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# Microtox® and *Spirillum volutans* Tests for Assessing Toxicity of Environmental Samples

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Many developed and developing countries today are facing serious ecological and toxicological problems resulting from the release of complex effluents and toxic substances to the environment. A wide range of bioassays using fish and other aquatic organisms from several trophic levels have been used for biological monitoring and toxicity assessment (Bringmann and Kuhn 1980; Cairns et al. 1976; Little 1978; Maciorowski et al. 1981). Unfortunately, most of these assays are relatively long, comparatively expensive, require sophisticated facilities, and also require a great deal of professional competence for data interpretation. Consequently, for quick screening of effluents and chemicals for toxicity and to determine if additional sophisticated tests are required, simple, rapid, sensitive and inexpensive assays could be more useful. Microorganisms, bacteria in particular, have several attributes which make them attractive for use in such tests for toxicity testing (Berkowitz 1979, Qureshi et al. 1984). In this regard, a variety of microbial and biochemical tests has been developed for use in determining chemical toxicity; these tests have been recently reviewed by Bitton (1983) and Liu and Dutka (1984).

Two short-term bacterial toxicity tests that have received a great deal of attention during the last few years are the Microtox and Spirillum volutans assays. The former involves monitoring changes in the light output from luminescent bacteria (Photobacterium phosphoreum) in a temperature controlled photometer when exposed to various concentrations of toxic substances. Toxicity is expressed as EC 50 which is the effective concentration of a toxicant causing 50 percent reduction of light output during the designated time intervals at 15°C. In the S. volutans assay, motility patterns of this large aquatic bacterium are used as the test endpoints. Relative toxicity is measured as MEC 90 which is the minimum effective concentration of a toxicant that causes loss of reverse or forward motility in greater than 90 percent of cells after exposure to the toxicant for different time periods. These two tests have been used and compared with conventional bioassays for assessment of aquatic toxicity (Bulich and Isenberg 1980;

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Curtis et al. 1982; Chang et al. 1981; Lebsack et al. 1981; McFeters et al. 1983; Qureshi et al. 1982). A good correlation between bacterial and fish and other bioassay data was shown in these studies which were largely concerned with the detection of toxicity of certain toxic compounds.

As part of our program in assembling and establishing a battery of short-term bacterial toxicity tests, we examined the use of Microtox and S. volutans for determining the toxicity of a wide variety of environmental samples. Samples examined included potable and surface waters, industrial effluents, soil/sludge extracts and landfill leachates. Some of the results are presented in this paper.

## MATERIALS AND METHODS

Several complex effluent samples were collected from various industries including an oil refinery, chemical storage tanks, building and chemical products, gas and fertilizer plants and food and beverage plants. In addition, a few sanitary landfill, sludge/soil extracts, landfill leachates, potable and surface water and mine tailings pond water samples were also tested. All samples were assayed by the two tests at their observed pH. The pH was determined potentiometrically on unmodified samples using a Fisher Accumet pH meter (Model 620) and standard buffers; bicarbonate/NaOH for pH 7.0 and phthalate for pH 4.0.

All Microtox assays were conducted using the model 2055 Toxicity Analyzer System (Beckman Instruments, Inc., Carlsbad, CA) with reagents and lyophilized luminescent bacteria supplied by the manufacturer. The bacterial reagent was diluted with Microtox reconstitution solution (distilled water free of organic compounds) and used fresh in all assays. Test samples were adjusted to 2% NaCl with the Microtox osmotic adjustment solution to provide osmotic protection for the luminescent bacteria. Further sample dilutions were made, as required, using the Microtox diluent (a solution of 2% NaCl in distilled water). Testing and data reduction followed the procedures described by Bulich and Isenberg (1980), Qureshi et al. (1984) and detailed in the Beckman Operating Manual (#015-55879). Initial light measurements were made for each cuvette containing 10 µL of bacterial reagent and 0.5 mL of Microtox diluent equilibrated to 15°C. Aliquots (0.5 mL) of sample dilutions were then added to individual cuvettes and light measurements were made after 5, 10, 15, 20 and 30 min. However, the 5 and 15 min measurements were used for calculating EC50 values as percent of original sample concentration. The Microtox diluent was used as a control to correct the time-dependent decrease in light output. For all EC 50 calculations gamma, the ratio of light lost to the light remaining, was used in place of simple percent light decrease as suggested by Bulich (1982). Furthermore, in all assays the gamma and sample concentration were used as the dependent and independent variables, respectively.

All S. volutans assays were performed using procedures described previously (Goatcher et al. 1984). In general, the procedure involved mixing 0.1 mL of Defined Test Medium (DTM), 0.8 mL sample (or sample dilution), distilled water (negative control) or $Hg^{+2}$ (1 mg/L) solution (positive control) and 0.1 mL of healthy (with >90 percent motility) bacteria from a 24 h culture grown at 25°C in Bacto-Casitone-Succinate Salts medium. Slide preparations from the mixture were examined using darkfield microscopy after 0, 5, 15, 30, 60, 90 and 120 min incubation. The toxic response (MEC 90) was measured as the percent concentration of original sample required to cause a loss of 90% motility after the designated incubation period.

## RESULTS AND DISCUSSION

The results of the Microtox and S. volutans assays of a wide range of environmental samples are given in Table 1. All the samples tested were aqueous and of unknown composition. With the exception of the T3 (slough #2) sample, all potable and surface water samples were found to be non-toxic. Similarly, only a few of the effluents were designated toxic by these tests. All four mine tailings pond water samples, however, were classified as toxic by at least one of these two tests.

In all samples the pH was not adjusted since it was previously found (Qureshi et al. 1984) that in metal salt solutions the dissolved components precipitated upon pH modification. Therefore, all samples were assayed at their observed pH to include any potential and meaningful effect of pH on toxicity. However, it was apparent from the data in Table 1 that the pH of the samples examined had no detectable effect on their toxicity response. The observed pH of most of these samples ranged between 5.8 and 11.0. Although for optimum sensitivity the desired pH for the Microtox tests is 6.7, these observations provide further support to suggestions made by Qureshi et al. (1984) that sample pH should be considered as part of the assay. In other words, environmental samples should be assayed by the Microtox test without pH adjustment to preserve sample stability and integrity, and also to eliminate introduction of another variable that may place the relevance and usefulness of the test in question.

Table 2 presents summary results of samples assayed by the Microtox and S. volutans tests. Of the 41 samples tested, 12 (29.3%) were found to be toxic by at least one of the two tests and the remaining 29 (70.7%) were determined to be non-toxic by these tests. Furthermore, 7 (17.1%) samples were designated toxic by the Microtox and non-toxic by the S. volutans assay, while only 5 (12.2%) elicited a toxic response to both of these tests. It is also interesting to note that in no instance the S. volutans was positive when the Microtox response was negative. These data indicate a general similarity and good agreement between the response of Microtox and S. volutans tests.

Table 1. Response (+ = toxic, - = non-toxic) of Microtox and S. volutans assays to environmental samples

| Sample | Sample No. | Sample pH | Assay Response Microtox | Assay Response S. volutans |
|---|---|---|---|---|
| Potable & Surface Water | | | | |
| Water Reservoir | T1 | 8.2 | -[a] | - |
| Tap Water | T6 | 7.4 | - | - |
| Slough #1 | T2 | 7.7 | - | - |
| Slough #2 | T3 | 7.4 | + | - |
| River #1 (Upstream) | T4 | 8.0 | - | - |
| River #1 (Downstream) | T5 | 7.8 | - | - |
| Landfill Surface Water | T12 | ND[b] | - | - |
| Man-made Lake | T13 | ND | - | - |
| River #2 | T14 | ND | - | - |
| River #3 | T48 | ND | - | - |
| River #3 (Upstream) | T49 | ND | - | - |
| River #3 (Downstream) | T50 | ND | - | - |
| Industrial Effluent | | | | |
| Chemical Storage Tanks | T7 | 6.0 | + | - |
| | T8 | 10.3 | + | - |
| | T9 | 11.0 | + | + |
| | T10 | 6.9 | + | - |
| Building Products | T25 | 8.4 | - | - |
| | T26 | 8.0 | - | - |
| Chemical Products | T29 | 6.8 | - | - |
| Gas Plants | T17 | 8.6 | - | - |
| | T18 | 8.9 | - | - |
| | T22 | 8.5 | - | - |
| | T23 | 8.2 | - | - |
| | T24 | 8.5 | - | - |
| Oil Refinery | T27 | 7.1 | - | - |
| Oil & Gas Production | T28 | 7.6 | - | - |
| Fertilizer Plant | T19 | 3.6 | + | + |
| Food & Beverage Plants | T15 | 7.1 | - | - |
| | T16 | 7.9 | - | - |
| | T20 | 7.4 | - | - |
| | T21 | 7.5 | - | - |
| | T30 | 8.1 | - | - |
| Sanitary Landfills | | | | |
| Effluent | T11 | 5.8 | + | + |
| Leachate #1 | T31 | 7.8 | - | - |
| Leachate #2 | T32 | 7.7 | - | - |
| Sludges & Soils | | | | |
| Sludge Extract (in Water) | T35 | ND | + | + |
| Soil Extract (in Water) | T36 | ND | - | - |
| Mine Tailings Pond | | | | |
| Surface Water (Non-Centrifuged) | T33[c] | 8.2 | + | - |
| Surface Water (Centrifuged) | T34 | 8.2 | + | - |
| Sub Surface Water (Non-Centrifuged) | T37 | 7.7 | + | + |
| Sub Surface Water (Centrifuged) | T38 | 7.7 | + | - |

a = Response may vary with residual chlorine concentration.
b = Not determined
c = Samples 33-38 were tested without Defined Test Medium.

Table 2. Summary of the comparison of Microtox and S. volutans assays of environmental samples

| Response category (+) Toxic, (-) Non-Toxic | Number of Samples | Percent of Samples |
|---|---|---|
| Microtox (-), S. volutans (-) | 29 | 70.7 |
| Microtox (+), S. volutans (+) | 5 | 12.2 |
| Microtox (+), S. volutans (-) | 7 | 17.1 |
| Microtox (-), S. volutans (+) | 0 | 0.0 |
| TOTAL | 41 | 100.0 |

A direct comparison of the Microtox and S. volutans tests is difficult and is complicated by inherent differences between these two systems. Nevertheless, the results of these assays were compared in an attempt to determine their relative sensitivity using a modified toxicity ranking method described by Bulich (1982). The Percent Rank Method (PRM) ranks each test results according to the relative index of toxicity (Table 3).

Table 3. The Percent Rank Method for determining toxicity by the Microtox and S. volutans tests

| Test Result EC 50 OR MEC 90 | Class | Rank |
|---|---|---|
| <25% | Very Toxic | 1 |
| 25-50% | Moderately Toxic | 2 |
| 51-75% | Toxic | 3 |
| >75% | Slightly Toxic | 4 |
| No Toxic Effect | Non Toxic | 5 |

Using this ranking method, all the Microtox and S. volutans data for the toxic samples were ranked and the results summarized in Table 4. Of the 12 samples, only two were ranked equally by the two tests and the remaining samples were classified differently showing individual toxicity sensitivity patterns. Despite the observed sensitivity differences, the data indicated that the Microtox was generally more sensitive than the S. volutans assay. These findings agree with others for the evaluation of toxicity from several chemical compounds using these tests (Dutka and Kwan 1981; Dutka et al. 1983; Qureshi et al. 1982).

Although the Microtox EC 50 values for all samples decreased between 5 and 15 minutes, the changes with one exception were not appreciable enough to change toxicity ranking. These results provide further support for the recommendation of Qureshi et al.

Table 4. Comparison of the sensitivity of Microtox and S. volutans tests for determining the toxicity of several environmental samples. All values are in percent of original sample

| Sample Type | Sample No. | Microtox EC50 5 min | Microtox EC50 15 min | S. volutans MEC 90 5 min | 15 min | 30 min | 60 min | 120 min | Toxicity Ranking[a] MX[b] | Toxicity Ranking[a] Sv[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| Slough #2 | T3 | 61.3 | 51.2 | No Toxic Effect (NTE) | | | | | 3 | 5 |
| Chem Storage Tank Effluent | T7 | 88.6 | 74.8 | | | NTE | | | 4→3 | 5 |
| | T8 | 34.4 | 27.8 | | | NTE | | | 2 | 5 |
| | T9 | 14.2 | 11.7 | NTE | 80 | 60 | 60 | 60 | 1 | 5→4→3 |
| | T10 | 65.2 | 57.0 | | | NTE | | | 3 | 5 |
| Fertilizer Plant Effluent | T19 | 1.2 | 1.0 | 15 | 15 | 15 | 15 | 15 | 1 | 1 |
| Sanitary Landfill Effluent | T11 | 0.7 | 0.8 | 8 | 4 | 4 | 4 | 4 | 1 | 1 |
| Sludge Extract | T35 | 3.1 | 3.3 | 50 | 50 | 50 | 50 | 50 | 1 | 2 |
| Mine Tailings Pond Water | T33 | 35.7 | 25.7 | | | NTE | | | 2 | 5 |
| | T34 | 39.1 | 28.0 | | | NTE | | | 2 | 5 |
| | T37 | 37.0 | 30.2 | NTE | 90 | 75 | 75 | 75 | 2 | 5→4→3 |
| | T38 | 40.0 | 38.6 | | | NTE | | | 2 | 5 |

a = Toxicity was ranked using the Percent Rank method as follows: 1, <25% (Very toxic); 2, 25-50% (Moderately Toxic); 3, 51-75% (Toxic); 4, >75% (Slightly Toxic), 5, No Toxic Effect (Non Toxic).

b = Microtox.

c = Spirillum volutans.

(1984) that the 5 and 15 min incubation time be used as a standard in the screening of environmental samples by the Microtox test. The S. volutans data indicated that in some instances the MEC 90 values decreased substantially over the 30 min assay period suggesting changes in toxicity rankings. The values, however, remained essentially the same after 30 and up to 120 min incubation for most samples. These observations suggest that for comparative purposes a 30 min MEC 90 be adopted as a standard in the testing of natural samples by the S. volutans assay. It should be emphasized, however, that as observed in two instances here a different and unpredictable toxicity pattern may emerge beyond 30 min incubation particularly for samples of unknown content. Therefore, for such samples, it is suggested that the S. volutans test should be conducted up to its maximum limit of 120 min to prevent reporting of erroneous results.

Since detailed chemical analysis was not attempted, it is difficult to postulate the nature and type of causative toxic agent(s) in toxic samples. A partial chemical characterization (unpublished data) indicated that different kinds of pesticides and fatty acids were present and may be responsible for the toxicity of T9 and T11 samples, respectively. Also, sample T19 yielded high concentrations of ammonia and aluminum and either one or both of them may be the toxic agent in this sample. Low concentrations of both aluminum and ammonia have been reported by previous investigators (Bulich and Isenberg 1980; Qureshi et al. 1982; Qureshi et al. 1984) to be highly toxic to fish, Daphnia, S. volutans and luminescent bacteria (Microtox). Furthermore, it should be emphasized that identification of the toxic agent in a complex mixture is not always possible due to potential additive, synergistic and antagonistic interactions among its constituents. In this regard, combining bacterial toxicity testing with chemical fractionation may prove to be useful in determining groups of toxic constituents, with similar chemical and physical characteristics, in complex environmental samples. Such an approach was recently evaluated by Delistraty (1984) for determining toxicity of synfuel by-product waters using the Microtox test.

The results of this study demonstrate that the Microtox and S. volutans tests are potentially useful for the detection of effluent or chemical toxicity. Similar results have been reported previously (EPA Quality Assurance Newsletter 1981; Bulich 1982; Curtis et al. 1982; Qureshi et al 1982) indicating good agreement and correlation between fish, Daphnia and bacterial assays for determining toxicity of complex effluents and organic chemicals. Furthermore, the comparative data (Table 4) indicated that Microtox was more sensitive than the S. volutans test for determining the toxicity of several environmental samples. In general, however, good agreement was found between the results of these two tests.

From this study and previously reported data, it is believed that there is no one universal test that can be used in all situations

for toxicity testing. Also, it is obvious that one bioassay cannot provide results equivalent to that of another bioassay due to their inherent biological differences. Furthermore, since each test has limitations such as pH effect and sensitivity, it is suggested that a battery of short-term tests employing organisms from various trophic levels be used for assessing toxicity in aquatic environments. The Microtox and S. volutans tests have a place in and should be considered as part of a battery of screening tests and their results may be used to supplement data obtained in other well established conventional toxicity bioassays. Major advantages of these two microbial test systems are that they are simple, inexpensive, sensitive, reproducible and provide cost effective results in a fast turnaround time. Moreover, they are especially useful as "Primary Screening" or "Early Warning" tests through which an investigator can rapidly ascertain which samples are non-toxic, toxic or very toxic. Also the results of these two tests can be used to determine if additional bioassays are required, and, if so, then they can be limited to only those significantly toxic samples that warrant further analysis.

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