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Use of the Microtox® Assay System for Environmental Samples

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Bioluminescence and modern electronic instrumentation allow measurement of light production from specific compounds with great precision. The involvement of enzymes and proteins in the bioluminescent reactions contributes biological specificity; and through enzymic coupling to bioluminescent reactions, the concentration of a variety of biologically important molecules can be measured with a sensitivity not achievable by other methods. A volume of Methods in Enzymology edited by DeLUCA (1978) presents many of these methods. HASTINGS (1979) states that the sensitivity of luminescent measurements is limited by only the thermal noise of the phototube, and corresponds to between 10^3 and 10^6 molecules depending upon the application.

The two bioluminescent systems most studied are those of the firefly and luminescent bacteria. Firefly luciferase is the basis for a sensitive assay for ATP and other nucleotides (DeLUCA & McELROY 1978). A whole technology for ATP measurement has been developed (BORUN 1975, 1977). The luciferase derived from luminescent bacteria utilizes reduced flavin, a long chain aldehyde, and oxygen to produce light (HASTINGS & NEALSON 1977). Biochemically, the bacterial bioluminescent pathway is a branch of the electron-transport chain (HASTINGS 1978). The process may give the bacteria an advantage in microacrophilic conditions (NEALSON & HASTINGS 1979).

Since the flow of electrons in the respiratory chain is an indication of the metabolic state of the cell, many bioassays can be based on light production by luminescent bacteria. For example, BEIJERINCK (1902) and OSHINO et al. (1972) have used luminescent bacteria for the measurement of oxygen. The effects of various drugs, of temperature, and of pressure on bacterial bioluminescence were determined by JOHNSON et al. (1954). Even anesthetic action has been measured using luminescent bacteria (WHITE et al. 1975). TCHAN et al. (1975) used such bacteria to determine the effect of herbicides on photosynthesis by measuring the oxygen produced. SERAT et al. (1965) developed a rapid biological assay based on bioluminescent bacteria growing on agar to measure air pollution. This process was further refined and patented (JORDAN et al. 1968).

From these observations Beckman Microbics Operation developed the Microtox® system for measuring water pollution. The assay is based on rehydrating freeze-dried luminescent

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bacteria and measuring the effect of potential inhibitors under defined conditions on light production. BULICH & GREENE (1979) have described the development of this system.

As part of our program in establishing biochemical indicators of ground water pollution we examined the use of luminescent bacteria as a bioassay system. We evaluated the response of the assay system to several standard toxic compounds, selected respiratory inhibitors, various pesticides and typical environmental water samples. This paper presents some of the results.

MATERIALS AND METHODS

Microtox® Reagent was purchased from Beckman (Carlsbad, CA). The pesticides were supplied by the Oklahoma State University Departments of Agronomy, Entomology, and Plant Pathology. Respiratory inhibitors were purchased from Sigma Chemical.

Limulus amebocyte lysate and lipopolysaccharide standards were obtained from Associates of Cape Cod (Woods Hole, MA). The clotting was determined spectrophotometrically using a Zeiss PM-6 spectrophotometer (COHEN 1979).

Light production from luminescent bacteria was measured in a Packard Pico-Lite Model 6100 photometer equipped with a Haake constant temperature water bath and a Houston Instruments Omni Scribe Model 35217-5 recorder. The temperature of the reaction chamber was maintained at 15 ± 0.1°C. The rehydrated bacteria were stored at 3°C and then they were diluted 50-fold with 2% NaCl just prior to use. Six 100-µL aliquots were placed in the photometer and light output followed for 15-20 min until light production stabilized. Light production was determined for each cuvette; then the samples were added (final concentration is 2% NaCl). The light production was determined for 5 min by cycling between cuvette positions and using the chart recorder to determine the times when readings were taken.

If proper sample concentrations are used, an approximate EC_{50} concentration of sample --causing a 50% reduction in light production in 5 min at 15°C-- can be determined by conventional procedures for treating bioassay data such as plotting log of sample concentration vs per cent of light loss. The light loss was obtained using a blank correction, normalized with control cuvettes, and expressed as per cent. When the sample is not very toxic, no EC_{50} can be obtained. In that case, the light loss, after 2 or 4 min is used and is designated LL_2 or LL_4, respectively.

RESULTS

Basic Measurements

Figure 1 shows the standard curve for determining the

toxicity of toluene. The experiment was done as described in the Materials and Methods section. The EC_{50} is 50 ppm. Table 1 shows the EC_{50} values for a number of compounds and the broad range of concentration of inhibitors that can be measured in the system -- ranging from 47,000 ppm for ethanol to 2.5 ppm for cyanide. Although the light output from a sample of bacteria was reduced to 1/3 during 5 h of storage after hydration, the EC_{50} determined for m-cresol with fresh and 5-h-old samples were 11 and 12 ppm, respectively. An example of the reproducibility between experiments is that with a concentration of 9 mg/L of m-cresol the normalized light loss was 44.0% for the first experiment and 44.9% for the second.

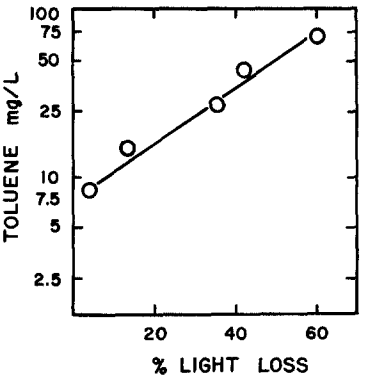

Figure 1. Inhibition of light production by various concentrations of toluene. The logarithm of toluene concentration is plotted against the per cent of light production loss by luminescent bacteria.

Table 1. Typical EC_{50}, LD_{50} and LC_{50} for Selected Compounds and Respiratory Inhibitors

Typical Compounds	Microtox® EC_{50} ppm	Rat (oral)[1] LD_{50} g/kg	Fish toxicity LC_{50} mg/L
Ethanol	47,000	14	13000[2]
1-Butanol	44,000	4.4	1900[2]
Benzene	200	5.7	50[3]
Toluene	50	5.0	23[2]
Phenol	26	0.53	5.0[2]
m-Cresol	11	2.0	19[2] (p cresol)
Formaldehyde	8.7	0.80	250[3]
Respiratory Inhibitors			
Amytal	1,000		
Thenoyltrifluoroacetone	3.5		
Cyanide	2.5		
Azide	400		
Arsenate	94		

[1] Lethal dose $_{50}$. From the Merck Index, Merck & Co., Rahway, N.J.

[2] Lethal concentration$_{50}$ (LC_{50}) BRUNGS, W. A., J. H. McCORMICK, T. W. NEIHEISEL, R. L. SPEHAR, C. E. STEPHAN, and G. N. STOKES: J. Water Poll. Control 49, 1425 (1977).

[3] McKIM, J. M., R. L. ANDERSON, D. A. BENOIT, R. L. SPEHAR, and G. N. STOKES: J. Water Poll. Control 48, 1544 (1976).

Environmental Samples

The results of typical application of the Microtox® method to environmental samples are shown in Table 2. In Part A various local water sources were tested, and in Part B various spring water samples from eastern Oklahoma were tested. The amounts of lipopolysaccharide present in these samples as determined by the Limulus amebocyte lysate assay are also shown. Part C shows the results of testing various oil refinery effluents. The LC_{50} for the fathead minnow 96-h bioassay is also shown for some.

Table 2. Analysis of Environmental Water Samples

Water Sample	Microtox® Assay		
	LL_2	LL_4	Lipopolysaccharide pg/mL
A. Local Water			
Barstead still system	15	27	5.0×10^2
Burned field water	100	100	1.0×10^5
Essenberg well	0	2	75
Glass-distilled water	0	0	0
Spring-obtained locally	93	96	1.0×10^4
Tap water	35	35	4.4×10^2
Water fountain	2	7	5.0×10^2
B. Eastern Oklahoma			
Park Spring	0	0	5.0×10^4
Sparrow Hawk Spring	-15	-15	2.5×10^7
Speed's Spring House	-24	-20	-
Stream	0	-1	2.5×10^4
Wilson Ranch Spring	-14	-14	-

%	LL_4	EC_{50} Microtox®	LC_{50} Fathead Minnow
		% Required	
C. Oil Refinery Effluents			
ETE - 55	46	58	65
17 - 51 - 80	34	74	65
LNX	0	100	65
UQB - 3	90	1.8	42
UQB - 4	0	100	75

The sample to be tested constituted 45% of the Microtox® assay volume. For the EC_{50} determination in part C the concentration of the effluent was varied.

Toxicity of Pesticides

A number of commonly used pesticides were tested in the Microtox® bioassay (Table 3). The luminescent bacteria were fairly sensitive to most of the compounds tested. Thus, the Microtox® assay is an effective method for their determination.

Table 3. Toxicity of Selected Pesticides

Common Name of Compound	EC_{50} ppm Microtox®	LD_{50} mg/kg[1] Rat (oral)
Captafol	7	6200
Carbaryl	2	500
Cyhexatin	10	540
Diazinon	1.7	300
Dichloran	3	5000
DDT	7	110
Glyphosate	7.7	4300
Malathion	10	1400
Paraquat	780	150
Ridomil[2]	120	670
Thiabendazole	3400	3100

[1]From Farm Chemical Handbook, 1980, Meister Publishing Co., Willoughby, OH.

[2]L-N-methyl(2,6-dimethyl phenyl)-N-(2'-methoxyacetyl)-alanine.

DISCUSSION

The sensitivity with which light production can be measured with commercially available instrumentation and the availability of suitable freeze-dried preparations of luminescent bacteria enhance the usefulness of the Microtox® bioassay. The ease of performance of the basic measurement and the range of concentrations of toxic and other compounds that can be determined are also attractive features of the Microtox® bioassay.

Table 1 Part A shows a variety of compounds with differing toxicities assayed in three systems. These three measures of toxicity have good correlations. The Microtox® has a corre-

lation coefficient of 0.90 with the rat toxicity and 1.0 with the fish toxicity. Advantages of the Microtox® test are the short time (30 min) required for an assay and the statistical advantage in using over 10^5 bacteria instead of a small number of rats or fish in the other bioassays. Because the luminescent pathway is a branch of the electron transport chain, the effects of various respiratory inhibitors were determined. The relationship between the electron transport chain and the luminescent pathway would suggest that inhibitors acting after the divergence of the two pathway should be less effective in blocking light production and even increasing it. This is not observed; therefore, the metabolic interrelationships must be more complicated.

The local water samples vary greatly in both their inhibition of light production and in their lipopolysaccharide contents. There is no correlation between these two parameters, but there is no reason to expect that there should be. Some of the eastern Oklahoma spring waters stimulate light production (shown by the minus sign). Certain of the compounds (at low concentrations) such as ethanol also stimulate light production. These compounds could either be substrates or change the availability of components (NEALSON et al. 1970).

Table 3 shows little relation between light inhibition by selected pesticides and their LD_{50} orally for the rat. The correlation coefficient between these variables is 0.09.

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