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Comparison of Three Microbial Toxicity Screening Tests with the Microtox Test

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A variety of test methods, criteria and procedures have been developed to assess the impact of chemical pollutants on aquatic biota. With the increasing awareness of the long term effects of many of those chemical pollutants, research efforts are being directed at short term bioassay tests, in an attempt to quickly alert dischargers as well as monitoring agencies of potential toxic conditions. One of the reasons for the time emphasis is that some effluents may be able to be stopped or contained for short periods for extra treatment, if necessary, but volume problems would make it unrealistic to attempt a 24-h much less a 96-h containment. Also by rapid assessment of changes in effluent quality, it may be possible to modify treatment before too great an environmental impact has occurred.

This report details our findings on the following microbiological acute toxicity screening tests; Microtox (developed by Beckman Instruments, Inc.), Spirillum volutans, (BOUDRE & KRIEG 1974). Pseudomonas fluorescens ATCC-13525 (based on the English standard NEN 6509-water-determination of the effect of toxic substances on the growth of a pure culture of bacteria) and Aeromonas hydrophila ATCC 23213, a typical water bacterium (using the P. fluorescens procedure).

MATERIALS AND METHODS

Chemicals: The following chemicals were tested at pH 6.7 (as the sensitivity and stability of the Microtox procedure is based on testing samples where the pH is close to 6.7): Hg^{++} ($HgCl_2$), Zn^{++} ($ZnSO_4 \cdot 7H_2O$), Cu^{++} ($CuSO_4$), Pb^{++} ($PbCl_2$), Ni^{++} ($NiCl_2 \cdot 6H_2O$), α-naphthol, sodium lauryl sulfate, sodium cyanide, 3,3'-dichlorobenzidine, phenol, N-nitrosodiethylamine, dichloromethane, and nitrotriacetic acid.

Toxicity Tests: The Microtox test was performed using the Microtox reagent and following the procedure detailed in the Beckman Instruments Interim Manual No. 110679B-9-80 with a 15 min incubation time. Spirillum volutans, a large aquatic bacterium with a rotating fascicle of flagella at each pole, was used to test the samples for toxicity, following a modification of the procedure developed in 1974 by BOWDRE & KRIEG (DUTKA 1978). P. fluorescens was inocculated into 100 mL of nutrient broth and incubated at 37°C for 16 to 18 h on a rotary shaker (100 rpm). Fifteen mL of

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this culture was inocculated into 1 L of nutrient broth in a 2-L Erlenmeyer flask which was constantly mixed with a stirring bar. This was the test inocculum which was dispensed into test flasks within 30 min. All chemicals spanned a minimum 4 log concentration gradient encompassing negative and positive effects. To test each chemical, 25 mL of sample was combined with 25 mL of cell inocculum in a 125-mL Erlenmyer flask, swirled to thoroughly mix and 5 mL of the sample was removed for optical density determinations for time 0. The flask was then placed on a 100 rpm rotary shaker for 18-h at 37°C. At the end of the incubation period, 5 mL aliquots were once again removed and tested for optical density (650 nm). All sample concentrations were tested in duplicate. Uninocculated media were used as a negative control and potassium dichromate (0.01 to 50 ppm) was the positive control. The data were graphed and EC_{50} values established. Similar procedures were followed using a culture of Aeromonas hydrophila, ATCC 23213.

RESULTS AND DISCUSSION

The great variation in sensitivity patterns of the four microbial toxicity screening procedures to the selected chemicals are shown in Table 1. Some of the chemicals have toxicity end point concentrations 100 to 1000 times greater from one test system to the next, e.g., the EC_{50} of Zn^{++} is 3.5 ppm in the Microtox system and 370 ppm in the P. fluorescens test, and sodium lauryl sulfate 1.8 ppm in the Microtox system and 3700 ppm in the A. hydrophila tests. With the exception of Hg^{++}, Ni^{++}, Pb^{++} and N-Nitrosodiethylamine the 15 min Microtox procedure (30 to 45 min total test) was the most sensitive to the chemicals tested with the P. fluorescens (20 h total test) procedure being the next most sensitive. In no instance were the 2-h Spirillum volutans test and the A. hydrophila toxicity tests the most sensitive to the chemicals being tested.

Table 1 data clearly illustrate the advantages (speed and sensitivity) of using the Microtox test in toxicity screening procedures. Table 1 is also very supportive of the battery approach for toxicity screening as each procedure is shown to react to different levels of chemicals.

The Microtox toxicity testing procedure, which is now undergoing a very thorough review by many laboratories, has shown to have some problems with reproducibility. For instance, in Table 2, six toxicants are compared with only two substrates (Hg^{++} and phenol) showing similar results by two laboratories (Beckman Instruments, Inc., and ours). Also, in a study (reported by Beckman Instruments, Inc.) using sodium pentachlorophenate, 30 separate assays were performed using 30 separate vials of Microtox reagent and recording 5 min EC_{50} and 15 min EC_{50} results, it was found: (a) 5 min EC_{50} concentrations varied from 0.57 to 0.38 ppm (65%); (b) the 15 min EC_{50} concentrations varied from 0.43 to 0.28 ppm (65%); and (c) the ratio's between 5 and 15 min EC_{50} readings varied from 1.3 to 1.5.

Table 1. Sensitivity of Four Acute Toxicity Screening Procedures to Various Chemicals

	Concentration in ppm to Give Typical Endpoint Reaction to Toxicants							
	15-min Microtox EC_{50}		120-min *S. volutans*		18 hour *P. fluorescens*		18 hour *A. hydrophila*	
Hg^{++} (Mercury Chloride)	0.046		0.2	b	0.031	a	0.049	
Zn^{++} (Zinc Sulfate)	3.5	a	12		360		500	b
Cu^{++} (Copper Sulfate)	3.8	a	10		17		21	b
Pb^{++} (Lead Chloride)	30		40		14	a	710	b
Ni^{++} (Nickel Chloride)	23		20	b	8.7	a	17	
α Naphthol	3.8	a	10		>100.	b	>100	b
Sodium Lauryl Sulfate	1.8	a	43		1700		3700	b
Sodium Cyanide pH 6.7	2.8	a	83		14		25	b
3,3 Dichlorobenzidine	0.058	a	16		>100	b	>100	b
Phenol	34	a	300		880		1600	b
N-Nitrosodiethylamine	140	a	570		>5000	b	>5000	b
Dichloromethane	>1000		>1600		>10000		>10000	b
Nitrotriacetic Acid	>1000		> 800		720	a	1500	b

a = most sensitive; b = most resistant

Table 2. Comparison of Microtox EC_{50} Values Obtained in Three Laboratories

	5-Minute EC50 (ppm)		
Toxicant	BULICH et al.*	Duluth EPA**	DUTKA-KWAN
Hg^{++}	0.065	0.052	0.064
Sodium lauryl sulfate	1.6	-	3.2
Zn^{++}	2.5	52	14
Cu^{++}	8.0	15	20
Phenol	25	41	28
Ethanol	31,000	57,000	-

* A.A. Bulich, M.W. Greene and D.L. Isenberg (1980).
** Data produced by Carolann Curtis, U.S. EPA, Environmental Research Laboratory, Duluth, MN. 55804

Thus, there is a problem of reproducibility of data within one laboratory, and also between laboratories (Table 2), probably related to variations in the cell suspension. Another example of this type of reproducibility problem is shown with EC_{50} values for ethanol. The 5 min EC50 value obtained for ethanol by the Beckman Instruments Laboratory was 31,000 ppm (BULICH et al. 1980) and by CHANG et al. (1981), 47,000 ppm. However, LC_{50} values in fish toxicity tests are also known to show similar reproducibility problems.

Reviewing the data from the four toxicity assessing techniques, it is obvious that each procedure has its own toxicity sensitivity pattern and cannot be readily correlated with the other procedures. There are areas of concurrence as well as areas of wide divergence in sensitivity to toxicants. Table 3 contains data from RYSSOV-NIELSEN's (1975) study which used TTC-dehydrogenase and short term Warburg tests for assessing toxicity and illustrates the variety of substrate concentrations that ellicit typical end points.

Similarly, in a U.S. EPA sponsored project (EPA Quality Assurance Newsletter, Vol. 4:2, April, 1981) of an effluent study comparing 24-h fathead minnow and Daphnia pulex LC_{50} tests with the 5 min Microtox test, it was found that Microtox indicated the presence of toxicity in 81% of the effluents that were toxic to the fathead minnows. The Microtox test indicated the presence of only 62% of the samples which were toxic to D. pulex. From the above, it would appear there is some overlap or "correct" guesses where all systems indicate a positive effect, but no system was able to predict the 100% presence of toxicant to another species.

From Tables 1 and 2 and the above EPA sponsored data, it is very obvious that no single biological testing procedure can predict the presence of all toxicants which might effect aquatic organisms or be eventually bioaccumulated and affect their predators or man.

In spite of the above, there is no doubt that the Microtox system is a sensitive toxicity assaying procedure which has as its major

TABLE 3. Comparison of Toxicity of Selected Chemicals by Six Microbial Toxicity Testing Techniques

Toxic Substrate	[1]TCC 50%* Inhibition Test (ppm)	[1]Warburg 50%* Inhibition Test (ppm)	Microtox 15-min EC_{50} (ppm)
Zn	26	1.4	3.5
Hg	1.5	0.6	0.046
Cn	0.47	4.7	2.8
	S. volutans 90% Inhibition 2-hour (ppm)	*P. fluorescens* EC_{50} (ppm)	*A. hydrophila* EC_{50} (ppm)
Zn	12	370	500
Hg	0.2	0.031	0.049
Cn	83	14	25

[1]RYSSOV-NIELSEN (1975).

benefit, a quick turnaround time which makes it an ideal member of a battery of screening tests. Used alone, we believe the Microtox system may have its most useful application in the monitoring of a supposedly consistent effluent stream. Thus, any deviations from the established norm could be easily and quickly noted and rectified. One major drawback of the Microtox test maybe its inability to test some samples at their natural pH.

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