replace, and certainly can reduce and refine *in vivo* techniques currently used for regulatory purposes. An obvious step in the validation process of a surrogate ecotoxicity protocol is to compare relative potencies attained with it to those using vertebrate organisms (Ball and Fentem 1999). While for aquatic-based systems, these organisms are principally fish, recent work by Huang et al. (2003) has used the *R. japonica* tadpole assay to test a series of benzene derivatives.

Benzene is the parent compound for a wide variety of derivatives many of which are among the more prevalent industrial organic chemicals in the world as defined by the High Production Volume Chemicals list (Green 2001). Therefore, toxicity data on benzene derivatives are important for use in the risk assessment process.

To assess further the potential of TETRATOX as a surrogate to toxicity assessment with aquatic vertebrates the current study was undertaken. Specifically, it was hypothesized that *Tetrahymena* population growth impairment data could predict the acute mortality of tadpoles of the frog *Rana japonica*. The aim of this investigation was to use regression analysis to compare organism-

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specific toxicity data within individual modes of action (i.e., class-based comparisons) prior to comparison of organism-specific toxicity data across modes of toxic action.

METHODS AND MATERIALS

Chemical toxicity toward tadpoles (*R. japonica*) for 47 benzene derivatives was taken from the literature (Huang et al. 2003). In this assay, three replicates of each treatment were run using 3.0 ± 0.1 cm tadpoles derived from naturally fertilized egg masses that were collected in the wild but reared in the laboratory (Huang et al. 2003). Tests were conducted at 25 ± 1° C in a six-step concentration series with a static renewal protocol. Specifically, ten tadpoles were placed in 300 ml of test solution in a 500 ml beaker, solutions were renewed after six hours exposure, and mortality recorded after 12 hours exposure. The concentration required to obtain 50% mortality for each chemical was calculated based on nominal concentrations and expressed as the 12-hr log (LC_{50}^{-1}) (mol/L).

TETRATOX data were secured from Schultz et al. (2003) or experimentally determined following the protocol described by Schultz (1997). Tests were conducted at 27 ± 1° C in a six- to ten-step concentration series with a static protocol. The endpoint, population density, of this static 40-hr assay was measured spectrophotometrically at 540 nm. The 50% growth inhibition concentration, IGC_{50}, was determined and expressed as the 40-hr log (IGC_{50}^{-1}) (mol/L).

Relationships were generated using log (IGC_{50}^{-1}) as the dependent variable and log (LC_{50}^{-1}) as the independent variable. Data were modeled using least squares regression (regression procedure of MINITAB release 13). The fit of the model was quantified with the squared correlation coefficient (r^2), predictivity was quantified by the leave-one-out cross-validated regression coefficient (q^2) calculated by the cross validation (PRESS) method. In addition, the standard error of the regression (s) and the Fisher's criterion (F) were recorded. Outliers were identified with reference to their standard residual values.

Mode of toxic action (MOA) was assigned based on chemical structure. Non-covalent toxicants were differentiated into neutral (MOA-1) or polar (MOA-2) interactions via the presence of electron-releasing groups (e.g., hydroxyl or amino group). Following the lead of Verhaar et al. (1992) in addition to phenols and anilines, nitrobenzenes that were not halogenated were classified as MOA-2. Aldehydes (Karabunarliev et al. 1996) or dinitroaromatics and halogenated nitroaromatics (Cronin et al. 1998) were designated as covalent reacting electrophiles (MOA-3). The single compound, 2,4-dinitrophenol, as a weak acid respiratory uncoupler was assigned MOA-4. Lastly, those derivatives containing a carboxyl-group, while not a recognized as a mode of toxicity, were assigned MOA-5.

Table 1. Mode of action and toxicity values for selected benzenes.

	Derivative	CAS number[a]	MOA[b]	Log (LC_{50}^{-1})	Log (IGC_{50}^{-1})
1	Chlorobenzene	108-90-7	1	3.20	3.06[c]
2	1,3-dichlorobenzene	541-73-1	1	3.68	3.56[c]
3	1,2-dichlorobenzene	95-50-1	1	3.79	3.55[d]
4	1,4-dichlorobenzene	106-46-7	1	3.85	3.53[c]
5	1,2,3-trichlorobenzene	87-61-6	1	4.43	4.21[c]
6	1-bromo-2,6-dichlorobenzene	19393-92-1	1	4.48	4.34[c]
7	1,2,4-trichlorobenzene	120-82-1	1	4.50	4.16[c]
8	1-bromo-2,3-dichlorobenzene	56961-77-4	1	4.56	4.30[c]
9	Resorcinol	108-46-3	2	2.07	2.35[d]
10	4-methoxyphenol	150-76-5	2	2.62	2.86[d]
11	2-methoxyphenol	90-05-1	2	2.65	2.49[c]
12	4-fluorophenol	371-41-5	2	2.69	3.02[d]
13	Phenol	108-95-2	2	2.77	2.79[d]
14	2-methylphenol	95-48-7	2	2.84	2.71[d]
15	2-chlorophenol	95-57-8	2	3.01	3.18[c]
16	4-methylphenol	106-44-5	2	3.06	2.84[d]
17	Nitrobenzene	98-95-3	2	3.29	3.14[bd]
18	2,6-dimethylphenol	576-26-1	2	3.32	2.92[ac]
19	4-chlorophenol	106-48-9	2	3.42	3.54[bd]
20	2-nitroresorcinol	601-89-8	2	3.49	3.66[bd]
21	2-nitrophenol	88-75-5	2	3.50	3.67[bd]
22	3-nitrophenol	554-84-7	2	3.51	3.51[bd]
23	2-nitrotoluene	88-72-2	2	3.53	3.26[bd]
24	4-nitrotoluene	99-99-0	2	3.62	3.65[bd]

[a]chemical abstract service number; [b]Mode Of Action, 1 = nonpolar narcosis, 2 = polar narcosis, 3 = covalent electrophilicity, 4 = weak acid respiratory uncoupling; 5 = carboxylic acid, [c]new data, [d]from Schultz et al. (2003)

RESULTS AND DISCUSSION

Regression-based comparisons of aquatic toxicity of *T. pyriformis* to *R. japonica* by MOA are presented in Table 2. The derivative, CAS number, assigned mode of action (MOA), toxicity data for *Rana japonica* (log (LC_{50}^{-1})) and *Tetrahymena pyriformis* (log (IGC_{50}^{-1}) are reported in Table 1.

Although based on only eight derivatives with very limited structural diversity, benzenes acting via neutral non-covalent narcosis (MOA-1) show an excellent relationship between *Tetrahymena* and *Rana* toxicity (Eq. [1]). The small number of compounds and their lack of diversity normally would be considered a noteworthy liability. However, since toxicity of neutral narcotics to aquatic organism is determined singularly by biouptake to the site of action (biomembranes) by passive diffusion and is particularly consistent between aquatic test protocols (Schultz et al. 1998), these restrictions are deemed a minor problem.

Similarly, Eq. [2] based on the derivatives with an ionized carboxyl moiety (MOA-5) exhibited a relationship with similar statistics to that of Eq. [1]. Studies of the aquatic toxicity of benzoic acids are limited. They are often separated into

Table 1. Continued.

	Derivative	CAS number[a]	MOA[b]	Log (LC_{50}^{-1})	Log (IGC_{50}^{-1})
25	4-bromophenol	106-41-2	2	3.66	3.68[d]
26	4-nitrophenol	100-02-7	2	3.66	4.42[c]
27	2-bromo-4-methylphenol	6627-55-0	2	3.72	3.60[d]
28	2,4-dichloroaniline	554-00-7	2	3.73	3.56[d]
29	1-naphthalenol	90-15-3	2	3.81	3.75[c]
30	2,4-dichlorophenol	120-83-2	2	3.87	4.04[d]
31	4-chloro-2-nitrophenol	89-64-5	2	3.88	4.99[c]
32	2-naphthalenol	135-19-3	2	3.89	3.79[c]
33	4-tert-butylphenol	831-82-3	2	4.03	3.91[d]
34	4-hydroxybenzaldehyde	123-08-0	3	3.08	3.27[c]
35	2-chloro-5-nitroaniline	6283-25-6	3	3.47	3.60[c]
36	1-chloro-4-nitrobenzene	100-00-5	3	3.93	3.73[d]
37	1,3-dinitrobenzene	99-65-0	3	4.02	3.76[d]
38	1,2-dinitrobenzene	528-29-0	3	4.05	4.25[d]
39	2,4-dinitrotoluene	121-14-2	3	4.06	3.87[d]
40	1-chloromethyl-4-nitrobenzene	100-14-1	3	4.32	4.18[d]
41	1-chloro-2,4-dinitrobenzene	97-00-7	3	4.34	5.16[d]
42	1-bromo-2,4-dinitrobenzene	584-48-5	3	4.46	5.31[c]
43	2,4-dinitrophenol	51-28-5	4	4.31	4.06[c]
44	Salicylic acid	69-72-7	5	2.84	2.49[c]
45	5-chlorosalicylic acid	321-14-2	5	3.01	2.60[c]
46	4-chlorobenzoic acid	74-11-3	5	3.42	3.11[c]
47	4-bromobenzoic acid	586-76-5	5	3.63	3.18[c]

[a]chemical abstract service number; [b]Mode Of Action, 1 = nonpolar narcosis, 2 = polar narcosis, 3 = covalent electrophilicity, 4 = weak acid respiratory uncoupling; 5 = carboxylic acid, [c]new data, [d]from Schultz et al. (2003)

their own group because in aqueous systems at neutral pH the equilibrium shifts to the right, so > 99.9% of the carboxyl group is in the dissociated form, which impacts biouptake.

Although not as statistically strong, a significant relationship was found for the polar non-covalent derivatives of MOA-2 (Eqs. [3]). It has been widely assumed that polar narcotics act through a different molecular mechanism than neutral narcotics (Schultz et al. 1998). Because polar narcotics can readily form hydrogen bonds, it has been speculated that they interact with a molecular site of action different from neutral narcotics.

Based on the standard residual values there was one outlier to Eq. [3]; this outlier, 4-nitrophenol, was more toxic in TETRATOX. The increased toxicity was related to abiotic transformation (Schultz 1992), which was attributed to tautomerism to a quinone-like substance (Roberts 1987). Removal of this data and subsequence reanalysis lead to Eq. [4]. While additional outliers to Eq. [4] and subsequent reanalyses could be identified, there was no obvious rational for their removal from the dataset for MOA-2.

Table 2. Toxicity regression equations.

MOA	Slope	Intercept	N	R^2	q^2	s	F	Eq.
1	0.99	0.23	8	0.98	0.96	0.07	312	1
5	1.02	0.32	4	0.97	0.89	0.08	67	2
2	0.86	0.46	24	0.79	0.71	0.24	81	3
2	0.91	0.31	23	0.83	0.80	0.18	100	4
3	0.36	2.54	11	0.41	0.00	0.32	5	5
3	0.89	0.56	8	0.68	0.06	0.27	10	6
All	0.96	0.26	43	0.87	0.85	0.20	258	7

Electrophiles (MOA-3) are chemicals, or their activated metabolites, which react covalently with nucleophilic sites in cellular macromolecules through nucleophilic substitution, Michael-type addition, or Schiff-base reactions (Jacobs 1997). The investigation of Karabunarliev et al. (1996) suggests that for modeling purposes electrophiles should be segregated by specific mechanism or chemical domain. However, due to the limited number of derivatives (ten) we elected to examine electrophiles as a single MOA. Initial regression analysis of the electrophilic chemicals of MOA-3 shows no relationship between toxic potencies (Eq. [5]). This is in large part due to the enhanced toxicity of 4-chloro-2-nitrophenol, 1-chloro-2,4-dinitrobenzene , and 1-bromo-2,4-dinitrobenzene in TETRATOX. It is worth noting that all these compounds can undergo neutrophilic substitution by replacement of a halogen, facilitated by electron-withdrawing nitro-groups (Jacobs 1997). Removal of the three above noted compounds and subsequence reanalysis lead to Eq. [6], which exhibits moderately good statistics. An additional outlier to Eq. [6] (4-hydroxybenzaldehyde) was identified. This derivative, the only aldehyde in the dataset, can react by the Schiff-base reaction (Jacobs 1997), but no further modification or analysis of the dataset for MOA-3 was undertaken.

Uncouplers of oxidative phosphorylation inhibit the synthesis of adenosine triphosphate within the mitochondria by "short-circuiting" hydrogen ion transport (Schultz et al. 1998). These chemicals are typically weak acids and are typified by phenols having multiple electronegative groups (i.e., more than one nitro-group substituents to the aromatic ring). Only one compound, 2,4-dinitrophenol represents this MOA and its toxicity is greater in *R. japanica.*

A plot of toxicity to *T. pyriformis* [log (IGC_{50}^{-1})] versus toxicity to *R. japanica* [log (LC_{50}^{-1})] is presented in Figure 1. The outlier noted for Eq [3] and the three outliers noted for Eq [5] are easily identified.

Regression analysis of the aquatic toxicities for the remaining 43 derivatives results in Eq. [7]. The slope of near unity supports the premise of a one-to-one correlation between the two sets of toxicity values. The intercept of 0.26 suggests that the TETRATOX protocol is slightly more sensitive.

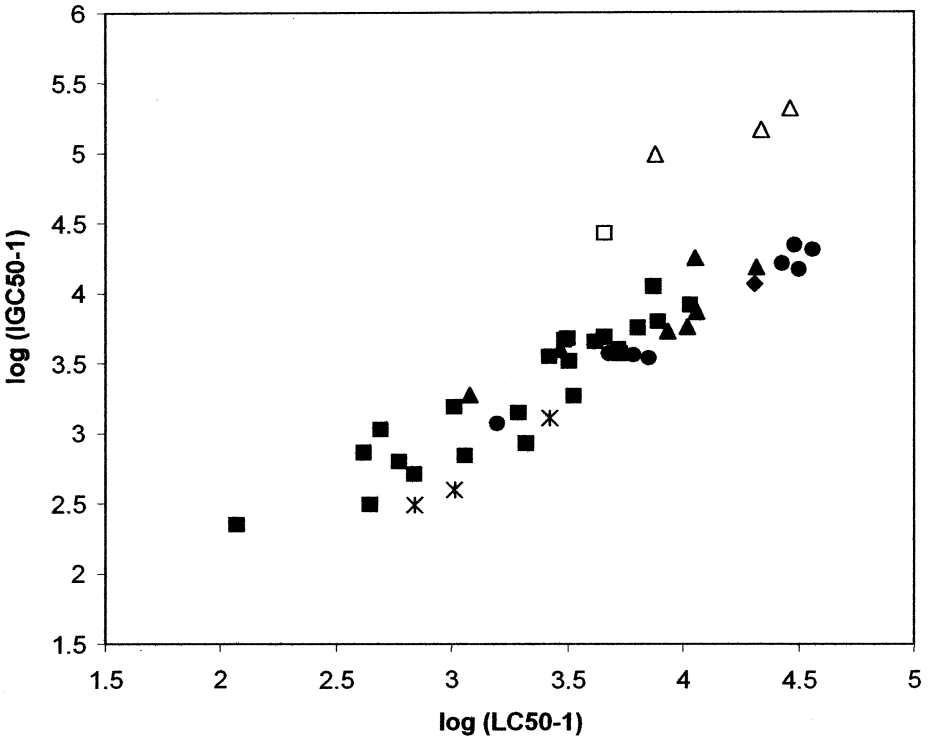

Figure 1. *Tetrahymena* toxicity [log (IGC_{50}^{-1})] versus *Rana* toxicity [log (LC_{50}^{-1})] Circles = MOA 1, Squares = MOA 2, Triangles = MOA 3, Diamond = MOA 4, Stars = MOA 5

In summary, with the exception of the neutrophilic substituting nitrobenzenes there is a superior overall relationship between *Tetrahymena* and *Rana* toxicity. While these analyses point to TETRATOX being a high-quality surrogate for another aquatic vertebrate (the *Rana* tadpole assay), more data regarding relative toxicity for other mechanisms of toxic action and more diverse molecular structures are necessary for a more specific evaluation of extrapolation of data between these two systems.

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