

---

---

# A SIMPLE, RAPID, INEXPENSIVE ASSAY FOR TOXIC CHEMICALS USING A BACTERIAL INDICATOR

J.L. Botsford<sup>1,2</sup>, T. Hillaker<sup>1</sup>, B. Robertson<sup>1</sup>, M. Gonzales<sup>1</sup>, M. Benavidez<sup>1</sup>, B. Jones<sup>1</sup>, R. Baker<sup>1</sup>, W. Steen<sup>1</sup>, F. Pacheco<sup>1</sup>, V. Homer<sup>3</sup>, O. Lucero<sup>4</sup>, M. Matthews<sup>4</sup>, and V. Koehler<sup>5</sup>, <sup>1</sup>Department of Biology, Box 3AF, New Mexico State University, Las Cruces, NM, 88003, <sup>2</sup>Phone: 505-646-3726, FAX: 505-646-5665, E-mail: jbotsfor@nmsu.edu; <sup>3</sup>Fort Stanton, New Mexico; <sup>4</sup>Gadsden High School and Las Cruces High School; and <sup>5</sup>DVM

**ABSTRACT** A simple test for toxic chemicals has been developed. *Rhizobium meliloti* is combined with the toxic chemical. A tetrazolium dye, MTT (3-[4,5-Dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide) is added. The bacterium reduces this dye, causing the optical absorbance to increase dramatically. The increase can be determined with a simple spectrophotometer. Toxic chemicals and minerals inhibit the reduction of the dye. Presumably the dye serves as a terminal electron acceptor for electron transport. Toxic substances presumably damage the electron transport system. The results compare favorably with published results of tests using the Microtox™ assay and with the Polytox™ assay. This assay is simpler and requires no specialized equipment. It should be possible to use this assay in a third world situation.

**KEYWORDS:** toxic chemicals, assay, student involvement, bioremediation

---

---

## INTRODUCTION

Any test for a toxic chemical requires an indicator organism. A compound is defined as being toxic if it damages living material. The tests used to define toxicity legally use rats, mice, dogs, rabbits, fish, or *Daphnia*, a sand flea. These tests are complicated and expensive. They require highly skilled personnel to carry out. It is necessary to determine if the animal died from the toxin or from other causes. Damage to the animal is usually determined by the death of the animal. Many toxins may damage the animal but may not kill the animal. Damage to the animal is not determined in these tests, only death. Animal tests require days if not weeks to carry out. Animal rights advocates object to using animals for this purpose. There is an obvious need for a simple, fast, and inexpensive method to assay for toxicity of chemicals. It would be useful to have a simple initial test to determine if a chemical

were toxic analogous to the Ames' Test for carcinogenicity [1].

The assay described here uses the bacterium *Rhizobium meliloti* as the indicator organism. This bacterium is non-pathogenic. The bacterium is mixed with the toxic chemical and a tetrazolium dye is added. Tetrazolium dyes have been used to isolate mutants unable to degrade carbohydrates [2]. The bacterium reduces the dye, causing the dye to precipitate and to become intensely colored. Presumably the dye is reduced by components of the electron transport system. The components of electron transport are associated with the cytoplasmic membrane in prokaryotes. If the toxic chemical damages the cytoplasmic membrane, this will inhibit electron transport, and the dye will not be reduced.

The assay is inexpensive. It is simple. High school students have been trained to run the assay. It is rapid—as many as 200 samples

can be assayed in a day. The assay can be carried out in third world situations. No specialized equipment is required, only a spectrophotometer and a water bath. The data can be analyzed with a simple pocket calculator. A detailed description of the assay will be published [3]. A patent has been sought. The assay should be available commercially in the near future.

## **MATERIALS AND METHODS**

### ***Strains***

*Rhizobium meliloti 102f34* has been used most extensively with the assay.

### ***Growth of bacteria***

*R. meliloti* was grown in CDM medium [4] with 0.1% casamino acids. Cells were grown in a rotary shaker at 30°. Cells were collected by centrifugation (6,000 x g in a refrigerated preparative centrifuge) and were washed once with 0.01 M potassium phosphate buffer, pH 7.5. After washing, cells were resuspended in the phosphate buffer to an absorbance (at 550 nm) = 0.3. In a few experiments, cells were resuspended at an absorbance of 3.0 in MOPS (pH 7.5, 0.1 M), and 0.1 ml rather than 1.0 ml was used. Once washed, cells were kept in an ice bath. Mannitol, the carbon source during growth, was added to the washed cells at a final concentration of 1%. It was found that the inoculum used was critical. An overnight culture resulted in cells best able to reduce the dye. If the cells used to inoculate the culture had been kept for a day in the refrigerator, cells grew normally but reduction of the dye was appreciably lower.

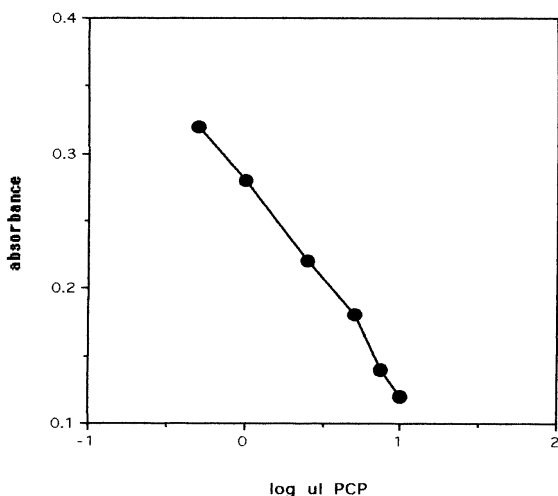
### ***Assays***

Assays were performed by combining 1 ml Tris HCl buffer (0.1 M, pH 7.5), 1.2 ml of the toxic chemical, and 1 ml cells in 13 x

110 mm test tubes. The absorbance was measured at 550 nm. The absorbance was measured at time = 0. 0.1 ml MTT (3-[4,5-Dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide) tetrazolium dye was added to yield a final concentration of 0.91  $\mu\text{M}$  and the tubes were mixed vigorously with a vortex mixer. It was found that the addition of the dye did not change the initial absorption appreciably. Tubes were then incubated at 30°C. The time = 0 absorbance was subtracted from the final absorbance. This difference between the time = 0 and the final absorbance is considered to be the "absorbance" used in calculations. Typically, samples were incubated 20 minutes. Samples could be incubated for longer periods of time. This did not change the apparent toxicity. It was found that for some chemicals, preincubating the cells at 30° for 20 minutes before adding the dye increased the sensitivity of the assay by about 30%. After this 20 minute interval, the MTT dye was added. But for other chemicals, preincubation had no effect. The interval between taking the t = 0 reading and adding the dye was not critical.

### ***Calculations***

The data were plotted, the concentration or the volume of the toxic chemical on the X-axis and the absorbance on the Y-axis. Regression lines were calculated, the slope of the line, the Y intercept, and the regression coefficient were determined. The equation  $Y = mx + B$  was used, where Y is the absorbance of the control, the sample without a toxic chemical; m is the slope of the regression line; B is the Y intercept (value for Y when  $x = 0$ ) calculated from the regression line; Y/2 is the value for the absorbance with reduction of the dye inhibited 50%; x is that concentration of toxic chemical resulting in 50% inhibition of electron transport. This equation is solved:



**FIGURE 1.** TOXICITY OF PENTACHLOROPHENOL. NOTE HOW THE CONCENTRATION OF PENTACHLOROPHENOL IS PLOTTED AS THE LOG OF THE CONCENTRATION. THE EQUATION FOR THE LINE IS  $Y = 0.278 - 0.153X$ ,  $R^2 = 0.995$ .

$$x = \frac{y/2 - B}{m}$$

This value,  $x$ , is equal to the concentration of toxic chemical resulting in 50% inhibition of reduction of the dye as estimated from the absorbance. This is referred to as the IC50 (inhibitory concentration, 50%).

With some chemicals, a linear plot did not provide usable data. However, when the absorbance was plotted vs. the log of the concentration of toxic chemicals, often a satisfactory regression could be fitted to the data. The equation was modified:

$$\log x = \frac{y/2 - B}{m}$$

The antilog of  $\log x$  is the concentration of chemical resulting in a 50% decrease in the absorbance. The data could be evaluated using a pocket calculator (Hewlett Packard

32SII). Alternatively, values were plotted and the regression line calculated on an Apple Computer using Cricket Graphics.

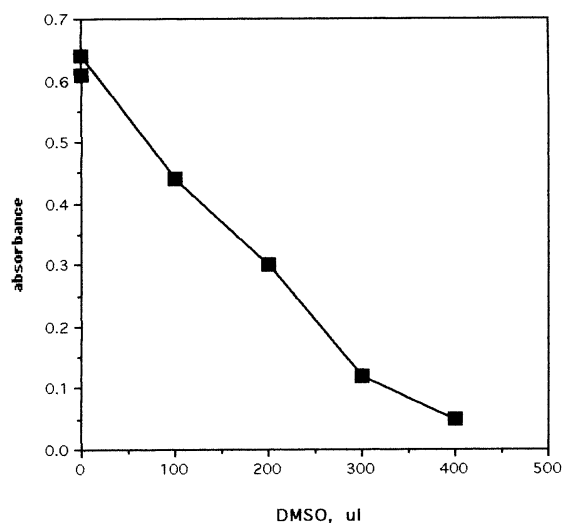
The data reported was calculated from the data with satisfactory values for the regression coefficient. If the  $R^2$  for the regression was less than 0.80, the data were not used. With  $R^2 = 0.8$ ,  $R = 0.894$ . No values are included unless there were at least 7 samples (five concentrations of the inhibitory chemical and two controls). The  $p = 0.01$  value, when  $v = 5$ , is 0.874 [5]. Thus any data from these plots is significant at the 0.01 level. All the values from experiments resulting in  $R^2 > 0.8$  were averaged and the standard deviation calculated. These values are presented in the results. For the results reported here, the concentration of the inhibitory compound varied at least tenfold. Experiments were repeated at least three times with three batches of cells. The mean and standard deviation were calculated. For nearly all the values presented, the regression coefficient was greater than 0.9. Results are expressed as ppm, part per million,  $\text{mg l}^{-1}$ , as is customary among toxicologists.

All chemicals were received from usual commercial sources and were used as received.

## RESULTS

### *Toxicity of organic chemicals*

Data for pentachlorophenol and DMSO are presented in Figures 1 and 2. These are typical results for the assay. Table 1 shows results of assays for the toxicity of a variety of organic compounds. Among the solvents normally used, DMSO was found to be the least toxic. This was used as a solvent for anything not readily soluble in water.



**FIGURE 2.** TOXICITY OF DMSO. NOTE HOW THE VOLUME OF DMSO IS PLOTTED LINEARLY. THE EQUATION FOR THE LINE IS  $Y = 0.610 - 1.500 \cdot 10^{-3} X$ ,  $R^2 = 0.985$ .

Benzene, toluene, and xylene were sampled using three approaches. First they were assayed directly—the chemical was simply added to the test tube in  $\mu\text{l}$  quantities. Water saturated solutions were prepared, the compounds were added to water, and the water saturated fraction collected with a separatory funnel. The amount of compound present in the water was determined from QSAR data [10, 11]. Finally, the volume required to make a solution with 1,000 ppm ( $1,000 \text{ mg l}^{-1}$ ) was added to water and this mixture assayed. The values varied about threefold depending on the method used. In all cases xylene was the most toxic. The data presented are for this last method. Dissolving BTX compounds in DMSO did not work.

### ***Toxic minerals***

In Table 2 results of experiments to determine the toxicity of minerals are presented. The assay is very sensitive to many minerals. However, lead, arsenic, lanthium, and iron were not inhibitory at less

than 1,000 ppm. Sodium and potassium were inhibitory but at very high levels, greater than 10,000 ppm. Calcium and magnesium were found to be toxic at concentrations below that typically used in bacteriological medium [12]. It is uncertain why these minerals inhibit reduction of the dye.

Inhibition of reduction of dye by calcium was of concern for measuring toxins in soil samples. It was found that the addition of 0.758  $\mu\text{moles}$  EDTA (0.5 ml of a 2.5 mM solution) in each tube relieved inhibition by 563  $\mu\text{moles}$  (62.5 ppm) of calcium.

Inhibition of reduction by all the metal ions was completely relieved by the addition of EDTA. The low concentration of EDTA had a slightly stimulatory effect on the reduction of the dye. EGTA, thought to be a more effective chelator of calcium, did not seem to provide protection against the effects of calcium. The EDTA relieved inhibition by all the minerals. The role of EDTA does not seem to be merely to chelate the calcium to remove it from the reaction.

While EDTA relieved inhibition by metal ions, it had little effect on inhibition by organic compounds. The effect of calcium and EDTA on toxicity was determined for pentachlorophenol, trichlorophenol, potassium cyanide, FCCP, TFFA, SDS, trichloroethylene, and tetrachloroethylene. Values were much the same in the controls and in samples with calcium and EDTA. The EDTA has little effect on inhibition of the reduction by organic compounds. Again, this suggests that the mechanism to account for inhibition of the reduction of the dye by minerals is by a different mechanism than inhibition by organic compounds.

Data are shown for experiments comparing the toxicity of trichloroethylene and tetrachloroethylene assayed in the presence and absence of calcium and EDTA. The values are comparable (Table 3). Again this

suggests two different mechanisms for reduction of the dye, one mechanism for inhibition by organic compounds and another for inhibition by minerals.

### ***Mechanism***

It has been proposed that MTT is reduced by

one of the early steps in electron transport [24]. The compounds that appear to be toxic are organic compounds known to affect membrane integrity, compounds known to inhibit electron transport, or metal ions that could compete with the iron found in cytochromes in electron transport. Aerating samples during the reduction inhibited

**TABLE 1. TOXICITY OF VARIOUS CHEMICALS<sup>a</sup>.**

Compound	n	mean	S.D.
<b>Solvents</b>			
Acetone	15	68,000	163206
DMSO	11	156,000	63960
Ethanol	8	73,362	21274
Methanol	9	66,027	27071
<b>BTX</b>			
Benzene <sup>b</sup>	4	799	143
Toluene	4	217	17.3
Xylene	4	131	55.0
<b>Organic compounds</b>			
Acetate	6	436	61.0
Phenol	7	1,433	444
Sodium lauryl sulfate	5	28.7	4.3
Chloroform	9	1,833	91.6
2,6-dinitrocresol	9	28.3	10.7
4,6-dinitrocresol	9	10.8	3.45
TNT	8	42.6	19.2
<b>Electron transport inhibitors</b>			
Dinitrophenol	11	45.4	19.06
FCCP <sup>c</sup>	16	2.13	0.894
Cyanide	13	21.4	10.7
Sodium azide	16	803	273
TFFA <sup>d</sup>	6	6.22	1.68
<b>Environmental pollutants</b>			
Trichlorophenol	23	4.49	3.23
Pentachlorophenol	8	0.397	0.189
Trichloroethylene	8	672	121
Tetrachloroethylene	5	356	60.5

<sup>a</sup>n = the number of samples used in the determination. Average is the value reported as ppm (mg ml<sup>-1</sup>). S.D. = standard deviation/mean.

<sup>b</sup>Benzene, toluene, and xylene measured in solutions with 1,000 ppm determined by the volume of compound added to water. These compounds gave much higher values when assayed directly. Water saturated solutions were prepared and the concentration of solute calculated from QSAR data [10, 23]. These values were lower than those measured directly but greater than these values presented.

<sup>c</sup>FCCP = carbonyl-cyanide p(trifluoromethoxy)phenylhydrozone, an inhibitor of electron transport.

<sup>d</sup>TFFA = thenoyltrifluoracetone.

reduction of the dye. Presumably oxygen competes with the dye for reduction. It was found that washing cells in buffer containing 100 mM EDTA inhibited reduction of the dye. EDTA removes divalent cations from the cytoplasmic membrane and alters the structure of the cytoplasmic membrane [6]. Presumably this disrupts electron transport sufficiently to preclude reduction of the dye. A bacterium, *Streptococcus lactis*, which totally lacks any electron transport [8, 9] was unable to reduce any of the dyes.

A series of experiments involving inhibitors of electron transport were carried out [7]. The results were not definitive (Table 1). These inhibitors are thought to inhibit a specific reaction in electron transport.

Presumably, if an early step of electron transport were blocked and MTT was still reduced, this would indicate that a later step was responsible for the reduction. Conversely, if the dye is reduced by a component involved in an early step of electron transport, inhibitors of later steps should have no effect. It was found that some inhibitors of early steps in electron transport also inhibited reduction of MTT (TFFA) while others did not (rotenone, antimycin A). Inhibitors of late steps, cyanide, and sodium azide also inhibited the reduction of the dye. In mitochondria, FCCP and dinitrophenol inhibit oxidative phosphorylation and stimulate oxygen consumption, that is stimulate electron transport [7]. Both these compounds were

**TABLE 2. INHIBITION OF REDUCTION OF MTT BY MINERALS<sup>a</sup>.**

Mineral	n	ppm	S.D.
Cadmium	12	0.791	0.03
Calcium	13	5.65	1.86
Cobalt	11	12.3	5.66
Copper	13	0.953	0.181
Magnesium	6	80.7	8.07
Manganese	11	1.44	0.50
Mercury	10	0.0159	0.0036
Nickel	7	58.6	6.45
Selenium	9	277	108
Zinc	10	0.847	0.059

<sup>a</sup>As in Table 1

**TABLE 3. EFFECT OF EDTA AND CALCIUM ON INHIBITION OF REDUCTION BY TRICHLOROETHYLENE AND TETRACHLOROETHYLENE<sup>a</sup>.**

	n	average	S.D.
Trichloroethylene, control	7	613	24.4
Trichloroethylene, + Ca <sup>++</sup> , EDTA	7	538	95.3
Tetrachloroethylene, control	9	335	96.5
Tetrachloroethylene, + Ca <sup>++</sup> , EDTA	8	246	50.9

<sup>a</sup>As in Table 2. The values presented in this table were not included in Table 3.

Calcium present at a concentration of 50 ppm in the reaction tube. EDTA was present at a concentration of 2.5 μmoles reaction tube<sup>-1</sup>.

found to inhibit MTT reduction.

A series of experiments were carried out to determine the nature of the inhibition of reduction of the dye by toxic compounds. The inhibition of reduction of the dye by pentachlorophenol, by copper, and by sodium dodecyl sulfate was examined by running experiments with varying concentrations of the substrate, MTT, and the toxic chemical [7]. The data were plotted with Lineweaver Burke plots. The data were fitted by regression analysis. The data showed the kinetics of the inhibition were "mixed." The inhibition does not appear to be competitive—the dye and the toxic chemical do not simply compete for electrons in the reduction. The inhibition is not non-competitive—the toxic chemical does not bind to the component responsible for reduction at a site distinct from the active site but influencing the reaction. The inhibition is not uncompetitive—the toxic chemical does not simply damage the component so it can't reduce the dye as effectively. Put simply, this kinetic analysis didn't provide any useful information concerning the nature of the reduction of the dye and its inhibition by toxic chemicals.

Alternatively, MTT could be reduced by a membrane-associated reductase. Phenazine methyl sulfonate (PMS) stimulates most reductases. PMS had no effect on the reduction of MTT but did stimulate reduction of another tetrazolium dye, INT.

MTT and INT have been shown to be reduced at an early step in electron transport in mitochondria [24]. However, since it was found that PMS does stimulate the reduction of INT, this suggests that this dye is reduced not only by electron transport but by a cytoplasmic reductase.

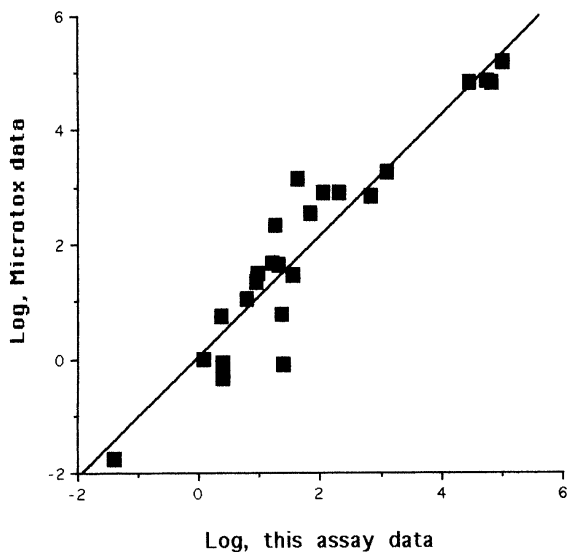
The MTT dye obviously enters the cells. After the reaction, cells can be removed from the reaction by centrifugation. The cells are blue with the reduced dye. The dye cannot be removed readily. It is possible that toxic chemicals block the transport of the dye into the cells.

Currently a series of experiments to isolate and characterize mutants unable to reduce the dye are underway. Given a mutant, it should be possible to clone the function responsible for MTT reduction. The cloned gene can be sequenced and identified.

## DISCUSSION

### *Comparisons with other tests*

The Microtox™ Test uses the bioluminescent marine bacterium *Photobacterium phosphoreum*. This bacterium uses a fluorescent pigment as a terminal electron acceptor. The bacterium emits light when growing. Toxic chemicals are thought to interfere with electron transport and to diminish the light produced by the bacterium. There are several reports in the literature comparing this test with others [15-19]. The assay has been used to measure toxicity in bioremediation sites for creosote and pentachlorophenol [13] and other wastes [14], 2-chlorophenol [19], detoxification of pesticides [20, 21], the toxicity of mycotoxins [22]. To the best of the author's knowledge, a complete description has not been published in peer reviewed literature. It is available in a kit including lyophilized bacteria, a luminometer, a refrigerated water bath, a computer, and a computer program to analyze the data obtained. It does not seem possible to carry out the assay without purchasing the kit. A similar assay using the bacterium *Vibrio harveyi* has been described [23]. A comparison of results with the Microtox™ assay and with this assay are



**FIGURE 3.** COMPARISON OF THE MICROTOX™ ASSAY AND THIS ASSAY. THE LOG OF THE VALUES IS PLOTTED. THE VALUES FOR THE MICROTOX™ ASSAY AS REPORTED IN THE LITERATURE [3, 18, 25]. WITH 24 POINTS, THE EQUATION FOR THE REGRESSION LINE IS  $Y = 1.06 - 6.55 \times 10^{-2} X$ ,  $R^2 = 0.866$ . VALUES FOR MICROTOX™ ARE FROM REFERENCE 25.

shown in Figure 3. These values were plotted and a regression line calculated. The regression coefficient for these values was 0.88, indicating that the assay methods give comparable results.

## THE POLYTOX™ TEST

The Polytox™ test is available commercially. With this assay samples are placed in flasks, an artificial consortium of 12 bacteria is added, and the utilization of oxygen is measured with the oxygen electrode or with respirometer. Toxic compounds reduce the consumption of oxygen. This has not been used extensively to assay for toxic chemicals but rather to design bioremediation programs using this consortium to degrade noxious chemicals. The toxicity of nine chemicals was compared with the two assays. The

regression coefficient for these values is 0.93. These data show that results with this assay using *R. meliloti* are comparable to results obtained with the Microtox™ and Polytox™ assays, two assays available commercially.

## The Rhizobium Assay

This assay with *R. meliloti* can be carried out in a minimally-equipped laboratory. No specialized equipment is required. A simple spectrophotometer and a 30° water bath are required. Growth of cells requires an incubator shaker. Harvesting and washing the cells requires a centrifuge. This is available in any laboratory equipped to work with bacteria. Hopefully, in the near future, lyophilized (freeze dried) bacteria will be available. Data can be analyzed with a pocket calculator. A computer is not needed.

The Microtox™ Assay requires that you purchase a kit from the vendor. This kit includes a luminometer to measure the light produced by the bacteria, a refrigerated 15° water bath, a computer, and a computer program to analyze the data. Lyophilized bacteria must also be purchased. This kit to set up the assay is quite expensive.

The Polytox™ Assay requires use of an oxygen electrode or a respirometer. Usually the data from the respirometer is analyzed with a sophisticated computer program [23]. Maintenance of respirometers can be a problem. Oxygen electrodes are expensive and are easily damaged. The consortium of 12 bacteria are purchased from the vendor. This cannot be duplicated readily in the laboratory.

In my experience, both the Microtox™ and Polytox™ assays are appropriate for graduate students committed to their research. Both require sophisticated laboratory techniques. In contrast, the assay

with *R. meliloti* has been carried out by high school students and by many undergraduates. It is a much simpler technique to learn. No special equipment is required. It can be carried out in a typical laboratory situation.

## CONCLUSIONS

This assay appears to provide an assay using a bacterium as an indicator organism that provides results comparable to the Polytox™ and Microtox™ assays yet requires fewer resources in the laboratory, is less expensive, is faster, and is much simpler than these two tests.

## ACKNOWLEDGMENTS

The author's research is funded by participation in the NIH MARC (5 T34 GM 07667-18) and MBRS (5S06 GM 08136-21) programs and a grant from the New Mexico Water Resources Research Institute. Three high school students, many undergraduates, and graduate students have contributed to the work. Dr. William Boecklen assisted with the development of the equations to calculate the toxicity.

## REFERENCES

1. B.N. Ames, J. McCann, and E. Yakasai, Methods for detecting carcinogens and mutagens with *Salmonella typhimurium* minisome test, *Mutation. Res.*, 31 (1975) 347-364.
2. B.R. Bochner and M. A. Savageau, Generalized indicator plate for genetic, metabolic and taxonomic studies with microorganisms, *Appl. Environ. Microbiol.*, 33 (1977) 434-444.
3. J.L. Botsford, Reduction of a tetrazolium dye by *Rhizobium meliloti*: A possible assay for toxic chemicals, *Can. Jour. Microbiol.*, (1996) submitted.
4. R. Gonzalez-Gonzalez, J.L. Botsford, and T.L. Lewis, Osmoregulation in *Rhizobium meliloti*: Characterization of enzyme involved in glutamate metabolism, *Can. Jour. Microbiol.*, 36 (1990) 469-474.
5. F.J. Rohlf and R.R. Sokal, *Statistical Tables*, W.H. Freeman and Co., NY, 1981, p. 106.
6. L. Leive, Studies on the permeability change produced in bacteria by ethylene-diaminetetracetic acid, *Jour. Biol. Chem.*, 243 (1968) 2372-2380.
7. C.K. Mathews and K.E. van Holde, *Biochemistry*, Benjamin/Cummings Publishing Co., Inc., Redwood City, CA, 1990.
8. J.L. Ingraham and J.C. Ingraham, *Introduction to Microbiology*, Wadsworth Publishing Co., Belmont, CA, 1995, p. 262.
9. D. White, *The Physiology and Biochemistry of Prokaryotes*, New York, Oxford University Press, 1995, p. 378.
10. N. Nirmalakhadndan and R.E. Speece, Aqueous solubility of organic chemicals: A compilation of experimental data, correlations and predictive models, *Environ. Sci. Technol.*, 22 (1988) 328-338.
11. K.L.E. Kaiser, QSAR of acute toxicity of 1,4-di-substituted benzene derivatives and relationships of the acute toxicity of corresponding mono-substituted benzene derivatives, In: K.L.E. Kaiser (Ed.), *QSAR in Environmental Toxicology II*,

- D. Reidel Pub. Co., Dordrecht, 1987, pp. 169-188.
12. J.L. Botsford, Osmoregulation in *Rhizobium meliloti*: Inhibition of growth by salts, Arch. Microbiol., 137 (1984) 124-127.
  13. J.G. Mueller, D.P. Middaugh, S.E. Lantz, and P.J. Capman, Biodegradation of creosote and pentachlorophenol in contaminated ground water: Chemical and biological assessment, Appl. Environ. Microbiol., 57 (1991) 1277-1285.
  14. B.D. Symons and R.C. Sims, Assessing detoxification of a hazardous waste using the Microtox™ bioassay, Arch. Environ. Contam. and Toxicol., 17 (1988) 497-505.
  15. J.C. Green, W.E. Miller, M.I.C. Debacon, M.A. Long, and C.L. Bartels, A comparison of 3 microbial assay procedures for measuring toxicity of chemical residues, Arch. Environ. Contam. and Toxicol., 14 (1985) 569-667.
  16. B.J. Dutka and K.K. Kwan, Comparison of three microbial toxicity screening tests with the Microtox test, Bull. Environ. Contam. Toxicol., 27 (1981) 753-758.
  17. G.A. McFeters, P.J. Bono, S.B. Olson, and Y.T. Tchan, A comparison of microbial bioassays for the detection of aquatic toxicants, Water Res., 17 (1983) 1757-1762.
  18. J.M. Ribo and K.L.E. Kaiser, Effects of isolated chemicals to photoluminescent bacteria and their correlations with acute and sublethal effects on other organisms, Chemosphere, 12 (1983) 1421-1442.
  19. M.-C. Lu, G.-D. Roa, J.-N. Chen, and C.-P. Huang, Microtox bioassay of photodegradation products from photocatalytic oxidation of pesticides, Chemosphere, 27 (1993) 137-1647.
  20. L. Somasudaram, J.R. Coats, K.D. Racke, and H.I.Y. Stahr, Application of Microtox system to assess the toxicity of pesticides and their hydrolysis metabolites, Bull. Environ. Contam. Toxicol., 44 (1990) 254-25.
  21. I.E. Yates and J.K. Porter, Bacterial bioluminescence as a bioassay for mycotoxins, Appl. Environ. Microbiol., 44 (1982) 1072-1075.
  22. K.W. Thomulka and D.J. McGee, Detection of biohazardous materials in water by measuring bioluminescent reduction with the marine organism *Vibrio harveyi*, Jour. Environ. Sci. Health, A28 (1993) 2153-2166.
  23. B. Sun, N. Nirmal-akhandan, E. Hall, X.H. Wang, J. Prakash, and R. Maynes, Estimating toxicity of organic chemicals to activated-sludge micro-organisms, Jour. Environ. Engin., 120 (1994) 1459-1469.
  24. G. Bitton and B.J. Dutka, Toxicity testing using microorganisms, CRC Press Inc., Boca Raton, FL, 1986.
  25. K.L.E. Kaiser and V.S. Palabrica, *Photobacterium phosphoreum* toxicity data index, Water Pollution Research Journal, Canada, 26 (1991) 361-431.