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Evaluation of the "Reference Toxicant" Addition Procedure for Testing the Toxicity of Environmental Samples

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Schaeffer (1987) proposed that the interpretability of toxicity tests made on environmental samples could be improved by using a "reference toxicant" addition procedure. The response curves for a "reference toxicant", designated **A**, are obtained for pure **A** and for **A** added at the same concentrations to a (uncharacterized) sample **B**. If statistical analysis of the slopes of the curves shows them to be parallel, the toxicity of **B** can be directly expressed in units of **A**. If the slopes are not parallel, the assay can be rerun using a dilution of **B** or a new reference toxicant **C**. The statistical test for parallel slopes is a direct test of the usual toxicological assumption of additivity. The context of work reported here was the development of a bioassay battery as an alternative to a "degree of hazard" toxicity scoring system proposed for the categorization of "special" industrial wastes (Reddy 1985). The bioassay battery used behavioral responses of the freshwater planarian *Dugesia dorotocephala* (Grebe and Schaeffer 1991a,b) and the Microtox test (Hankenson and Schaeffer 1991).

MATERIALS AND METHODS

Approximately 70 effluent and receiving stream water samples provided by the Illinois Environmental Protection Agency (IEPA). These samples had been stored by IEPA at 4 C for 4-8 weeks prior to our toxicity testing. Although storage could have affected the original toxicity of these samples, and hence their true degree of hazard, such changes would not affect the test development and demonstration goals of this research. Chemical and source data from IEPA's testing is given in Table 1 for the samples reported here.

The standard Microtox (Microbics Inc., Carlsbad, California) procedure (Microbics 1988) was followed except that reconstitution of the bacteria was accomplished by rapidly adding chilled reagent to a vial of bacteria maintained in ice on a vortex mixer. The vortex mixer increased the initial light output by a factor of 5-10. Bioluminescence intensity at 15 C was determined before (I_0) and 15 min after (I_t) the introduction of toxicants into the test medium. Defining the blank ratio as $BR = I_{t(b)}/I_{O(b)}$ and the reduction in light intensity for the sample as $\beta = I_t/[I_0\ (BR)]$, the light output is expressed as $\Gamma = (1-\beta)/\beta$; $\text{Log}_e\ \Gamma$ is a logit.

Samples which were not toxic in the standard Microtox assay were not

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tested further. Toxic samples were diluted to 50% and re-tested. Further dilutions were made as required to achieve a no-effect concentration. The dilution data were used to estimate an approximate EC50. Dilutions below or near the EC50 were then tested using additions of known concentrations of phenol. Some studies also used manganous sulfate, cadmium sulfate, or pentachlorophenol (PCP) as reference toxicants.

Table 1. Chemical Composition of Samples Provided by the Illinois Environmental Protection Agency

Sample	Type[1]	pH	Ca mg/L	Mg mg/L	K mg/L	Al µg/L	Ba µg/L	B µg/L	Cd µg/L	Cr µg/L	Cu µg/L	Fe mg/L	Pb µg/L	Mn µg/L	Ni µg/L
1234	EFFL	6.9						7100	7200	12	7	7	200	310	44
4508	STRM	7.3											5		
4470	STRM	5.9	107	50	5.9	80	73	275	10	10	5	89	50	3800	5
4879	LPWT	8.0	135	60	6.7	672	110	284	3	8	6	2.2	100	164	16
5248	LPWT	7.7	110	7	5	9514			3	23	36	19.2	50	4058	33
5301	AGRI	8.0													
5594	EFFL	7.4													
5831	EFFL	No analytical data													
5833	FACL	No analytical data													
5834	FACL	No analytical data													
5835	EFFL	8.2													
5836	FACL	No analytical data													
5837	FACL	No analytical data													
5855	EFFL	No analytical data													
8444	EFFL	8.0													
8566	EFFL	8.0													
8691	EFFL	8.1													
16571	LPWT	9.5													
17330	LPWT	1.3													

[1] AGRI = agricultural facility
EFFL = sewage treatment plant effluent
FACL = industrial facility
LPWT = land pollution waste treatment
SLDG = sewage treatment plant sludge
STRM = receiving stream

EC50's were computed using the trimmed logit method (Sanathanan et al. 1987). Reference (R) and sample (S) EC50s were compared using the upper fiducial limits (UL) of the EC50s (Litchfield and Wilcoxon (1949). Thus, if

$$H = \text{antilog}\,[(\log UL(R)/EC50(R))^2 + (\log UL(S)/EC50(S))^2]^{1/2}$$

is smaller than Z = (larger EC50)/(smaller EC50), the chances are that the EC50 values are from different populations. However, as explained next, a slopes were used to determine whether addition of the reference toxicant contributed additively or nonadditively to a sample's toxicity.

The response curve for the reference toxicant in each sample was compared to the curve for the reference toxicant alone using analysis of covariance. In Tables 2 and 3, the column "Equality of Slopes" is the test of the applicability of the covariance model. Samples marked "NS" had a slope for the reference toxicant which did not differ significantly from the slope for the pure reference toxicant. For these samples, the "Intercept" column is interpreted as showing whether the lines were coincident ("NS") and showed (e.g., Table 2 §2-1) or significantly separated but parallel (e.g., 25% Sample 1234+Phenol in Table 3). When the test for the equality of slopes is significant, intercept equality cannot be tested using analysis of covariance. A sample having a parallel slope and a significantly different intercept exhibits "strictly additive interaction" (Warren 1971, p. 208). A sample having a nonparallel slope can exhibit either supra-additivity (synergism or potentiation; e.g., Table 2 §2-2) or infra-additivity (antagonism; e.g., Table 2 §2-3) (Warren 1971, p. 209).

The equivalent toxicity, ET, is obtained from the coefficients of the regression model $\ln \Gamma = B_0 + B_1 \ln$ (Reference Toxicant Concentration) $+ B_2 D$. In this expression, B_0 is the average intercept, B_1 is the pooled slope, and B_2 is the displacement of the intercepts between, and D is a dummy variable which is assigned a value of 1 for the reference and 2 for the unknown. The equivalent toxicity is:

$$ET = \exp[(-b_0 - b_2)/b_1] - \exp[(-b_0 - 2\,b_2)/b_1].$$

For example, for 2% 16571 + phenol, b_0=-2.175, b_1=0.536, and b_2=0.343, so ET = exp[(2.175-0.343)/0.536)-exp[.175-2(0.343))/0.536] = 14.4 mg/L.

Dugesia dorotocephala were obtained from Carolina Biological Supply (Gladstone, Oregon) and maintained at 19 C in synthetic media (Kostelecky et al. 1989). Animals were fed once-weekly for 4 h on liver and then placed in fresh media. Routine behavioral tests (Kostelecky 1988) using reference toxicants (Grebe and Schaeffer 1990a,b) and environmental samples were carried out by adding 5 or 10 planarians to the undiluted sample and, when the sample was toxic, to dilutions of the sample with media. Behavioral responses were recorded at 1, 2, 3, 4, 5 10, 20, 30, 40, 50, and 60 min of exposure and were pooled into the 3 groups of 1-5 min, 10-30 min, and 40-60 minutes. Several distinct behavioral responses were scored. The principle analysis of toxicity combined separate responses into the categories of **Loc**omotive [(1) restlessness, (2) hyperkinesia, (5) swims upside down], **Morph**ological [(3) spiraling, (4) head/nose twist, (7) shape change, (16) ornamentation, (17) banana curl or coil], **Neuro**logical [(6) convulsions, (8) nervous signs], **Morb**idity [(12) labored movement, (13) depression, (14) unconsciousness, (15) death], and **Prot**ective [(9) pharynx protrusion, (10) vomiting, (11) mucus covering body, (18) lesions].

Three criteria (R1, R2, R3) were developed for evaluating the relative behavioral toxicity to Dugesia of the environmental samples given in the tables.

R1: Strong samples gave a response at 90% dilution. Moderate samples gave a response at 50% but not 90 % dilution. Weak samples gave a response only at 100% dilution. Not toxic (NT) samples gave no response at full strength.

R2: Results from tests at full strength were compared to simultaneous

tests with phenol. The rating is the concentration of phenol giving the closest set of responses to the sample. This rating is not an equality but is a subjective estimate that the sample was at least equivalent to the specified concentration of phenol but was not as toxic as the next higher concentration of phenol.

R3: Relative toxicity of a mixture of phenol+sample to sample alone. Sample concentrations tested were the highest (0%, 50%, or 90% dilution) producing no responses, or greatly reduced responses (as in the case of some of the lethal samples). A phenol concentration of 1 mg/L was used, which is the highest phenol concentration not showing an effect. All environmental samples were tested without the addition of phenol. Samples which were directly toxic were further tested by the addition of phenol per R3. Samples which were not directly toxic were randomly tested (1 in 5 selection) per R3.

RESULTS AND DISCUSSION

Tables 2 and 3 are organized into "sets" corresponding to a given reference toxicant standard curve. Each table gives the EC50 for the reference toxicant alone and in the designated sample. Table 2 shows samples that were not directly toxic in the Microtox test. Samples in group 1-1 did not enhance the toxicity of added phenols whereas those in group 1-2 did enhance the toxicity of added phenol. Samples in the group 1-3 inhibited the toxicity of added phenol. Table 3 shows samples that were directly toxic in the Microtox test and that enhanced the toxicity of added phenol, Cd^{2+}, Mn^{2+} or pentachlorophenol (PCP).

Tables 2 and 3 demonstrate the basic premise of the testing approach proposed by Schaeffer (1987). Some samples which were not directly toxic in the assays still contained components at concentrations which enhanced the toxicity of the added reference toxicant. The toxicity of the samples showed no apparent relationship to sample chemistry provided by IEPA, other than pH (<4 or >9).

The representative data in Tables 2 and 3, and other data, suggest that the planarian bioassay is comparable to the standard Microtox test in identifying toxic samples and in providing an estimate of the toxicity. The Microtox data also show that the "reference toxicant" procedure is not limited to a specific reference toxicant. We routinely use phenol, but other reference toxicants with different modes of toxic action or solubilities are used when appropriate.

The testing approach used here is an environmentally realistic approach for identifying, and quantifying, hidden toxicity. Table 2 shows that samples which were not directly toxic in the Microtox test could either substantially enhance or inhibit the toxicity of a reference toxicant. This is very important from an environment perspective because it shows that the directly measured toxicity of the sample could misrepresent its potential environmental toxicity. For example, the hidden equivalent toxicity of nontoxic samples usually exceeded an Illinois water quality standard of 1 mg/L phenol. The reference toxicant addition procedure is thus shown to improve assessment of the potential environmental hazard of chemically uncharacterized samples by expressing sample toxicity as the concentrations of toxicants for which a regulatory agency has established limits.

Table 2. Reference Toxicant Toxicity of Samples Not Directly Toxic in the Microtox Test

Sample[1]	EC50[2] (95% CI[2])	Slope	Equality of Slope	Equality of Intercept	Reference Equivalent[3]	R1	R2	R3
§2-1. Samples Showing No Additional Toxicity with Added Phenol								
Phenol	38.0 (23.0,62.7)	0.950						
4508	38.1 (27.1,53.5)	1.364	NS	NS	0	NT	NT	NT
5301	39.3 (29.2,52.8)	1.862	NS	NS	0	NT	--	NT
5470	29.9 (22.4,39.8)	1.193	NS	NS	0	NT	--	--
5594	32.2 (26.6,39.0)	2.422	NS	NS	0	NT	--	NT
Phenol	21.8 (16.1,29.5)	0.900						
5831	22.7 (17.9,28.7)	1.200	NS	NS	0	NT	--	--
5833	22.5 (17.4,29.1)	1.102	NS	NS	0	NT	--	yes
§2-2. Samples Showing Additional Toxicity with Added Phenol[4]								
Phenol	39.6 (24.5,64.0)	1.027						
5834	35.3 (28.5,43.7)	2.441	**	nC	nC	NT	--	--
5835	39.7 (29.8,52.9)	2.131	**	nC	nC	NT	--	--
5836	32.7 (27.0,39.6)	2.311	**	nC	nC	weak	2.06	yes
5837	36.7 (28.5,47.2)	1.927	**	nC	nC	NT	--	--
Phenol	38.0 (23.0,62.7)	0.950						
5855	35.1 (29.5,41.8)	3.226	**	nC	nC	weak	1.03	no
(Sample 16571) (Planarian Responses: Not tested.)								
Phenol	17.6 (13.5,22.9)	1.190						
2%	14.6 (12.0,17.8)	1.284	NS	**	14.4			
§2-3. Samples Masking Phenol Toxicity in the Microtox Test[4]								
Phenol	20.0 (15.8, 25.4)	1.015						
4879	24.9 (20.3, 30.6)	1.181	NS	**	-4.9	weak	--	--
5248	22.7 (18.3, 28.3)	1.109	NS	**	-2.6	mod	--	--
8444	25.6 (20.6, 31.9)	1.095	NS	**	-5.6	mod	--	--
8566	24.7 (20.1, 30.3)	1.145	NS	**	-4.7	NT	--	--
8691	26.3 (22.4, 30.8)	1.498	**	nC	nC	NT	--	--

Footnotes for Tables 1 and 2.

[1] Samples are 100% unless dilution (%) is denoted. Reference toxicant is mg/L.

[2] EC50 is mg/L phenol or stated reference toxicant; CI = Confidence Interval.

[3] Toxicity of sample expressed in mg/L of reference toxicant.
NS = P > 0.10; * = 0.05 < P ≤ 0.10; ** = P ≤ 0.05.
nC = Test for equality of intercepts was not Computed when slopes were not parallel.
NT = Not toxic in 1 h planarian test. # = Sample and reference EC50s differ at P ≤ 0.05.
Negative ET value implies inhibition by the sample.
Bracketed ET valued was computed assuming parallel slopes, so estimate may low.

[4] Increase or decrease in toxicity is based non-equality of slopes. See text.

Table 3. Reference Toxicant Toxicity of Samples Toxic in the Microtox Test

Sample[1]	EC50[2] (95% CI[2])	Slope	Equality of Slope	Equality of Intercept	Reference[3] Equivalent
(Sample 1234) (Planarian Responses: R1=Strong, R2=97.9, R3=Yes.)					
Phenol	34.9 (20.1,60.8)	1.139			
25%	24.6 (15.5,39.1)	0.900	NS	**	18.7
30%	15.8 (11.6,21.6)#	0.829	NS	**	28.8
35%	7.5 (4.9,11.5)#	0.603	**	nC	[43.1]
Cadmium	61.1 (51.2,72.9)	1.789			
10%	208 (79.4,546)#	1.620	NS	**	-132
15%	349 (60.4,2016)	0.887	**	nC	nC
20%	366 (31.2,4291)	0.508	**	nC	nC
PCP	0.84 (0.58,1.23)	1.148			
25%	0.90 (0.49,1.66)	0.681	**	nC	nC
30%	0.69 (0.37,1.32)	0.498	**	nC	nC
(Sample 17330) (Planarian Responses: R1=Strong, R2=97.9, R3=No.)					
Phenol	25.6 (15.5,42.4)	1.080			
0.5%	17.6 (13.1,23.7)	0.996	*	**	9.0
1.0%	15.3 (10.5,22.2)	0.703	**	nC	16
1.25%	9.1 (5.8,14.3)#	0.524	**	nC	24
Mn	39.1 (20.7,74.0)	1.253			
0.50%	30.7 (20.5,45.9)	1.674	NS	NS	0
1.00%	44.6 (20.8,95.9)	1.071	NS	NS	0
1.25%	83.9 (7.5,945)	0.180	**	nC	nC
Cd	79.0 (59.2,105.3)	1.508			
0.5%	80.3 (55.6,116.0)	1.162	NS	*	14.3
1.0%	37.9 (33.2,43.3)#	1.844	NS	**	36.0
1.4%	20.2 (17.2,23.9)#	2.398	NS	**	42.9
PCP	0.60 (0.44,0.80)	0.869			
1.4%	0.94 (0.37,2.37)	0.435	**	nC	nC
1.6%	0.48 (0.30,0.78)	0.464	NS	NS	0
2.0%	0.44 (0.24,0.81)	0.344	**	nC	nC

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