



A SIMPLE, INEXPENSIVE, AND RAPID METHOD TO DETERMINE TOXICITY USING A BACTERIAL INDICATOR ORGANISM

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ABSTRACT

A simple, inexpensive and rapid method to determine the toxicity of compounds, isolated chemicals, soil samples, and water samples has been developed. The test uses the bacterium, *Rhizobium meliloti*, as the indicator organism. Toxicity values obtained with this test are comparable to those obtained with more familiar tests like the sand flea *Daphnia magna*, Microtox™, and tests using animal and human cells grown in culture. No specialized equipment is required, only a spectrophotometer and a water bath. No specialized training is required. Only rudimentary chemical skills are required to perform the assay which takes less than an hour. The test could be adopted in developing nations without sophisticated laboratories, with very modest budgets and with relatively unskilled personnel. It could be included as an alternative test for toxicity in sophisticated laboratories. A patent has been obtained and we are seeking a firm to market the assay. However, the test could be used by any laboratory able to grow bacteria. Detailed information is available in the published literature.

Key words: toxicity, compounds, *Rhizobium meliloti*

INTRODUCTION

The author has developed a test for toxic chemicals and for water and soil samples that is simple, inexpensive, and rapid. It uses a bacterium as the indicator organism so animal rights advocates are in favor of it. This test can be used by any laboratory able to grow bacterial cells. We hope to market the assay in the near future. This presentation will outline how the test is conducted, how the test deals with divalent cations that are perceived as toxic, and how the test compares with other more familiar tests.

THE TEST

Rhizobium meliloti can reduce the thiazole tetrazolium dye MTT very readily. The dye turns dark blue when reduced and this can be followed with a simple spectrophotometer. Toxic chemicals inhibit the reduction. The utility of the test has been discussed (Botsford et al., 1997, 2001); the

mechanics of the test have been described in detail (Botsford, 1998); and protocols for carrying out the assay have been published (Botsford, 1999). Cells are grown in a simple defined medium (Gonzalez-Gonzalez et al., 1990). Cells are collected by centrifugation in a refrigerated preparative centrifuge. Cells are washed with phosphate buffer and are diluted with the buffer. Varying volumes of the toxin to be tested of a known concentration are added to each tube, and the tubes are brought to a constant volume. Buffer and cells are added to each tube. The absorbance is read (550 nm) at time = 0. The MTT dye is added, and the tubes are shaken vigorously and incubated at 30°C for 20 minutes. The tubes are shaken again and placed in an ice bath. The absorbance is read again. The change in absorbance for each sample during the 20-min. incubation is plotted against the volume or log volume of the sample tested. A regression line is fit to the data, and

from the values for the absorbance in the control receiving no toxin, the Y-intercept, the slope for the regression line, and the volume of toxin inhibiting reduction of the dye by 50% is determined. If the regression coefficient is less than 0.8, the experiment is discarded as being statistically questionable (Rolf and Sokal, 1981). From this volume and the initial concentration of the toxin, the concentration of the toxin inhibiting the reduction of the dye by 50% is calculated. This provides the IC50 (50% inhibitory concentration). This parameter is comparable to an LD50 value. These calculations can be carried out with a pocket calculator able to determine regressions (Hewlett Packard 32SII) but more often a computer graphics program, Cricket Graphics, is used to plot the data and to carry out the calculations.

A method to lyophilize cells has been developed and cells can be reactivated months after lyophilization (Robertson, 1996). The lyophilized (freeze dried) cells can be suspended in water and in a few minutes, these cells can perform the assay as well as fresh cells. Cells remain active, if kept on ice, all day. Sensitivity to toxins is increased in cells from the exponential phase as contrasted with cells in the stationary phase. It is planned to market a kit with instructions, a training video, a computer program to permit data to be analyzed more simply, and chemicals and lyophilized cells required for several hundred assays.

COMPARISONS WITH OTHER TESTS

As noted, more than 200 chemicals have been tested using the *Rhizobium* assay and this

has permitted values for toxicity with this assay to be compared with values from the literature. For example, values for 50 MEIC chemicals (multicenter evaluation of in vitro cytotoxicity) were tested by Calleja's group in Belgium (Calleja et al., 1993) and by Lilius' group in Finland (Lilius et al., 1994), using the standard *Daphnia magna* assay technique. With *Daphnia*, this would provide LD50 data, the amount of toxin killing half the animals. These authors did not compare the two assays. In the principal investigators' laboratory, the values were entered into the computer, the log of each value determined, and the results plotted—the log of values from the Calleja work on one axis, the log of values from the Lilius work on the other. A regression line was fit to the data; the regression coefficient was determined and from this the correlation coefficient was calculated. For this comparison, a correlation coefficient of 0.895 was calculated. Had the results been identical, the correlation coefficient would have been 1.000. This shows that even with a very well-defined method using a selected indicator organism, two laboratories with different personnel and different lots of chemicals could not achieve identical results.

Results of comparisons of the *Rhizobium* assay with 24 other tests are shown in Table 1. Most of these tests are LD50 tests. Rats, mice, *Daphnia magna*, the various fish, and the bacteria *E. coli* and *B. subtilis* are LD50 tests in which the animals are divided into groups and the concentration that results in the death of half the animals is noted. HLD (human lethal dose)

is data from human autopsies obtained in Scandinavian countries of individuals dying of poison. The concentrations are only estimates. IC50 determinations, studies with hepatocytes,

Table 1. Comparison of the *Rhizobium* test with other methods.

Comparison	n	CC	references
rats LD50	31	0.742	Calleja et al., 1993
mice LD50	29	0.686	Calleja et al., 1993
<i>Daphnia</i> 1	34	0.859	Calleja et al., 1993;1994
<i>Daphnia</i> 2	34	0.890	Lilius et al., 1994
<i>Daphnia</i>	19	0.817	Munkittrick et al., 1993
<i>E. coli</i>	20	0.641	Kerzman 1993
<i>B. subtilis</i>	20	0.619	Kerzman 1993
guppies	8	0.950	Konneman 1993
trout	12	0.788	Munkittrick et al., 1993
fathead minnow	16	0.740	Munkittrick et al., 1993
fathead minnow	20	0.872	Geiger et al., 1991
HLD	31	0.741	Calleja et al., 1993
IC50	22	0.827	Halle, et al., 1994
rat hepatocytes	33	0.788	Shrivastava et al., 1994
trout hepatocytes	31	0.760	Lilius et al., 1994
ascites tumor cells	34	0.870	Romert et al., 1994
QSAR	19	0.779	Sun et al., 1993
Polytox™ 1	19	0.810	Sun et al., 1993
Polytox™ 2	11	0.796	Elnabarawy et al., 1994
sludge	16	0.853	Sun et al., 1994
Biotox™	24	0.913	Kahru and Borchardt 1994
Microtox™	15	0.758	Munkittrick et al., 1993
Microtox™	36	0.819	Kaiser and Palabrica, 1991
Microtox™	34	0.793	Calleja et al., 1993
average	24	0.796	

n = number of toxic compounds in the comparison

CC = correlation coefficient for the comparison

and ascites tumor cells are determinations using animal and human cells grown in the laboratory. The effect of toxic chemicals is estimated using a variety of methods in the isolated cells. Specific enzymes can be assayed. There are morphological changes that can be followed. Cells stressed with a toxin cannot take up some dyes readily. QSAR is a method to predict the toxicity of a compound, knowing its structure, and how hydrophobic (water insoluble) it is. Polytox™ and tests using sewage sludge note the oxidation of glucose. Toxic compounds inhibit the oxidation. Microtox™ and Biotox™ note the inhibition of light production by bioluminescent marine bacteria by toxins.

These data show that if a compound is toxic in one test, in all probability this same compound will be toxic in the *Rhizobium* test. The average correlation coefficient for all these comparisons with *Rhizobium* data was 0.800. The *Rhizobium* test is most comparable to *Daphnia* and fish among the animals, to tests using isolated human and animal cells, and to Microtox™. The *Rhizobium* test is not only simple, inexpensive, and rapid, but it provides results comparable to other tests. From the few comparisons in the toxicological literature, it cannot be determined what is an acceptable correlation coefficient.

In these comparisons, we are asking, if a chemical is toxic by one test, will it be found to be toxic by the second test? In Table 2, values for 34 chemicals measured, using eight different tests, are presented. This shows the type data that were utilized to develop the data in Tables 1 and 3. The data in this table show the immense

diversity observed in different assays. Ten of the chemicals tested with rat LD50 had the highest values and were the least toxic of the eight assays. But two chemicals, Warfarin and caffeine, were lowest, and most toxic for the rat LD50 system. This is typical. Many tests are insensitive to a few chemicals and don't perceive them as toxic, and these same tests will be extremely sensitive to other chemicals. However, with the exception of NaCl and KCl in some tests, all the chemicals in this table were toxic for all of the test systems used.

In Table 3, the correlation coefficients generated comparing the eight test methods with the 34 chemicals tested are presented. These values were generated using the data presented in Table 2. The results include the slope of the regression line. If two tests provide identical results, the slope should be = 1.00. Similarly, if the two determinations are identical, the regression coefficient and the correlation coefficient (square root of the regression coefficient) should be = 1.00.

Values from the literature from one test could be compared with both the *Rhizobium* test and the Microtox™ test. Twenty-one tests were compared. The average correlation coefficient for the *Rhizobium* tests was 0.800, and the average correlation coefficient for the tests with Microtox™ was 0.705. This indicates that the *Rhizobium* test provides results that are more comparable to other methods than does the Microtox™ test. It should be noted that the results with the *Rhizobium* test do compare well with results from the Microtox™ test (Table 3).

MECHANISM FOR REDUCTION OF THE DYE

It was found that many bacteria can reduce the dye more readily than can *R. meliloti*, but in none of these bacteria were the reduction of the dye inhibited by common toxic chemicals (Bochner and Savageux, 1977).

Eleven strains were screened with six toxic chemicals and for some, one or two would appear to be a little toxic, to inhibit reduction a little, but only in *R. meliloti* were all six toxic.

It is thought that tetrazolium dyes were reduced by cytochromes (Altman, 1976), but

Table 2. Comparison of 8 tests.

	Rhizobium	Microtox™	IC50	Daphnia	rat hepatocytes	ascites tumor cells	rat LD50	HLD
1,1,1-trichloroethane	0.583	0.324		6.71	75.2	7.63	77.6	1.48
acetomenaphen	1.649	2.187	1.45	0.269	10.7	0.57	15.8	1.698
acetylsalicylic acid	1.106	0.145	1.7	0.933	2.66	1.26		2.818
amitriptyline	0.015	0.0776	0.1	0.018	0.07	0.062	1.15	0.2
barium chloride	0.109			0.794	0.47	1.29	1.35	
caffèine	1.02	3.46	1.58	3.39	1.6	5.57	1	1
carbon tetrachloride	1.91	4.79	22.1	126	4	24.6	15.1	0.447
chloroform	5.29	12.9		2.63	6.2	9.08	7.58	1.74
chloromaphenicol	4.33	1.12	0.54	5.25	0.402	0.82	7.7	1.23
copper II sulfate	0.007	0.01		0.001	0.048	0.144	1.86	0.316
dichloromethane	4.122	37.1		10.5	109.1	19.8	18.6	4.17
digoxin	0.231		0.12		0.271	0.165	0.043	1.4 e-3
ethanol	1643	691	158	234	451	311	151	109.6
ethylene glycol	3427	1778	322	1202	358	589	75.8	25.7
hexachlorophene	2 e-4	0.02	6.8 e-3	2.7 e-4	0.002	0.005	0.138	0.11
iron II sulfate	0.32	0.708		0.302	1.62	2.19	2.089	3.02
isopropyl alcohol	950	380	90.5	155	304	175	83.2	41.7
lindane	0.141	21.9	0.36	0.005	0.144	0.1	0.263	
malithion	0.112	0.479				0.13	0.871	0.741
mercury II chloride	6 e-5	1.5 e-4		1.3 e-4	0.003	0.018	3.72	0.107
methanol	2130	912	673	661	906	673	178	52.5
nicotine	6.1	0.224	4.52	0.023	3.581	2.5	0.309	0.011
oprphenadrine HCl	1.168	0.005		0.033	0.114	0.35	0.832	0.098
paraquat	0.262	2.344		0.1	1.77	0.002	0.224	0.166
pentachlorophenol	5.4 e-4	0.02	0.025	0.002	0.05	0.007	0.1	0.109
phenol	13	0.186	4.25	0.077	0.797	1.75	3.39	1.48
potassium chloride	290	490		490	92.2	53.7	34.6	0.288
potassium cyanide	0.225	0.275	1	0.014	0.783	0.62	0.078	0.044
quinidine sulfate	0.422	1.202	0.036	0.079	0.129	0.23	0.617	
sodium chloride	288	562		60.3	102	107	51.3	17
sodium oxalate	>13	5.43			0.582	17.4		
theophylline	1.926	13.8		2.63	2.18	3.84	1.349	0.724
warfarin	1.781	0.209	0.67	1.549	0.139	0.27	0.005	0.0219
xylene	1.24	0.0794		0.851	17.47	1.34	34.6	0.468

Bold face type indicates method with lowest (most toxic) value.

this has been questioned (Marshall et al., 1993; Berridge and Tan, 1993). In eucaryotic cells, the cytochromes are in the mitochondria, and Marshall's group has found that the dyes are reduced in preparations from cells with the mitochondria removed. It has been found that a mutant of Escherichia coli, lacking one of

two major cytochromes found in this bacterium, is unable to reduce MTT (Botsford, unpublished).

A cytochrome may not be involved. There are enzymes that could reduce the dye. These enzymes could be membrane associated, and toxic chemicals affecting the membrane could

Table 3. Comparisons among the tests based on data presented in Table 2.

test		slope	reg coef	corr coef
Rhizobium	Rat LD50	0.369	0.378	0.614
Rhizobium	Microtox	0.767	0.717	0.846
Rhizobium	HLD	0.364	0.346	0.588
Rhizobium	Daphnia	0.883	0.789	0.888
Rhizobium	IC50	0.654	0.870	0.933
Rhizobium	rat hepatocytes	0.678	0.764	0.874
Rhizobium	ascites tumor cells	0.672	0.754	0.868
Daphnia	IC50	0.618	0.773	0.879
Daphnia	rat hepatocytes	0.689	0.780	0.883
Daphnia	ascites tumor cells	0.673	0.767	0.875
rat LD50	Microtox	0.900	0.332	0.576
rat LD50	HLD	0.839	0.656	0.809
rat LD50	Daphnia	1.185	0.473	0.688
rat LD50	IC50	0.838	0.599	0.774
rat LD50	rat hepatocytes	0.980	0.575	0.758
rat LD50	ascites tumor cells	0.958	0.557	0.746
Microtox™	HLD	0.408	0.530	0.728
Microtox™	Daphnia	0.917	0.701	0.837
Microtox™	IC50	0.730	0.686	0.828
Microtox™	rat hepatocytes	0.701	0.679	0.824
Microtox™	ascites tumor cells	0.649	0.578	0.760
HLD	Daphnia	1.345	0.492	0.701
HLD	IC50	0.659	0.494	0.702
HLD	rat hepatocytes	0.659	0.494	0.702
HLD	ascites tumor cells	0.822	0.477	0.690
IC50	rat hepatocytes	1.034	0.910	0.953
IC50	ascites tumor cells	1.057	0.944	0.971
rat hepatocyte	ascites tumor cells	0.842	0.720	0.848
average		0.781	0.608	0.790

reg coef, regression coefficient; corr coef, correlation coefficient

These are based on the 34 comparisons using the data in Table 2.

affect the activity of the enzyme. Toxic chemicals could also interact with the enzyme *per se* and inhibit its activity. It has been observed that the dye is taken into the cells before it is reduced. In the microscope, small dark blue spheres of the reduced dye can be seen inside the cells. Toxic chemicals could interfere with the uptake of the dye into the cells. Uptake of compounds is catalyzed by membrane-associated proteins. The toxic chemicals could affect the relationship of the membrane components and the transport proteins.

Five transposon-insertion mutants, unable to reduce the dye, have been isolated. All grow very slowly in minimal media supplemented with 0.1% casamino acids, and obviously all have lost a critical function. With these mutants, it should be possible to clone, to sequence, and to identify the function responsible for reduction of the dye.

It would be useful to understand the mechanism responsible for reduction of the dye. The test would be accepted more readily by the scientific community. Experiments could be designed to determine why some compounds are more toxic than others. *Rhizobium* is easily manipulated genetically, and it would be possible to find mutants in which toxic chemicals no longer inhibited reduction. This could delineate critical regions of the function. It might also be possible to “engineer” new strains more sensitive to some classes of toxic compounds. A firm involved in studies of arsenic has asked if a strain able to determine toxicity of arsenic might be constructed.

TOXICITY OF DIVALENT CATIONS

Reduction of the dye is inhibited by divalent cations in the *Rhizobium* system. Mercury and cadmium were found to be the most toxic with this assay. Copper and zinc were also toxic. Common ions, calcium and magnesium, inhibit the reduction of the dye. Water and soil samples typically contain calcium and magnesium, so in order to analyze water and soil samples for toxic organic chemicals, a method to eliminate this inhibition by metal ions was sought. This has not been examined carefully with the Microtox™ assay. There is data in the literature indicating that mercury, cadmium, copper, and zinc, metals ions thought to be toxic, are toxic for the Microtox™ assay. However, there is nothing in the literature reporting if calcium and magnesium are toxic for Microtox™. Microtox™ may not provide useful data with water samples.

EDTA is used routinely in biochemistry to chelate divalent metal ions, and it was thought this might chelate the calcium and remove it from the system. It was found that 2.5 μmoles EDTA would eliminate toxicity of 4 μmoles calcium but of only 0.015 μmoles mercury. Most organic compounds could be assayed in the presence of this much EDTA. EGGA and EGTA, also chelators, did not relieve inhibition by divalent cations. It may not be simple chelation; there may be some specific interaction between EDTA and the function responsible for reduction of the dye. This work has been published (Botsford, 2000b).

Soil samples with as much as 5 gm (45 mM) calcium per kg soil have been assayed using 2.5 μ moles EDTA in each sample (Hillaker, 1996). The calcium is complexed with sulfate and phosphate ions and the calcium is not available to the cell, and it is not seen by the mechanism that reduces the dye. Levels of free soluble calcium and magnesium in water are very low. We have found that 2.5 μ moles of EDTA relieves the inhibition caused by divalent cations in all water and soil samples tested thus far.

OTHER METHODS TO DETERMINE TOXICITY

Animal tests for toxicity

Typically the toxicity of chemicals is determined using animals, usually rats and mice. Animals of comparable age and sex are divided into groups of 10 animals. Each group receives a different dose of the chemical, and it is determined which concentration of the toxic chemical kills half the animals in the group (Rodericks, 1992). At the very least, the survivors are ill from receiving the toxin. The animals are maintained until death, and this can take weeks. As a consequence, the tests are very expensive. Animal facilities must be maintained, and highly skilled personnel are required. The autopsy can require a veterinarian. The results are often complex and can require sophisticated statistical analysis. In the United States it is estimated that as many as 30 million animals are used each year to test for toxic chemicals.

Rodents have an organ, the caecum, between the large and the small intestine. In a

rodent this can be vary large, almost as large as the small intestine, and is full of bacteria. It is not known what the bacteria in this pouch could do with toxic chemicals. Animals are force fed the toxic substances; toxins are forced into their stomach with a syringe. The chemicals come in immediate contact with the highly acidic contents of the stomach, and it is uncertain what the effect of this acidity has on toxic compounds.

One disadvantage of these sorts of animal tests is that they are not always definitive. Ruelius (1977) has questioned use of animals in testing. Ekwall et al. (1988) found poor agreement between tests with rats and mice. Bradie and Reid (1967) report that many drugs are much less toxic for rats and mice than for humans. There are anecdotal reports on the Web about tests being negative for one animal yet toxic for another ([www.animal tests for toxic chemicals.com](http://www.animaltestsfortoxicchemicals.com)). It is significant that nine drugs have been removed from the American market this year. These include Rezullin, a drug for Type II diabetes, and the very popular allergy drug Seldane. These drugs were all tested thoroughly with animals but once released, proved to be toxic for humans. And we remember the drug Thalidomide many years ago that was tested carefully yet had tragic results with humans.

Fish, the fathead minnow, *Pimphales promeleas*, and trout hatchlings are often used to test for toxic chemicals. Minnows are grown in huge tanks and the young are isolated, sexed, and used in tests. Trout hatchlings are obtained from trout hatcheries. Typically chemicals are

more toxic for fish than for animals. Fish and the water flea *Daphnia magna* are tested by having the animals swim in solutions of the toxin. Animals seem to be more sensitive to toxins administered this way. These are typical LD 50 tests but are rarely run for more than 96 hours. The fish are not autopsied; it cannot be determined which organ has been harmed by the toxin.

Daphnia magna

Tests for toxicity are also run using the water flea *Daphnia magna* (Stephenson et al., 1990). This very small crustacean can be grown in the laboratory using alga grown in the laboratory as food. The animals are isolated using a dissecting microscope. Animals 48 hours after hatching are isolated and are combined in water with different concentrations of the toxic chemical. The animals are observed for 48 hours, and it is noted when animals stop swimming, indicative of death of the animal. They are very inexpensive; the crustaceans can be grown readily in the laboratory. However, the tests require highly skilled laboratory personnel. These tests require skills not normally developed by those comfortable with chemical analysis. Any laboratory wishing to use this assay must make a commitment to running the tests and to having an individual trained in the technique.

Alternatives to animal testing

Animal rights advocates and those who question the value of animal tests seek "alternative tests" for toxic chemicals. In Europe there has been tremendous interest in developing tests

using animal and human cells grown *in vitro*, in laboratory culture (Clemendson et al., 1993). Many different cell lines are used; different techniques to determine the effect of the toxin on the cell are used. For the most part, the different tests agree well; a compound found to be toxic with one test will be toxic for another test. The tests can take as much as a week to complete. And the tests require sophisticated laboratory facilities to grow the cells and highly trained technical personnel to run the tests. This is not third world technology.

Bacterial methods

There have been countless tests developed using various bacteria as the indicator organism (Bullich and Dutka, 1986). However, only one, Microtox™, has been adopted by the toxicological community (Ribo and Kaiser, 1987). This test for toxic chemicals uses the bioluminescent marine bacterium *Photobacterium phosphoreum*, which produces light. There is a pigment in the bacterium that serves as a terminal electron acceptor making the pigment luminescent. Toxic chemicals inhibit the production of light presumably because toxic chemicals impair electron transport. The test is rapid; results can be obtained in as few as 5 minutes, but most laboratories incubate the cells for at least 20 minutes. Cells must be held in a refrigerated incubator shaker. And the light produced must be measured with a luminometer, an instrument unfamiliar to most laboratory personnel. A kit to perform the test is offered by the Azure Biochemical Company, a subsidiary of Beckman Instruments, for

\$20,000. The kit includes a refrigerated incubator, a luminometer, and a sophisticated computer program to analyze the data (Bullick et al., 1990). Despite the complexity, Microtox™ was selected as the best assay among a number of tests (Toussaint et al., 1992).

This method has two disadvantages. The cost of the kit to run the test is more than most laboratory budgets can readily absorb. The method requires that at least one person in the laboratory master the test, and make a commitment of time and effort to learn to run the assay. At this university, graduate students master the test but it takes time and effort. This commitment of funds and manpower indicate that the laboratory must be committed to assays of toxicity. It should be pointed out that the *Rhizobium* assay gives results more comparable to other tests than does Microtox™. The *Rhizobium* assay requires no special equipment, only equipment normally found in analytical laboratories, and the *Rhizobium* test is simple enough for high school students to master. It is learned readily.

Polytox™ is a method offered by the Polybac Corporation to determine toxicity. It utilizes a consortium of bacteria isolated from sewage sludge. The bacteria oxidize a carbon source (glucose) and oxygen consumption is measured with a respirometer or with oxygen electrodes. This is not used frequently to determine toxicity (Sun et al., 1994) but to determine if sewage effluent will harm a sewage system. Both respirometers and oxygen electrodes are expensive, difficult to maintain, and

require careful training of the personnel who use them. Again a laboratory must make a firm commitment to this methodology.

Toxitrac™ is marketed by the Hach Chemical Company. This method uses a reductase from a bacterium and toxic chemicals inhibit the reductase (Liu, 1988). Hach has not marketed it successfully and it is not used often.

QSAR

QSAR is a method of predicting the toxicity of a compound (Nirmalakhanda and Speece, 1988). The structure of the molecule, the hydrophobicity, and other parameters are combined in a very sophisticated computer program to predict the toxicity. It has been compared with values from Polytox™ and activated sewage sludge (Sun et al., 1994). This technique has been used to estimate the toxicity of many chemicals, but QSAR data is not often encountered in the toxicology literature.

It should be noted that no one organism can be considered to be the ideal indicator. Algae are more sensitive to toxic metal ions than are vascular plants, but vascular plants are more sensitive to herbicides than are algae. The choice of indicator depends on the nature of the toxic chemical examined. It was found that the *Rhizobium* assay was much more sensitive to the toxicity of 30 herbicides tested than were rats or birds, but was not as sensitive as *Daphnia magna* or trout hatchlings (Hillaker, 1996).

SUMMARY

This work shows the utility of this simple test with *R. meliloti*. Any laboratory able to grow bacteria should be able to adopt it.

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