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COMPARISON OF THE SENSITIVITY OF FOUR MICROBIOLOGICAL PROCEDURES USED IN TOXICITY SCREENING TESTS

by

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ABSTRACT

This report details invetigations, findings and recommendations about the following microbiological acute toxicity screening tests: Microtox, Spirillum volutans, Pseudomonas fluorescens and Aeromonas hydrophila. In the single substrate-toxicant study, the Microtox test was the most sensitive procedure followed by the Pseudomonas aeruginoas EC_{50} density test.

Data from single and mixed toxicant studies imply that it is unwise to try to assess the presence of toxicants in waters or effluents by a single species test. The battery approach encompassing two or three genera and involving two to four species is recommended, to more thoroughly assess the potential presence of toxicants. The Spirillum volutans and Pseuodomonas fluorescens tests are recommended as potential candidates for the battery approach to toxicity testing. The Spirillum volutans test is recomended for inclusion in a battery approach over the Microtox procedure because of its ability to test samples over a wide pH range. The Microtox procedure requires all samples to be pH adjusted (approximately 6.7) and thus the toxicity of a sample may be influenced by this pH adjustment.

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EXECUTIVE SUMMARY

A variety of test methods and criteria have been developed to assess the impact of chemical pollutants on aquatic biota. With increasing awareness of the long term effects of many of these chemical pollutants, research efforts are being directed at short term bioassay screening tests, the majority of which are unstandardized microbial tests. To try to bring some standardization to toxicity screening procedures used by Canadian governmental laboratories, a study was undertaken to evaluate the following four microbiological acute toxicity screening tests using single and mixed chemical solutions; Microtox, Spirillum volutans, Pseudomonas fluorescens and Aeromonas hydrophila.

One of the major findings of the study was that it is dangerous or unwise to try and assess the presence of toxicants in waters or effluents by a single species test. The battery approach, encompassing two or three genera and involving two to four species is

recommended to more thoroughly assess the potential presence of toxicants. The Spirillum volutans (2 hr) and the 18 hr Pseudomonas fluorescens EC_{50} density tests are recommended as potential candidates for the battery approach to toxicity testing. The Spirillum volutans test is recommended for inclusion in a battery approach over the Microtox procedure because of its ability to test samples over a wide pH range.

INTRODUCTION

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 these 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. The majority of short term bioassay tests are bacterial based and vary from laboratory to laboratory. As there are, as yet, no internationally standardized bacterial procedures for testing aquatic environmental samples for toxicity, a wide variety of tests are used. These tests vary from commercially available procedures (Microtox - Beckman Instruments, Inc.) to procedures adapted from growth inhibition and stimulation tests as well as specialized procedures based on the research interest of the laboratory or scientist. The time required to perform these bacterial aquatic acute toxicity assays range from 30 minutes to 24 hours.

As industrial effluent pollutants and also such toxicants as herbicides, pesticides, fertilizers, car emissions etc., affect aquatic biological systems at different levels and in a variety of ways, it is acknowledged that the battery approach utilizing several different short term biological indicators would be preferred in any monitoring scheme. At present, the most commonly used aquatic

toxicity testing systems are the 24 or 96-hour reactions of larval or fingerling fish in static or flow through systems and the 24-hour EC Daphnia immobilization test. However, present technological needs are for indicator systems which can assess the toxicant levels in effluents or samples in as short a time frame as possible. One of the reasons for this time emphasis is that some effluents may be able to be stopped or contained for short periods for the extra treatment, if necessary, but volume problems would make it unrealistic to attempt a 24-hour, much less a 96-hour, 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.

The microbiology laboratories at the National Water Research Institute have investigated the use of, and potential of, several microbiological acute testing procedures whose initial result time varies from 30 minutes to 18 hours.

This report details our investigations, findings, and recommendations about the following microbiological acute toxicity screening tests; Microtox (developed by Beckman Instruments, Inc.), Spirillum volutans (Bowdre and 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 Pseudomonas fluorescens procedure).

METHODS

Microtox Test

Beckman Instruments, Inc. have devised a test for acute toxicants in water in which specialized strains of luminescent bacteria (Photobacterium phosphoreum) are used as the bioassay organism. This test is functional because the metabolism of the luminescent bacteria is influenced by low levels of toxicants and, occasionally, stimulants. Any alteration of metabolism affects the intensity of the organisms' light output. By sensing these changes in light output, the presence and relative concentration of toxicants can be obtained by establishing the EC_5 levels from graphed data; EC being, in this case, that concentration of toxicant (or dilution of unknown) causing a 50% reduction in light from the base level (Beckman Instruments, Inc., 1980). Bascially, the test involves the addition of luminescent bacteria into a vial of precooled dilutent solution (15°C) and allowed to stabilize. Approximately 15 minutes later, a measured amount of sample or sample dilution is added to this vial, which is then transferred to a light-tight turret where it is exposed to a photomultiplier tube. Total light output is read, usually over a 5, 10 or 15-minute period, from a digital panel meter attached to an accessory recorder. By testing several concentrations of sample, an EC_{50} can be established. Microtox reagent lot No. M006009 was used almost exclusively.

Spirillum volutans Test

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 and Krieg (Dutka, 1978). The procedure involved pipetting 0.1 mL of Defined Test Medium into 13 x 100 mm tubes, and adding 0.8 mL of the sample plus 0.1 mL of healthy bacteria from an overnight culture. The tube is swirled, a drop removed via Pasteur pipette and placed on a slide and quickly examined under a darkfield or phase contrast microscope (125X). This is the 0-minute reading. Samples from the tube were examined at 5, 10, 30, 60, 90 and 120-minute intervals. If, during any one examination, the reversing motility had been eliminated in more than 90% of the cells, a positive toxic effect was recorded. Negative controls in distilled water were routinely used to ensure inhibition was due to the tested sample. Positive controls were used only to verify percentages of lost motility in doubtful reactions.

Pseudomonas fluorescens and Aeromonas hydrophila Growth Inhibition

Inhibition Tests

Pseudomonas fluorescens ATCC strain 13525 was inoculated into 100 mL of nutrient broth (Oxoid Lab Lemco) and incubated at 37°

for 16 to 18 hours on a rotary shaker (100 rpm). Fifteen mL of this culture was inoculated into 1 L of nutrient broth in a 2 L Erlenmyer flask which was constantly mixed with a stirring bar. This was the test inoculum which was dispensed into test flasks within 30 minutes.

All chemical tests spanned a minimum 4 log concentration gradient encompassing negative and positive effects. To test each sample, 25 mL of sample was combined with 25 mL of cell inoculum 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 hours at 37°C. At the end of the incubation period, 5 mL aliquots were once again removed and tested for optical density in a Spectronic 20 set at 650 nm. All samples were tested in duplicate, with a minimum of five dilutions spanning the 4 log concentration gradient. Distilled water was used as the negative control and potassium dichromate (0.01 to 50.0 ppm) in five concentrations was the positive control. The data were graphed, concentration versus density readings and EC_{50} values (concentration of chemical required to decrease optical density values 50% from the negative effect values) established. The results may also be reported in potassium dichromate equivalents, i.e., that concentration of chemical equivalent to concentration of potassium dichromate which produces a 50% decrease in optical density within 24 hours. This method of reporting was not followed in this study.

Similar procedures were followed using a culture of Aeromonas hydrophila ATCC #23213.

Chemicals Tested

The following chemicals alone, or in combination, were used to compare the sensitivities of the four microbiological procedures. All chemicals, unless otherwise noted, were brought to 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^{++} ($HgCl_2$), Cu^{++} ($CuSO_4$), (Zn^{++} $ZnSO_4 \cdot 7H_2O$), Pb^{++} ($PbCl_2$), Ni^{++} ($NiCl_2 \cdot 6H_2O$) Al^{+++} ($Al_2(SO_4)_3 \cdot 16H_2O$) ($Al(NO_3)_3 \cdot 9H_2O$) ($AlCl_3 \cdot 6H_2O$), arsenite ($Na_2AsO_2$), arsenate ($Na_2HAsO_4 \cdot 7H_2O$), cetyl trimethyl ammonium chloride, 3,5 dichlorophenol, α-naphthol, sodium lauryl sulfate, 2,4 dichlorophenoxyacetic acid, CN-(NaCN), 3,3 dichlorobenzidine, phenol, N-nitrosodiethylamine, dichloromethane, nitrotriacetic acid, 1-bromo-2 chloroethane.

RESULTS

Initially, during this study, the Microtox test was performed using a five-minute incubation-contact step, i.e., the bacterium, Photobacterium phosphoreum, and sample were mixed and

incubated in the cuvette incubator wells for five minutes before the first readings of light level are made in the reaction chamber. However, it soon became apparent that the readings obtained after the five-minute incubation time were often very difficult to reproduce because of the continuing action between sample and cells. Thus, ten and fifteen minute incubation readings were added to the procedure in hopes of achieving a more stable, reproducible set of results.

Table 1 presents a summary of all the chemicals tested for their acute toxicity effects as measured by the 5, 10 and 15-minute incubation Microtox test and the two-hour Spirillum volutans test. From Table 1 it can be seen that the majority of chemicals tested via the Microtox procedure have an increasing toxic effect with increased incubation (contact), while others such as Al^{+++} (pH 3.3) and 3,5 dichlorophenol were fairly stable in their toxicity effect over the 15-minute contact period. A few of the organic chemicals tested (i.e., phenol and 3,3-dichlorobenzidine) decreased in toxicity over the 15-minute contact period.

In Table 1, it can be seen that the Microtox procedure is much more sensitive to the tested chemicals than the two-hour Spirillum volutans test.

TABLE 1. COMPARISON OF THE SENSITIVITY OF THE MICROTOX AND SPIRILLUM VOLUTANS TEST TO ASSESS THE TOXICITY OF VARIOUS CHEMICALS

Chemical	Concentration in ppm to Give Typical Endpoint Reaction to Toxicants			
	Microtox EC_{50}			Spirillum volutans
	5-min Incubation	10-min Incubation	15-min Incubation	120-min Incubation
Hg^{++} (Mercury Chloride)	0.064	0.049	0.046	0.2
Al^{+++} (Aluminum Nitrate pH 3.3)	1.6	1.65	1.6	10.0
Al^{+++} (Aluminum Nitrate pH 4.5)	2.9	2.7	2.6	20.0
Zn^{++} (Zinc Sulfate)	13.8	6.1	3.45	11.6
Cu^{++} (Copper Sulfate)	19.5	9.4	3.8	10.0
Pb^{++} (Lead Chloride)	31.5	29.9	30.1	40.0
Ni^{++} (Nickel Chloride)	155.0	59.0	23.0	20.0
Arsenite (Na Salt)	202.0	119.0	103.0	20.6
Arsenite (Na Salt)	491.0	132.0	89.0	98.0
Cetyltrimethylammonium chloride	1.15	0.98	0.86	1.45
3,5 Dichlorophenol	3.2	3.0	2.9	5.0
α Naphthol	4.15	3.9	3.8	10.0
Sodium Lauryl Sulfate	3.19	2.1	1.8	43.0
2,4 Dichlorophenoxyacetic Acid	28.0	20.0	16.8	95.0
Sodium Cyanide pH 6.7	16.0	3.9	2.76	83.0
Sodium Cyanide pH 10.2	16.0	6.1	3.0	33.0
3,3 Dichlorobenzidine	0.442	0.051	0.058	16.0
Phenol	28.0	31.9	34.3	300.0
N-Nitrosodiethylamine	107.0	124.0	138.0	570.0
Dichloromethane	1000	1000	1000	neg at 1600
Nitrotriacetic Acid	1000	1000	1000	net at 800
1-Bromo-2-Chloride	270	270	270	300.0

The sensitivity of the four acute toxicity screening procedures to selected chemicals are shown in Table 2. Here it can be seen that the tests show a wide variety of sensitivities, i.e., 1.8 ppm sodium lauryl sulfate in the Microtox test and 3690 ppm sodium lauryl sulfate in the Aeromonas hydrophila procedure, with the 15-minute Microtox procedure usually being the most sensitive with the exception of Hg^{+++}, Ni^{+}, and Pb^{++} and N-Nitrosodiethylamine where the Pseudomonasfluorescens procedure was the most sensitive. Variations in the sensitivity levels of the four toxicity testing procedures to the chemicals tested at pH 6.7, indicated, with the exception of Cu^{++} and Ni^{++}, that sensitivity to toxicity ranged from 10 to more than 1000 times the most sensitive procedure. In no instance were the two-hour Spirillum volutans test and the Aeromonas hydrophila toxicity tests the most sensitive to the chemicals being tested.

Aluminium which, next to silicon and oxygen, is the commonest constituent on the earth's crust (7 to 8%) and functions to bind metals and compounds together into soil and rock, plays an important role in the buffering mechanisms of land and water subjected to acid rain.

In areas where acid rains have inundated the buffering capacity of soils and lakes; increased concentrations of aluminum are found in the rivers and lakes. It has been reported that trace amounts of aluminum in pH 5 waters are harmful to fish (Howard and

TABLE 2. SENSITIVITY OF FOUR ACUTE TOXICITY SCREENING PROCEDURES TO VARIOUS CHEMICALS

Chemical	Concentration in ppm to Give Typical Endpoint Reaction to Toxicants							
	5-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)	3.45	a	11.6		367.0		500.0	b
Cu^{++} (Copper Sulfate)	3.8	a	10.0		16.75		21.25	b
Pb^{++} (Lead Chloride)	30.2		40.0		13.8	a	705.0	b
Ni^{++} (Nickel Chloride)	23.0		20.0	b	8.7	a	16.80	
α Naphthol	3.8	a	10.0		100.0	b	100	b
Sodium Lauryl Sulfate	1.8	a	43.0		1650.0		3690.0	b
Sodium Cyanide pH 6.7	2.76	a	83.0		14.0		25.25	b
3,3 Dichlorobenzidine	0.058	a	16.0		100	b	100	b
Phenol	34.3	a	300.0		875.0		1620.0	b
N-Nitrosodiethylamine	138.0	a	570.0		5000	b	5000	b
Dichloromethane	1000		1600		10000		10000	b
Nitrotriacetic Acid	1000		800		719.0	a	1500.0	b

a = most sensitive.
b = most resistant.

Perely, 1980), therefore studies were carried out to assess the sensitivities of the four testing procedures to Al^{+++} in three pH ranges.

The results of these studies are tabulated in Tables 3 and 4. In Table 3, the three aluminium salts tested show similar patterns when tested by the Microtox procedure, with toxicity increasing with lower pH values, but not increasing significantly with increased incubation. The two-hour Spirillum volutans test also shows that there is increased toxicity with lowering pH. The Spirillum test is less sensitive than the 15-minute Microtox test to Al^{+++}.

Table 4 data presents the results of the four acute toxicity testing procedures when challenged by Al^{+++} from $AlCl_3$ at three pH levels, 6.7, 4.5 and 3.5. All four procedures were most sensitive to Al^{+++} in pH 3.5 water and the 15-minute Microtox procedure is the most sensitive while the Pseudomonas fluorescens and Aeromonas hydrophila optical density tests were the least sensitive. The pH control tests indicated that Spirillum volutans are not affected by pH 4.5 and 3.5 solutions while the Microtox test is extremely sensitive to pH 3.5 and slightly sensitive to pH 4.5 and solutions of Pseudomonas fluorescens and Aeromonas hydrophila were not affected by control samples of pH 3.5 and 4.5.

TABLE 3. COMPARISON OF THE SENSITIVITY OF THE MICROTOX AND *SPIRILLUM VOLUTANS* TEST TO ALUMINIUM SALTS AT 3 pH REGIMES

		Concentration in ppm to Give Typical Endpoint Reaction to Toxicants			
		Microtox EC_{50}			*Spirillum volutans*
		5-min Incubation	10-min Incubation	15-min Incubation	120-min Incubation
$AlCl_3 \cdot 6H_2O$	pH 6.7	10	10	10	70
$AlCl_3 \cdot 6H_2O$	pH 4.5	2.4	2.1	2.06	15
$AlCl_3 \cdot 6H_2O$	pH 3.5	1.98	1.99	1.99	4.6
$Al_2(SO_4)_3 \cdot 16H_2O$	pH 6.7	10	10	10	10
$Al_2(SO_4)_3 \cdot 16H_2O$	pH 4.5	2.03	2.05	2.06	10
$Al_2(SO_4)_3 \cdot 16H_2O$	pH 3.5	1.57	1.47	1.51	16
$Al(NO_3)_3 \cdot 9H_2O$	pH 6.7	20	20	20	40
$Al(NO_3)_3 \cdot 9H_2O$	pH 4.5	2.9	2.7	2.6	20
$Al(NO_3)_3 \cdot 9H_2O$	pH 3.5	1.6	1.65	1.6	10

TABLE 4. COMPARISON OF THE SENSITIVITY OF THE FOUR TESTING PROCEDURES TO Al^{+++} IN THE FORM OF $AlCl_3 \cdot 6H_2O$

Chemical and pH	Concentration in ppm to Give Typical Endpoint Reaction to Toxicants			
	Microtox EC_{50} 15-min Inc.	*S. volutans* 120-min Inc.	EC_{50} *P. fluorescens* 18-hour Inc.	EC_{50} *A. hydrophila* 18-hour Inc.
Al^{+++} pH 6.7	10	70	100	100
Al^{+++} pH 4.5	2.06	15	162.5	320
Al^{+++} pH 3.5	1.99	4.6	34.25	27.5
Distilled water pH 6.7	Negative	Negative	Negative	Negative
Distilled water pH 4.5	12-20% light inhibition	Negative	Negative	Negative
Distilled water pH 3.5	100% light inhibition	Negative	Negative	Negative

When assessing the acute toxicity of natural samples, one is aware that the sample is a mixture or solution of one or many chemicals which may be (a) antagonistic to each other thus perhaps decreasing the toxicity effect compared to the effect of a single chemical in the sample, (b) synergistic, so that there is an increase or magnification of the toxic effects of one or more of the chemicals in the sample, or (c) neutral, so that the effect noted, if any, is due to the action of one chemical. Thus, when testing a natural water sample or effluent, the toxic effect seen, if any, could be a, b, or c, and invariably the tester never knows the active chemical(s) which is (are) producing the effect, unless the chemical is so concentrated that its effect blurs all other effects, i.e., caustic soda spilled into a small creek or a chlorine mixture applied to sewage effluent.

To try and assess the effects of mixtures of toxic chemicals on acute toxicity screening systems, two types of studies were carried out using the Microtox system; one to evaluate the sensitivity of the Microtox system, and the other to observe the various interactions between some toxic chemicals.

In one series of tests, chemicals were mixed and incubated with the Microtox reagent and their inhibitory effect was noted and recorded as percent light inhibition after 5, 10 and 15 minutes incubation. These results were then compared to the individual chemicals making up the mixture. Some of these data are presented in

Table 5. In the other series of tests, specific chemicals were considered key chemicals and were tested in a variety of concentrations to establish EC_{50} values by the Microtox system. Then, to the various concentrations, consistent concentrations of one or more chemicals were added, tested by the Microtox procedure with 5, 10 or 15-minute incubation, and the results recorded as new EC_{50} values of the key chemical. These data are presented in Table 6.

In the first study, recorded in Table 5, different concentrations of phenol were combined with the same concentrations of sodium lauryl sulfate. From the controls of this study, it can be seen that phenol, in the concentrations used, has a decreasing toxic effect with increased incubation (contact) time, while the toxic effect produced by sodium lauryl sulfate appears stable over the 15-minute incubation period. However, combining the two chemicals produces an increasing toxic effect with increased incubation with the 20 ppm phenol combination appearing to have a direct additive effect, while the 2.5 ppm phenol plus 0.5 ppm sodium lauryl sulfate complex appear to produce a magnified toxicity effect in the Microtox system. Thus, the concentration of individual chemicals in a solution or mix greatly affect the toxicity level shown by the Microtox test.

TABLE 5. COMPARISON OF ADDITIVE EFFECTS OF CHEMICALS ON MICROTOX TEST

Chemical ppm	Percentage Light Inhibition		
	Incubation		
	5-min	10-min	15-min
20 phenol + 0.5 sodium lauryl sulfate	59.1	63.2	66.5
2.5 phenol + 0.5 sodium lauryl sulfate	45.9	57.1	64.2
20.0 phenol	40.6	35.5	35.1
2.5 phenol	4.2	2.4	1.3
0.5 sodium lauryl sulfate	21.5	21.6	22.0
2.5 sodium cyanide + 0.5 sodium lauryl sulfate	17.5	21.3	40.5
2.5 sodium cyanide	18.2	22.4	41.6
0.5 sodium lauryl sulfate	21.5	21.6	22.0
10.0 Cu^{++} + 0.0025 Hg^{++} + 2.0 Z^{++}	38.4	71.6	88.8
2.5 Cu^{++} + 0.0025 Hg^{++} + 2.0 Zn^{++}	10.7	34.4	58.5
10 Cu^{++}	3.7	63.8	83.9
2.5 Cu^{++}	5.3	14.4	36.3
0.0025 Hg^{++}	1.9	6.8	8.5
2 Zn^{++}	3.3	11.0	13.6
0.005 Hg^{++} + 5 Pb^{++}	18.7	25.3	20.2
0.075 Hg^{++} + 5 Pb^{++}	54.0	84.1	94.5
0.005 Hg^{++}	32.2	37.9	35.6
0.075 Hg^{++}	63.8	92.6	96.1
5.0 Pb^{++}	18.7	17.4	17.2
0.005 Hg^{++} + 5.0 Ni^{++} + 5.0 Pb^{++}	26.7	29.8	30.0
0.075 Hg^{++} + 5.0 Ni^{++} + 5.0 Pb^{++}	57.8	85.6	95.5
0.005 Hg^{++}	32.2	37.9	35.6
0.075 Hg^{++}	63.8	92.6	96.1
5.0 Ni^{++}	17.9	18.5	25.9
5.0 Pb^{++}	18.7	17.4	17.2

In comparison, the sodium cyanide and sodium lauryl sulfate mixture does not produce an enhanced or antagonistic toxic effect, instead a neutral type effect is seen. The percent light inhibition pattern for the NaCN and sodium lauryl sulfate complex is almost a direct replica of the NaCN control. Thus, in one instance, a chemical (sodium lauryl sulfate) can act synergestically and in another neutrally. However, in the case of phenol, sodium lauryl sulfate may enhance the solubility of phenol thus increasing its activity.

In the study with 10.0 ppm Cu^{++} plus 0.0025 ppm Hg^{++} and 2.0 ppm Zn^{++}, a slight additive to neutral toxicity effect can be seen; however, when 2.5 ppm Cu^{++} is used there is a strong additive toxicity effect which, after 15 minutes incubation (contact) with the Microtox reagent, is much greater (in percentage light inhibited) than the sum of the individual chemical, toxicity effects. This example again illustrates the importance of toxicant concentration and how lower concentrations of toxicants in combination have toxicity effects much greater than suspected when assumed toxicity effects are based on the toxicity pattern of complexes made up of higher concentrations of metals.

The Hg^{++} and Pb^{++} combination shown in Table 5 are a good example of a strong antagonistic effect by Pb^{++} to the toxicity of Hg^{++} at the lower Hg^{++} concentrations. At the higher Hg^{++} concentration (0.075 ppm) there is still a slight antagonistic to

neutral effect by Pb^{++} to Hg^{++}, even after 15 minutes incubation. The concentration of individual chemicals within a complex are again shown to play an important role in the displayed toxic effect (inhibition of light). The addition of 5.0 ppm Ni^{++} to the above combination slightly increases the toxic effect of the complex, but it is still well below the toxicity effect of 0.0025 ppm Hg^{++} alone. A similar additive effect is noted with the higher concentration of Hg^{++} (0.075 ppm) and the complex toxicity reaction after 15 minutes is similar to that produced by Hg^{++} alone. Thus, in these studies it can be seen that the original concentration of chemicals is very important to the toxicity level exhibited by the complex at pH 6.7. Furthermore, the total toxic effect varies greatly from a "simple" addition of parts to a magnification where the whole effect is greater than the parts and to the other extreme where there is obvious antagonism or neutralization of toxic effects.

In Table 6, which presents some of Table 5 data in another form, Zn^{++}, which is the first chemical illustrated, follows the typical pattern of increased toxicity with increased incubation with the Microtox reagent with its EC_{50} falling from 13.8 to 3.45 ppm. The Zn^{++} plus Hg^{++} combination was the most toxic Zn complex tested, and with the addition of Pb^{++} to this complex, the toxicity was slightly neutralized, even though when Pb^{++} is added to Zn^{++}, it enhances the toxicity of Zn decreasing the 15-minute EC_{50} from 3.45 ppm to 2.3 ppm.

TABLE 6. COMPARISON OF EFFECTS OF COMPLEXES ON MICROTOX EC_{50} CONCENTRATIONS EXPRESSED AS ppm OF KEY COMPARED CHEMICAL

Chemical ppm	EC_{50} ppm		
	5-minute Incubation	10-minute Incubation	15-minute Incubation
*Zn^{++} + 5.0 Pb^{++}	9.4 Zn	3.85 Zn	2.3 Zn
Zn^{++} + 0.025 Hg^{++}	5.7	3.0	2.15
Zn^{++} + 5.0 Pb^{++} + 0.025 Hg^{++}	6.1	3.38	2.6
Zn^{++} + 1.25 3,5 Dichlorophenol	7.5	3.65	2.65
Zn^{++}	13.8	6.1	3.45
*Cu^{++} + 0.025 Hg^{++}	15.4 Cu	5.3 Cu	2.2 Cu
Cu^{++} + 0.025 Hg^{++} + 2.0 Zn^{++}	17.0	5.7	1.6
Cu^{++}	19.5	9.4	3.8
*Hg^{++} + 5.0 Pb^{++}	0.070 Hg	0.052 Hg	0.05 Hg
Hg^{++} + 5.0 Pb^{++} + 5 Ni^{++}	0.066	0.051	0.0385
Hg^{++}	0.064	0.049	0.046
*Ni^{++} + 10.0 Pb^{++}	235 Ni	95 Ni	59 Ni
Ni^{++} + 0.025 sodium lauryl sulfate	122	45	34
Ni^{++}	155	59	23
*Phenol + 0.5 sodium lauryl sulfate	6.9phenol	3.7phenol	0.25phenol
Phenol	28	31.9	34.3

* Key chemical

In the next series, it can be seen that the Hg^{++} increases the toxicity effects of Cu^{++}. This study illustrates a curious reaction which was noted with other samples in that for the first 10 minutes of incubation, the Cu^{++} plus Hg^{++} complex was the most toxic, but after 15 minutes of contact with the Microtox reagent, the Cu^{++} Hg^{++} Zn^{++} complex showed the greatest toxicity and lowest Cu^{++} (1.6 ppm) EC_{50} value (Table 6). These results may be indicative of several toxicity factors being involved, perhaps two or more different enzyme systems are being affected.

In the Hg^{++} and Pb^{++} study, there appeared to be an antagonism by Pb^{++} to the toxicity of Hg^{++} to the Microtox reagent and with the addition of Ni^{++}, the Pb^{++} effect appears to be neutralized and the Hg^{++} Ni^{++} Pb^{++} complex toxicity approaches that of the metal Hg^{++} alone. The important role that incubation plays is again illustrated within this study. The Hg^{++} Ni^{++} Pb^{++} complex produces the most toxic reactions, i.e., lowest EC_{50} Hg value after 15-minute incubation (contact) while the 5 and 10-minute incubation between the Hg^{++} and the Microtox reagent produces the lowest Hg^{++} EC_{50} values (Table 6).

As observed in Table 5, and illustrated again in Table 6, phenol decreases in toxicity effect with increased contact in the Microtox system, perhaps serving as a nutrient, while the addition of sodium lauryl sulfate greatly increases the toxicity of phenol,

lowering the 15-minute phenol EC_{50} to 0.25 ppm from 34.3 ppm and instead of the toxicity decreasing with time, it increases.

Thus, the data from Table 6 illustrates the importance of incubation (contact) time. For comparison studies, a consistent incubation period must be used, preferably one where a few seconds variation in incubation does not involve a variation in EC_{50} values. On the basis of these data, 15 minutes is suggested as the ideal time for comparison studies.

Several combinations of metal ions were tested for their toxicity via the Spirillum volutans test and the results are shown in Table 7. In the first series, it can be seen that the combination of Zn^{++} plus Hg^{++} at pH 6.7 produces a strong additive, almost magnification type toxicity effect. This is well illustrated by the combination of 2.5 ppm Zn^{++} (non toxic after 24 hours contact) with 0.2 ppm Hg^{++} (toxic effect noted after 120 minutes of contact) which produces a toxic effect after only 15 minutes of contact. In the second series, it can be seen that Pb^{++} plus Ni^{++} in combination produce a toxic effect, while alone they are not toxic to Spirillum volutans after 120 minutes incubation at pH 6.7.

TABLE 7. COMPARISON OF ADDITIVE TOXIC EFFECTS OF HEAVY METALS ON SPIRILLUM VOLUTANS

Chemical and Concentration (ppm)	Incubation Period in Minutes Before 90% of Spirillum volutans Exhibit Distress or Death						
	0	5	15	30	60	90	120
20 Zn^{++}	-	-	-	-	+	+	+
10 Zn^{++}	-	-	-	-	-	-	-
2.5 Zn^{++}	-	-	-	-	-	-	-
0.2 Hg^{++}	-	-	-	-	-	-	+
0.1 Hg^{++}	-	-	-	-	-	-	-
10 Zn^{++} + 0.2 Hg^{++}	-	+	+	+	+	+	+
10 Zn^{++} + 0.1 Hg^{++}	-	+	+	+	+	+	+
2.5 Zn^{++} + 0.1 Hg^{++}	-	-	+	+	+	+	+
10 Pb^{++}	-	-	-	-	-	-	-
10 Ni^{++}	-	-	-	-	-	-	-
10 Pb^{++} + 10 Ni^{++}	-	-	-	-	+	+	+
5 Cu^{++}	-	-	-	-	-	-	-
10 Cu^{++}	-	-	-	-	-	-	+
5 Cu^{++} + 10 Ni^{++}	-	-	-	-	-	-	+
10 Cu^{++} + 10 Ni^{++}	-	+	+	+	+	+	+
10 Ni^{++} + 10 Zn^{++}	-	-	-	-	-	+	+
5 Ni^{++} + 5 Cu^{++} + 5 Zn^{++}			+	+	+	+	+
5 Ni^{++} + 5 Cu^{++} + 5 Zn^{++} + 0.1 Hg^{++}	+	+	+	+	+	+	+
10 Ni^{++} + 10 Cu^{++} + 10 Zn^{++}	-	+	+	+	+	+	+

TABLE 8. COMPARISON OF ADDITIVE TOXIC EFFECTS OF CHEMICALS ON SPIRILLUM VOLUTANS TEST

Chemical and Concentration (ppm)	Incubation Period in Minutes Before 90% of Spirillum volutans Exhibit Distress or Death										
	0	5	10	15	30	60	75	90	120	150	180
200 Pehnol	–	–	–	–	–	–	–	–	–	–	+
300 Phenol	–	–	–	–	–	–	–	–	–	+	+
40 Sodium lauryl sulfate (NaLS)	–	–	–	–	–	–	–	–	–	–	+
5 3,5 Dichlorophenol (Dich)	–	–	–	–	–	–	–	–	–	–	–
20 Pb^{++}	–	–	–	–	–	–	–	–	–	–	–
40 Pb^{++}	–	–	–	–	–	–	–	–	+	+	+
5 Cu^{++}	–	–	–	–	–	–	–	–	–	–	–
10 Cu^{++}	–	–	–	–	–	–	–	–	+	+	+
200 Phenol + 40 NaLS	–	–	–	–	–	–	–	–	–	–	+
200 Phenol + 20 Pb^{++}	–	–	–	–	–	–	–	–	–	–	+
200 Phenol + 20 Pb^{++} + 5 Cu^{++}	–	–	–	–	–	+	+	+	+	+	+
200 Phenol + 20 Pb^{++} + 5 Cu^{++} + 40 NaLS	–	–	–	+	+	+	+	+	+	+	+
5 Cu^{++} + 20 Pb^{++}	–	–	–	–	–	–	–	–	–	+	+
5 Cu^{++} + 5 Dich	–	–	–	–	–	+	+	+	+	+	+
5 Cu^{++} + 20 Pb^{++} + 40 NaLS	–	–	+	+	+	+	+	+	+	+	+
5 Cu^{++} + 5 Dich + 40 NaLS	–	–	–	–	–	+	+	+	+	+	+
5 Cu^{++} + 5 Dich + 40 NaLS + 20 Pb^{++}	–	+	+	+	+	+	+	+	+	+	+
40 Sodium cyanide	–	–	–	–	–	–	–	–	–	–	–
40 Sodium lauryl sulfate (NaLS)	–	–	–	–	–	–	–	–	–	–	–
40 NaCN + 40 NaLS	–	–	–	–	–	–	+	+	+	+	+
0.1 Hg^{++}	–	–	–	–	–	–	–	–	–	+	+
5 α naphthol	–	–	–	–	–	–	–	–	–	–	–
15 α naphthol	–	–	–	+	+	+	+	+	+	+	+
5 α naphthol + 0.1 Hg^{++}	–	–	–	–	+	+	+	+	+	+	+
15 α naphthol + 0.1 Hg^{++}	+	+	+	+	+	+	+	+	+	+	+

In the next series, Ni^{++} is shown to add to or magnify the toxic effect of Cu^{++}. The addition of Zn^{++} to the Ni^{++} plus Cu^{++} mixture produces an increased toxicity effect which is again made more toxic by the addition of Hg^{++}. Thus, in Table 7, it can be seen that the metal ions tested, when combined at levels which are minimally toxic or non-toxic as measured by the Spirillum volutans 120-minute test, become much more toxic with the effect appearing to be greater than a simple additive effect.

In Table 8, the toxicity of mixtures of metallic ions and organic chemicals to Spirillum volutans are shown. The addition of minimally toxic levels of phenol (200 ppm pH 6.7) to non toxic levels of Pb^{++} or sodium lauryl sulfate (NaLS) does not magnify or add to the toxicity of the phenol. However, the addition of non toxic concentrations of Cu^{++} to the phenol plus Pb^{++} combination greatly increases the toxicity fo the complex. Similarly, when NaLS in a non toxic concentration is added to Pb^{++} Cu^{++} phenol complex, there is an added toxicity effect.

When 3,5-dichlorophenol is added in non toxic amounts to non toxic concentrations of Cu^{++}, a magnified toxicity effect is produced, and yet when a non toxic level of NaLS is added to the above mixture, no increase in toxicity occurs, as indicated by the Spirillum volutans 120-minute test. Adding Pb^{++} to the above complex greatly increases

the toxicity. Thus, in this series, neutral, additive, and magnification of toxic effect to Spirillum volutans are shown.

Again, in the next series (Table 8), it can be seen that NaLS enhances the toxic effect of sodium cyanide pH 6.7. The combination of Hg^{++} and α-napthal illustrates magnfiication and additive effects, depending on the initial concentrations of the individual chemicals in the complex.

With the Spirillum volutans test for toxicity, the subtle variations in effects noted with the Microtox test to the toxic effects of single chemicals or mixtures, were not seen, i.e., decrease in sensitivity with increased incubation. The main effects noted with individual chemicals tested via Spirillum volutans in the 120 minute test were the production of typical dose response effects and with chemical mixtures, are that additive toxicity effects were usually noted when some sub-toxic concentrations of chemicals were mixed. However, when various concentrations of substrates were tested at concentrations not toxic in 120 minutes, the neutralization of toxicity of one toxic chemical by another was readily observed, similar to the effects noted with the Microfox test (Table 9).

Density inhibition test using Pseudomonas fluorescens and Aeromonas hydrophila were tested with mixtures of chemicals at concentrations equivalent to or approximately equal to their

TABLE 9. EFFECTS OF NON LETHAL* CONCENTRATIONS OF SUBSTRATES (ALONE AND IN COMBINATION) ON SPIRILLUM VOLUTANS WHEN INCUBATED FOR 24 HOURS

Substrate(s) and Concentration (ppm)	Time in Hours Required Before 90% of Spirillum volutans Exhibited Distress or Death					
	0	2	4	6	18	24
Hg^{++} 0.10	-	-	+	+	+	+
Hg^{++} 0.05	-	-	+	+	+	+
Hg^{++} 0.04	-	-	+	+	+	+
Hg^{++} 0.03	-	-	-	+	+	+
Hg^{++} 0.02	-	-	-	-	-	-
**Ca^{++} 60	-	-	-	-	-	-
Ca^{++} 40 + Hg^{++} 0.04	-	-	-	-	-	-
Ca^{++} 40 + Hg^{++} 0.03	-	-	-	-	-	-
Pb^{++} 40	-	+	+	+	+	+
Pb^{++} 25	-	-	-	-	-	-
Pb^{++} 10 + Hg^{++} 0.04	-	-	-	-	-	+
Pb^{++} 10 + Hg^{++} 0.03	-	-	-	-	-	+
Pb^{++} 10 + Hg^{++} 0.04 + 50 Phenol	-	-	-	-	-	-
Ni^{++} 20	-	+	+	+	+	+
Ni^{++} 10	-	-	-	+	+	+
Ni^{++} 5	-	-	-	-	-	-
Zn^{++} 10	-	-	+	+	+	+
Zn^{++} 7.5	-	-	-	-	+	+
Zn^{++} 5	-	-	-	-	-	-

* Concentrations not lethal in the routine 2-hour toxicity test.

** Ca^{++} from $CaCl_2$

Continued...../

TABLE 9 (CONT'D). EFFECTS OF NON LETHAL* CONCENTRATIONS OF SUBSTRATES (ALONE AND IN COMBINATION) ON SPIRILLUM VOLUTANS WHEN INCUBATED FOR 24 HOURS

Substrate(s) and Concentration (ppm)	Time in Hours Required Before 90% of Spirillum volutans Exhibited Distress or Death					
	0	2	4	6	18	24
Zn^{++} 10 + Ni^{++} 10	-	-	-	-	-	-
Zn^{++} 7.5 + Ni^{++} 10	-	-	-	-	-	-
Zn^{++} 7.5 + Ni^{++} 10 + Hg^{++} 0.05	-	-	+	+	+	+
Zn^{++} 7.5 + Ni^{++} 10 + Hg^{++} 0.05 + 75 Phenol	-	-	-	-	-	+
Zn^{++} 7.5 + Ni^{++} 10 + Hg^{++} 0.05 + 20 Sodium lauryl sulfate	-	-	-	-	-	+
Phenol 75	-	-	-	-	+	+
Phenol 50	-	-	-	-	-	-
Sodium lauryl sulfate 25	-	-	-	-	-	-

* Concentrations not lethal in the routine 2-hour toxicity test.

individual EC_{50} concentrations. Similar to the Microtox and Spirillum tests, a definite additive toxicity effect was noted in some combinations (Table 10) and in other combinations a neutralizing or antagonistic effect to toxicity was noted. As shown in Table 10, a number of chemicals were placed in solution together, at their individual EC_{50} concentrations and these mixtures were found to produce solutions with EC_{50} concentrations equivalent to 1/70 to 1/1000 the original EC_{50} values, thus indicating a strong additive effect. These findings are very similar to those observed with the Microtox test and again illustrate the importance of toxicant concentration (i.e.) that lower concentrations of some toxicants in combination have toxicity effects much greater than higher individual toxicant concentrations.

When some combinations of toxicants (i.e., EC_{50} concentrations of Cu^{++}, Zn^{++}, Pb^{++}, and Ni^{++}) are mixed, the maximum EC value obtained by this concentration of chemicals is equivalent to EC_{45}. Also, when EC_{50} concentrations of Pb^{++} and Ni^{++} are combined, the resulting EC value is EC_{30}. Similar to the observations with the Spirillum and Microtox procedures, Cu^{++} and Pb^{++} combinations appear to be antagonistic in density inhibition toxicity tests using Pseudomonas fluorescens and Aeromonas hydrophila. An EC_{50} concentration of Pb^{++} (13.8 ppm), combined with an EC_{50} concentration of Hg^{++} (0.049 ppm) produce a toxic solution equivalent to EC_{28}, i.e., the density of the microbial populations is only reduced 28%. Both Pseudomonas fluorescens and Aeromonas hydrophila react similarly. However, when 6.9 ppm or 3.45 ppm Pb^{++} are combined with an EC_{50}

TABLE 10. ILLUSTRATING THE ADDITIVE TOXIC EFFECT OF CHEMICALS BY COMBINING EC_{50} CONCENTRATIONS OF CHEMICALS AND ESTABLISHING THE EC_{50} OF THE MIXTURE

Chemical	EC_{50} Concentation (ppm)	Concentration of Chemicals (ppm) in New EC_{50} Based on Combining Individual Chemicals at Original EC_{50} Concentrations					
		P. fluorescens	A. hydrophila	P. fluorescens	A. hydrophila	P. fluorescens	A. hydrophila
Hg	0.05	0.0001*	0.0001*	0.0006	0.0007		
Zn	367.00	0.3288	0.2666	4.5813	5.0305		
Cu	16.80	0.0170	0.0138	0.2372	0.2604		
Pb	13.80	0.0124	0.0100	0.1723	0.1892	0.234	0.239
Ni	8.70	0.0078	0.0063	0.2086	0.1193	0.148	0.151
NaLS	1660.00	1.4840	1.2032			28.118	28.709
Total Concentration of Chemicals in EC_{50} in ppm		1.8501	1.50	5.17	5.91	28.50	29.10

* Rounded to closest decimal.

concentration of Hg^{++}, the EC value increased to EC_{32}, indicating a slight increase in toxicity and a less dense bacterial suspension.

The Microtox toxicity testing procedure, which is now undergoing a very thorough review by many North American laboratories, has, in our laboratory, and in many other laboratories (Table 11), shown to have some problems with reproducibility. For instance, in Table 11, 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-minute EC_{50} and 15-minute EC_{50} results, it was found: (a) 5-minute EC_{50} concentrations varied from 0.574 to 0.375 ppm (65.3%); (b) the 15-minute EC_{50} concentrations varied from 0.425 to 0.275 ppm (64.7%); and (c) the ratio's between 5 and 15-minute EC_{50} readings varied from 1.27 to 1.45.

Thus, there is a problem of reproducibility of data within one laboratory, and also between laboratories (Table 11), probably related to the variations in the cell suspension. Another example of this type of reproducibility problem is shown with EC_{50} values for ethanol. In one study, performed by the U.S. EPA Duluth Laboratory, a 5-minute EC_{50} of 56,706 ppm was found, and in another study by the

TABLE 11. COMPARISON OF MICROTOX EC_{50} VALUES OBTAINED IN THREE LABORATORIES

Toxicant	5-Minute EC_{50} (ppm)		
	Bulich et al.*	Duluth EPA**	Dutka-Kwan
Hg^{++}	0.065	0.052	0.064
Sodium lauryl sulfate	1.6	-	3.19
Zn^{++}	2.5	52	13.8
Cu^{++}	8.0	15.1	19.5
Phenol	25.0	40.7	28.0
Ethanol	31,000	56,706	-

* A.A. Bulich, M.W. Greene and D.L. Isenberg (1980). The reliability of the bacterial liminescence assay for determination of toxicity of pure compounds and complex effluents. Beckman Instruments Inc.

** Data produced by Miss Carolann Curtis, U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota 55804.

same laboratory, an EC_{50} value of 44,000 ppm was obtained (Curtis et al., 1981). For comparison, the 5-minute EC_{50} 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} for fish toxicity tests are also known to show similar reproducibility problems.

In spite of the above, there is no doubt that the Microtox system is a sensitive toxicity assaying procedure which has as its major benefit, a quick turnaround time which makes it an ideal member of a battery of screening tests. Used alone, we believe the Microtox 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.

In the single substrate-toxicant study, the Microtox procedure (30 to 45 minutes) was the most sensitive procedure followed by the Pseudomonas fluorescens EC_{50} density procedure (20 hours), the Spirillum volutans test (20 to 140 minutes), and the Aeromonas hydrophila EC_{50} procedure (20 hours). The cost of these tests, based on $10.00 per hour technician costs (including setting up, preparing glassware, and recording or reporting results) were: (a) Spirillum

TABLE 12. 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)	*S. volutans* 90% Inhibition 2-hour (ppm)	*P. fluorescens* EC_{50} (ppm)	*A. hydrophila* EC_{50} (ppm)
Zn	26	1.4	3.45	11.6	367.0	500.0
Hg	1.5	0.6	0.046	0.2	0.031	0.049
CN	0.47	4.7	2.76	83.0	14.0	25.25

[1] H. Ryssov-Nielson (1975). Measurement of the inhibition of respiration in activated sludge by a modified determination of the TTC-Dehydrogenase activity. Water Research, 9:1179-1185.

* tested at pH 7.5.

volutans $34.50 for one test or $13.60 each for three tests: (b) Microtox $40.00 for one test and for three tests, $17.60 each; and (c) Pseudomonas fluorescens $57.60 for one tes, or six tests at $17.75 each.

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 toxicant sensitivy. Table 12 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 an U.S. government EPA sponsored project (EPA Quality Assurance Newsletter, Vol. 4:2, April, 1981) of an effuent study comparing 24-hour fathead minnow and Daphnia pulex LC_{50} tests with the 5-minute 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 Daphnia pulex.

From the above, it would appear there is some overlap or "correct" guesses where all three systems indicate a positive effect, but no system was able to predict the 100% presence of toxicant to

another species. By reviewing Tables 2 and 11, and the above EPA sponsored data, it is very obvious that no single biological testing procedure can predict the presence of a toxicant which might effect aquatic organisms or be eventually bioaccumulated and affect their predators or man.

Again, by examination of Tables 5, 6, 7, 8 and 9, 10, it also becomes very apparent that some mixtures of compounds have greatly enhanced toxicity levels and thus the setting of water quality toxicant standards based on the maximum allowable level of single substrates, does not protect the most sensitive users, the microbiota, the first phase of the food chain.

From the data obtained during this study, Table 10 presents the best illustration of the "added substrate" effect of chemicals. Hg^{++}, which alone varies in "toxicity effect" from 0.031 ppm to 1.5 ppm by various screening tests, while in combination with other substrates (Table 10) it has a toxic effect at 0.000044 ppm. Also, from these studies, it would appear that the 18-hour Pseudomonas fluorescens and Aeromonas hydrophila density inhibition tests are the most practical sensitive tests for studying the effects of toxicant mixtures.

Thus, we believe the data summarized in Tables 5 to 10, and the quoted EPA study, imply that it is dangerous or unwise to try and assess the presence of toxicants in waters or effluents by a single species test. The battery approach, encompassing two or three genera and involving two to four species is recommended, to more thoroughly assess the potential presence of toxicants.

From the data obtained in this study, the Spirillum volutans (inexpensive, simple, quick, reproducible), and the 18-hour Pseudomonas fluorescens EC_{50} density tests (economical, simple, and very sensitive to mixtures), are recommended as potential candidates for the battery approach to toxicity testing. The Spirillum volutans test is recommended for inclusion in a battery approach over the Microtox procedure because of its ability to test samples over a wide pH range. The Microtox procedure requires all samples to be pH adjusted (approximately 6.7) and thus the toxicity of a sample may be influenced by this pH adjustment.

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