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ANALYZING AND MODELING TOXICITY OF MIXTURES OF ORGANIC CHEMICALS TO MICROORGANISMS

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ABSTRACT

Inhibition concentrations of 50 organic chemicals acting singly and jointly in different combinations on activated sludge microorganisms and a surrogate test microbial culture, Polytox, were measured using the respirometric technique. The suitability of the surrogate test culture in toxicity studies was evaluated against activated sludge microorganisms. The toxicity correlation between the two organisms was highly significant with coefficient of determination of 0.922. The joint toxic effects of several 2-, 8-, and 10-component mixtures on the surrogate cultures were found to be simply additive. Using the single chemical toxicity data, Quantitative Structure Activity Relationship (QSAR) models were developed to relate toxicity to molecular structures of the chemicals. The application of these QSAR models in predicting joint effects in six 2-, six 8-, and ten 10-component mixtures was demonstrated. These predictions agreed with the experimentally measured values with coefficient of determination of 0.866 at $p < 0.0001$.

KEYWORDS

Joint effects; microorganisms; mixtures of organic chemicals; Polytox; QSAR models; toxicity.

INTRODUCTION

Toxicity of synthetic, xenobiotic organic chemicals to aquatic life forms and microorganisms is a key consideration in chemical hazard management and control. Even though considerable toxicity research has been done on various aquatic species, only a few studies have focused on the effects on microorganisms. Environmental engineers in particular are concerned about toxicity to activated sludge (A/S) cultures because of their use in biological unit processes in municipal wastewater treatment plants. Since these plants were not originally designed to receive such toxic chemicals, plant upsets may result from influent toxicity. Upsets caused by influent toxicity may result in costly effluent discharge permit violation penalties, process downtime and degradation of receiving water quality.

For efficient and dependable design and operation of biological waste treatment plants, advance knowledge of chemical toxicity to microorganisms would be highly desirable. However, such toxicity data are not readily available: according to a Worldwatch Report, toxicity data are not available for about 80% of the 70,000 chemicals that are in commercial use today; only 10% of the new chemicals submitted for approval to the Environmental Effects Branch, EEB, of the Office of Toxic Substances of US Environmental Protection Agency (EPA) have contained toxicity data (EPA-560-6-88-001). This problem is further

compounded by the fact that wastewater streams have been known to carry mixtures of several toxicants causing joint effects (Volskay and Grady, 1988). Because of the large number of possible combinations of chemical mixtures and the limited resources available for toxicity testing, methods for rapid prediction of toxicity would be of considerable benefit. During the past decade, effects of multiple chemicals on fish have been extensively studied by aquatic toxicologists to understand, analyze and predict the joint effects. This paper presents a study on joint effects of multiple chemicals on microorganisms in which a methodology is developed to rapidly predict toxic effects of single and multiple chemicals on activated sludge microorganisms.

Prediction of Toxicity

Two common approaches to predicting toxicity to A/S microorganisms are 1) use of standardized surrogate test organisms and, 2) use of Quantitative Structure Activity Relationship (QSAR) models (Blum and Speece, 1990). In the first approach, experimentally measured 50% inhibition concentrations (IC_{50}) of a selected number of chemicals to a standardized microbial culture are compared with those of A/S cultures to establish a correlation. Then, toxicity of "new" chemicals to A/S cultures may be estimated using rapid measurements using the standardized cultures and the correlation. From a stand point of toxicity measurement, these standardized cultures have some advantages over direct testing of A/S cultures: consistency, reproducibility, and ease of measurement.

The second approach is based on the premise that activities and properties of organic chemicals are closely related to their molecular constituents and structures. In this approach, a limited number of chemicals are first tested for toxicity. Then, a correlation (or QSAR model) is derived between the numerical molecular descriptors and the measured toxicity. For untested chemicals, one can then use the QSAR model to estimate the toxicity in terms of the chemicals' molecular descriptors. The advantage of this approach over the first one is that, once the correlation has been established, no further experimentations are necessary. Thus, the QSAR approach can be quite useful in estimating toxicity of the large number of untested chemicals that are in commercial use today. The EEB, for example, has adapted about 50 QSAR models for use on a regular basis to estimate toxicity to aquatic organisms such as fish (EPA-560-6-88-001). However, only a very few well documented and demonstrated models for microbial toxicity have been published. The study reported here was designed to develop an internally consistent microbial toxicity database to be used as the training set for building QSAR models.

Analyzing Joint Effects of Multiple Chemicals

Joint effects of two or more chemicals on fish have been studied extensively by ecotoxicologists. Some of the pioneering work in this area has been done by Bliss (1939) and Gaddum (1948). Following them, Plackett and Hewlett (1948, 1952, 1963, 1967); Sprague and co-workers (1964, 1965, 1970); Ashford and co-workers (1958, 1964, 1965, 1974); Konemann and co-workers (1981a, b, c), and Hermens and co-workers (1984a, b, c, 1985a, b, c) have conducted several experimental and theoretical studies to interpret and formalize the analysis of joint effects of multiple chemicals in mixtures.

Three concepts have been proposed by the above workers to quantify and analyze joint effects: the concepts of Toxic Units, TU; Additivity Index, AI; and Mixture Toxicity Index, MTI. These descriptors are calculated as follows for a mixture containing N chemicals with each component, i, having an individual IC_{50} of $IC_{50,i}$:

$$TU_i = C_i/IC_{50,i}$$

$$AI = M-1 \quad \text{if } M = 1$$

$$= 1/M - 1 \quad \text{if } M < 1$$

$$= M(-1)-1 \quad \text{if } M > 1$$

$$\text{and, MTI} = \{\log M_0 - \log M\}/\log M_0$$

where, $M = \Sigma TU_i$ and $M_0 = M \div \max \text{ of } TU_i$.

Using these concepts, joint effects may be classified into simple additivity ($\Sigma TU_i = 1$), antagonism ($\Sigma TU_i >$ M0), synergism ($\Sigma TU_i < 1$), independent action ($\Sigma TU_i = M_0$), and partial addition ($M_0 > \Sigma TU_i > 1$).

Objectives of This Study

The major objectives of this study were to: evaluate the suitability of the Polytox cultures as simple-to-use surrogates and to establish the toxicity correlation with activated sludge organisms; develop QSAR models to predict single chemical toxicity to the above two organisms; to analyze the joint effects of several 2-, 8-, and 10-component mixtures using TU, AI, and MTI; and, to develop and validate a QSAR-based approach to predict the concentrations in equitoxic mixtures that would cause 50% inhibition of the surrogate organisms.

MATERIALS AND METHODS

Test Chemicals

Of the 50 organic chemicals selected for toxicity assays, 17 are listed as priority pollutants by the US EPA. The selected chemicals represented typical environmental contaminants with a wide range of molecular structures including simple and halo-substituted alkanes and aromatics, alcohols, esters, ketones, amines etc. In the six 2-component mixture studies, octanol was used as one of the components. The mixtures were all designed to be "equitoxic" (i.e. each component was of the same TU in the mixture).

Polytox Test Cultures

One vial of 8 gr of Polytox freeze-dried cultures was dispersed in 250 ml of buffered dilution water, prepared according to Standard Methods. This medium was mixed for 30 minutes and then allowed to settle for 5 minutes. The supernatant was then separated for placement in eight 123 ml reactors. Each of the eight reactors was fed with 6 ml of the supernatant and topped with buffered dilution water to bring up the final volume to 60 ml. The control reactor was topped up to 62 ml. All the reactors were then capped with potassium hydroxide pellets in holders attached to the caps.

Activated Sludge Test Cultures

The A/S test cultures were obtained daily from the aeration tank of the nearby Las Cruces Municipal Wastewater Treatment Plant. The MLSS and MLVSS of activated sludge varied from 1,200 to 2,600 [mg/l] and 1,020 to 1,970, [mg/l] respectively. All the 123 ml reactors received 10 ml of activated sludge each. The test reactors were topped with tap water to bring up to a final volume of 60 ml while the control reactors were topped up to 62 ml. All the reactors were then capped with potassium hydroxide pellets in holders attached to the caps.

Respirometric Test Procedure

The toxicity tests were run on a 20-reactor, computer-interfaced Comput-OX Respirometer (N-CON Corporation, NY). Details of this system have been presented elsewhere (Cadena *et al.*, 1988). Each reactor, except the control, was spiked with different volumes [µl] of the toxicant being assayed. The toxicant dose was prepared by dissolving the toxicant in 2 ml of acetone. The capped reactors were placed in the respirometer water bath maintained at 25°C with continued supply of oxygen. The data acquisition system was then initiated to monitor and record the oxygen uptake of each reactor for the next 6 hours for the Polytox tests and 12 hours for the activated sludge tests.

Determination of IC_{50} Values

The reductions in oxygen uptake rates of the spiked reactors compared to that of the control reactor were taken as the percent inhibition [%] at different concentrations of the toxicant (Blum 1989; Elanbaraway *et*

al., 1988). These percentage inhibition values were then plotted against the respective concentrations, and from these plots, the concentration causing 50% inhibition, IC_{50} [mg/l], was determined.

RESULTS

Reproducibility of IC_{50} Values

Preliminary studies were conducted to establish and compare the reproducibility and the variability in the experimental results for the two cultures. Four "cold start" runs were made for four selected chemicals to evaluate the variations in the final IC_{50} values for each of the cultures. The mean of the IC_{50} values from the four runs for the four chemicals and the corresponding standard deviations are presented in Fig. 1. The Polytox testing procedure can be seen to be consistent in yielding IC_{50} values with an average standard deviation of 16.4 while the A/S testing procedure yielded slightly higher variations with standard deviation of 22.6. These variations are comparable to those reported by Blum (1989) for activated sludge test, and may be considered acceptable for microbial toxicity work.

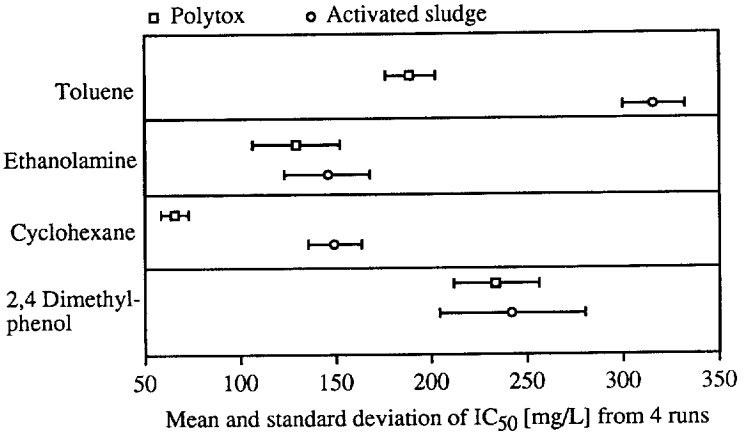

Fig. 1. Comparison of reproducibility between Polytox and activated sludge tests.

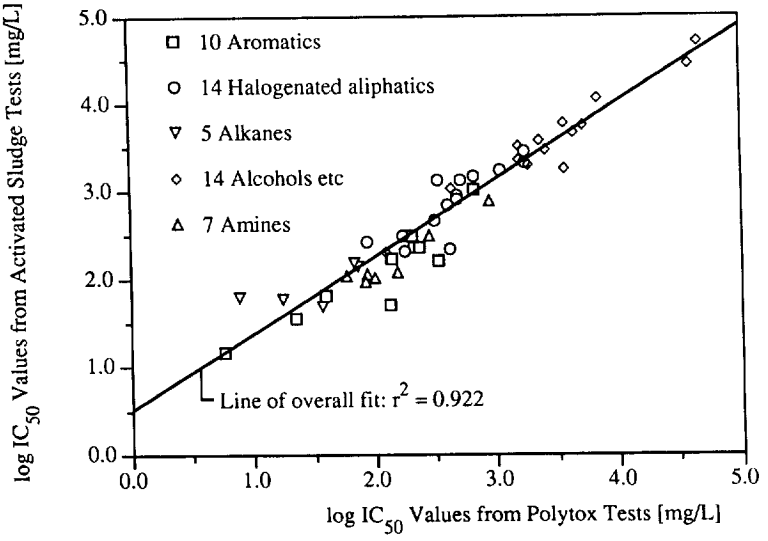

Fig. 2. Correlation between activated sludge tests and Polytox tests.

TABLE 1. Comparison of IC_{50} Values for Polytox and Activated Sludge

ID #	Chemical	Type*	IC50 Values [mg/L]	
			Polytox	Act. sludge
1	Benzene	Aro	685	993
2	Toluene	Aro	207	292
3	Xylene	Aro	140	166
4	Ethylbenzene	Aro	220	222
5	Chlorobenzene	Aro	350	155
6	1,2 Dichlorobenzene	Aro	135	49
7	1,3 Dichlorobenzene	Aro	40	63
8	1,4 Dichlorobenzene	Aro	6	14
9	1,2,4 Trichlorobenzene	Aro	23	35
10	2,4 Dimethyl phenol	Aro	240	224
11	Methylene chloride	Hal	1,750	1,994
12	Dibromomethane	Hal	1,110	1,572
13	Carbon tetrachloride	Hal	325	432
14	1,2 Dichloroethane	Hal	685	1,385
15	1,1,1 Trichloroethane	Hal	415	659
16	1,1,2,2 Tetrachloroethane	Hal	180	197
17	1,2 Dichloropropane	Hal	500	861
18	Bromochloromethane	Hal	1,800	2,672
19	Bromodichloromethane	Hal	90	249
20	Chlorodibromomethane	Hal	425	206
21	Ethylene dibromide	Hal	520	1,271
22	1,2 Dichloroethylene	Hal	350	1,249
23	Trichloroethylene	Hal	500	770
24	Tetrachloroethylene	Hal	175	299
25	Cyclohexane	Alk	74	133
26	Pentane	Alk	70	150
27	Hexane	Alk	38	47
28	Heptane	Alk	18	58
29	Octane	Alk	8	60
30	Bis (2-chloroethyl) ether	Alc	1,600	3,025
31	Ethanol	Alc	40,000	26,311
32	Propanol	Alc	7,200	10,875
33	Pentanol	Alc	2,325	3,528
34	Octanol	Alc	126	194
35	n-Butyl acetate	Alc	3,750	1,649
36	Isobutyl acetate	Alc	1,600	2,156
37	n-Amyl acetate	Alc	440	1,031
38	Ethyl acetate	Alc	5,400	5,427
39	Acetone	Alc	48,000	48,619
40	Methyl ethyl ketone	Alc	1,900	1,873
41	Methyl isobutyl ketone	Alc	2,600	2,811
42	Methyl n-propyl ketone	Alc	4,500	4,267
43	Cyclohexanone	Alc	3,750	5,452
44	n-Butyl amine	Ami	90	111
45	t-Butyl amine	Ami	85	90
46	Diethylamine	Ami	104	100
47	Acetic acid	Ami	287	299
48	Cyclohexylamine	Ami	60	103
49	Ethanolamine	Ami	160	115
50	Triethanolamine	Ami	900	741

* Aro- aromatic; Hal- halogenated aliphatic; Alk- alkanes; Alc- alcohols, esters, ketones, and ethers; Ami- amines

Correlation Between Polytox and Activated Sludge Test Results

Good correlation was found between Polytox IC_{50} values and A/S IC_{50} values. Figure 2 illustrates this correlation for the 50 chemicals belonging to the different classes, with an overall coefficient of determination of 0.922 and standard error of 0.225. Experimental IC_{50} values for the 50 chemicals from the two tests are tabulated in Table 1.

Thus, for chemicals belonging to congeneric classes similar to those tested here, Polytox may be used as a surrogate test culture for rapid estimation of toxicity to A/S cultures. The relationship between the IC_{50} values [mg/l] for the two cultures is given by:

$$\log IC_{50,\,A/S} = 0.412 + 0.889 \log IC_{50,\,Polytox} \quad n = 50;\ r = 0.960;\ r^2 = 0.922;\ SE = 0.225. \tag{1}$$

The relationship given by Eq. 1 shows that, in general, the Polytox cultures are more sensitive than the A/S cultures. When a paired t-test was done to test if the difference of the means of the IC_{50} values from the two tests was significant, the A/S log IC_{50} values were found to be greater than Polytox log IC_{50} values by 0.129 at 95% confidence level. Thus, the toxicity predicted by Polytox could be considered a conservative estimate. Having established the correlation between the two tests, the mixture toxicity testing was done using the surrogate cultures to take advantage of the shorter test durations (6 hrs vs. 12 hrs) and the reproducibility.

QSAR Models for Polytox and Activated Sludge Test Results

Results shown in Table 1 were used to develop QSAR models for IC_{50} for the two cultures. As described elsewhere, (Nirmalakhandan *et al.*, 1994), the molecular connectivity indexes (calculated according to Nirmalakhandan, 1988) correlated with the log IC_{50} values [in mM/l] yielding the following models for Polytox cultures:

Alcohols, ketones and esters:

$$\log IC_{50} = 3.690 - 0.896\ {}^{1}\chi^{v} \quad n = 14;\ r = 0.954;\ r^2 = 0.910;\ SE = 0.246. \tag{2}$$

Alkanes:

$$\log IC_{50} = 1.851 - 0.765\ {}^{1}\chi^{v} \quad n = 5;\ r = 0.999;\ r^2 = 0.999;\ SE = 0.018. \tag{3}$$

Amines and acids:

$$\log IC_{50} = 1.045 - 0.470\ {}^{1}\chi^{v} \quad n = 6;\ r = 0.957;\ r^2 = 0.915;\ SE = 0.101. \tag{4}$$

Aromatics:

$$\log IC_{50} = 3.258 - 1.133\ {}^{1}\chi^{v} \quad n = 9;\ r = 0.852;\ r^2 = 0.726;\ SE = 0.311. \tag{5}$$

Halogenated aliphatics:

$$\log IC_{50} = 2.670 - 0.448\ {}^{0}\chi^{v} \quad n = 12;\ r = 0.942;\ r^2 = 0.887;\ SE = 0.141. \tag{6}$$

The results of the activated sludge tests yielded very similar QSAR models:

Alcohols, ketones and esters:

$$\log IC_{50} = 3.532 - 0.801\ {}^{1}\chi^{v} \quad n = 14;\ r = 0.951;\ r^2 = 0.904;\ SE = 0.229. \tag{7}$$

Alkanes:

$$\log IC_{50} = 1.103 - 0.381\ {}^{1}\chi^{v} \quad n = 5;\ r = 0.839;\ r^2 = 0.703;\ SE = 0.181. \tag{8}$$

Amines and acids:

$$\log IC_{50} = 0.819 - 0.326\ {}^{1}\chi^{v} \quad n = 6;\ r = 0.852;\ r^2 = 0.726;\ SE = 0.142. \tag{9}$$

Aromatics:

$$\log IC_{50} = 3.254 - 1.135\ {}^{1}\chi^{v} \quad n = 9;\ r = 0.852;\ r^2 = 0.726;\ SE = 0.314. \tag{10}$$

Halogenated aliphatics:

$$\log IC_{50} = 2.924 - 0.481\ {}^{0}\chi^{v} \quad n = 14;\ r = 0.886;\ r^2 = 0.785;\ SE = 0.222. \tag{11}$$

While the QSAR models for the two cultures are remarkably similar in form and significance, the quality of the Polytox models is somewhat better than that of the A/S models. This is as expected because of the higher variability in the A/S data as discussed earlier. While supporting the earlier finding of good correlation between the two cultures, the similarity of the QSAR models also indicates that these chemicals act on these two organisms by very similar mechanisms (Konemann, 1981).

Analysis of Joint Effects

The joint effects of multiple chemicals acting together were analyzed using the three concepts that have been used in fish toxicity studies: the Toxic Units (TU) Concept; the Additivity Index (AI) Concept; and the Mixture Toxicity Index (MTI) Concept. From the 2-, 8-, and the 10-component mixture tests, the average ΣTU, AI and MTI values were found to be 1.05 ± 0.12; 0.04 ± 0.12, and 0.98 ± 0.05 respectively, while the expected values for ideal simple additivity are 1; 0; and 1 respectively. The variations of observed ΣTU for the 2-, 8-, and 10-component mixtures are presented in Fig 3. A one sample t-test was used to compare the observed mean ΣTU against the hypothesized value of 1 for simple additivity. This test confirmed that the mean of the observed ΣTU value of 1.05 was not different from 1 at 95% confidence level. Thus, it can be concluded that the chemicals assayed in this study act jointly by simple addition. These findings are in agreement with those reported for fish toxicity of similar chemicals (Hermens and co-workers, 1984a, b, c, 1985a, b, c; Broderius and Kahl (1985).

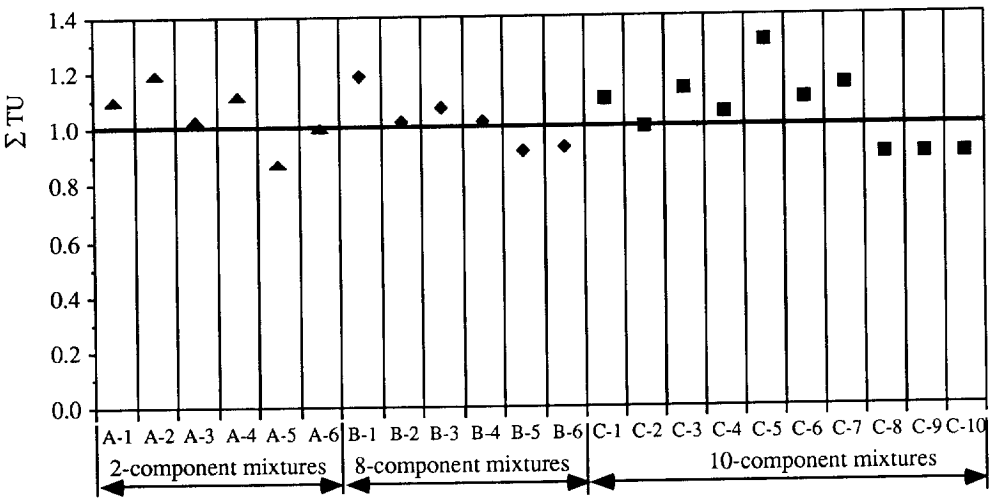

Fig. 3. Observed variations from ΣTU = 1 in the 2-, 8-, and 10-component mixtures.

Predictions Using QSAR Models

The use of the QSAR models in predicting joint effects in equitoxic mixtures by simple additivity is illustrated next. In such an N-component mixture, the sum of the toxic units of all the N components should equal 1, and the toxic unit of each component should be 1/N. Then, the concentration of any component in that mixture that would result in 50% inhibition can be obtained as (1/N) times the individual IC_{50} of that component. Thus, if the single chemical IC_{50} values of each of the N components of an N-component mixture can be predicted by a QSAR model, then the concentrations of each of the N components that would jointly result in 50% inhibition can be estimated.

This hypothesis was tested using the six 2-, the six 8-, and the ten 10-component mixture results. From each of the different N-component mixtures (i.e N = 2, 8, and 10), the concentration of the N^{th} component that

would contribute to 50% inhibition was estimated using the QSAR models developed in this study and assuming perfect additivity. Then, these predicted concentrations were compared against the respective concentrations found experimentally. The components of the mixtures used for this study and the results are tabulated in Table 2. Satisfactory agreement was found between the observed and predicted concentrations as illustrated in Fig. 4 with an overall coefficient of determination of 0.866. The deviations of the points from the line of perfect prediction are due to minor inadequacies of the QSAR models, slight deviations from simple additivity as well as due to the experimental errors. Nevertheless, this degree of prediction can be considered more than adequate for microbial toxicity work.

TABLE 2. Comparison of Observed vs. Predicted Concentrations

Mixture type	ID # of chemicals in mixture		Obs.	Obs. IC50 of	Conc. of Nth chemical	
	N-1 chemicals	Nth	ΣTU	Nth chemical [mg/l]	Observed [mg/l]	Predicted [mg/l]
A-1	34	16	1.11	180	100	112
A-2	34	44	1.20	90	54	41
A-3	34	20	1.04	425	221	145
A-4	34	37	1.12	440	246	287
A-5	34	5	0.88	350	154	162
A-6	34	25	1.01	74	37	37
B-1	40,41,35,36,32,33,12	30	1.21	1600	242	124
B-2	40,41,35,36,12,18,1	2	0.95	207	24	39
B-3	40,41,35,36,32,33,22	23	1.08	500	68	76
B-4	4,36,32,33,12,18,34	17	1.03	500	64	60
B-5	4,10,36,32,18,23,22	33	0.92	2325	267	298
B-6	40,35,21,15,4,5,2	10	0.96	240	29	12
C-1	4,5,10,36,32,33,12,18,2	1	1.10	685	75	77
C-2	4,5,10,36,32,33,12,22,23	18	0.98	1800	176	120
C-3	40,41,35,36,32,33,5,10,17	4	1.14	220	25	8
C-4	41,35,36,32,33,4,5,10,2	40	1.05	1900	200	582
C-5	40,41,35,36,32,33,31,43,17	34	1.31	126	17	16
C-6	40,41,35,36,31,43,18,1,2	12	1.10	1110	122	69
C-7	40,41,43,31,33,12,18,22,23	32	1.15	7200	828	1279
C-8	40,41,35,36,4,5,17,34,23	43	0.90	3750	338	333
C-9	40,41,35,4,5,17,18,1,2	36	0.90	1600	144	195
C-10	40,43,4,5,17,12,18,22,23	41	0.90	2600	234	220

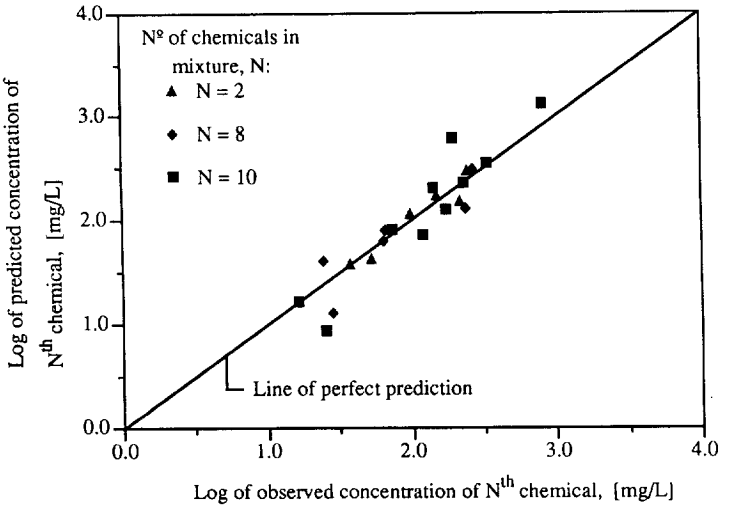

Fig. 4. Agreement between observed and predicted concentration of the N^{th} chemical in N-component mixtures.

CONCLUSIONS

The surrogate culture evaluated in this study, Polytox, was found to be easy to use and yielded more consistent results than the activated sludge organisms. A highly significant correlation (coefficient of determination = 0.922) was found between the 50% inhibition concentrations of the 50 organic chemicals assayed. This correlation was further confirmed by the very similar single-variable QSAR models relating the toxicity to the two organisms to the same molecular descriptors. Different combinations of the 50 chemicals assayed in this study were found to act jointly by simple addition in several 2-, 8-, and 10-component mixtures. An approach was developed for predicting concentrations of components in mixtures that would cause 50% inhibition. The validity of this approach was demonstrated by comparing the predicted concentrations against those measured experimentally.

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