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Toxicity of Herbicides Determined with a Microbial Test

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The assay used to determine the toxicity of herbicides involves a chemical reaction in a bacterium, *Sinorhizobium meliloti.* This bacterium readily reduces a thiazole tetrazolium dye, MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide). Toxic chemicals inhibit the reduction of the dye. The test is quantitative, the inhibition of reduction of the dye is proportional to the concentration of the toxin. This method is inexpensive, rapid and simple to carry out (Botsford, 1998). With this assay, more than 250 chemicals have been assayed and results are comparable to 23 other tests (Botsford, 2002).

Many tests to quantify toxicity using bacteria have been proposed (Bitton and Dutka, 1986). Only one, a method using a bioluminescent marine bacterium *Photobacterium phosphoreum,* is used widely. The ability of the bacterium to produce light is inhibited by toxic chemicals, presumably because of inhibition of electron transport and the concomitant reduction of the luminescent pigment. A kit, Microtox™ to perform this test, marketed by Strategic Diagnostics Inc., is available commercially. More than 1000 chemicals have been tested (Kaiser and Palabrica, 1991).

The toxicity of 30 herbicides used on the Lyendecker Plant Science Research Center farm at New Mexico State University was determined using this test. The manufacturers of the herbicides include in the MSDS (Material Safety Data Sheets), reports of the toxicity of the herbicides tested with rats, mallard ducks, quail, the sand flea *Daphnia magna,* and trout fingerlings. These values were compared with the results using this test with *S. meliloti.* The assay was also used to estimate the half-life of 3 herbicides in soil.

MATERIALS AND METHODS

The assay used in this study has been described in detail (Botsford et al. 1997, Botsford 1998, Botsford, 1999). The bacterium, *S. meliloti*, has been studied for its role in biological nitrogen fixation. The reduction of the dye MTT can be

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followed spectrophotometrically. The reduced dye absorbs light strongly at 550 nm. The unreduced dye does not absorb light. The concentration of the toxin inhibiting the reduction of the dye 50% is determined. This IC50 (inhibitory concentration, 50%) is comparable to the LD50 (lethal dose, 50%), the value obtained with traditional animal tests. It was observed that nearly all bacteria can reduce MTT. But only in *S. meliloti* was reduction of the dye inhibited by toxic compounds. There is something unique about the mechanism affording reduction of the dye in this bacterium that makes it sensitive to toxic chemicals.

Cells were grown in a semidefined medium (Botsford, 1998) and were washed in dilute phosphate buffer. In an assay, cells, buffer and varying volumes of the toxic chemical were mixed. The optical absorbance of the sample was measured at time = 0 and the dye was added. The samples were incubated 20 minutes at 30 C and the absorbance was measured again. The data were plotted, the change in absorbency versus the volume of toxin used. A regression line was fit to the data. With values for controls not receiving the toxin, the Y intercept and the slope of the regression line it is possible to calculate the volume of toxin inhibiting reduction of the dye 50%. Knowing the concentration of the toxin used, the concentration comparable to the critical volume can be calculated. More than 250 chemicals have been assayed and results are comparable to other tests (Botsford, 2002)

Seven of the herbicides were dissolved in DMSO: cyanazine, diuron, EPTC (s-ethyl disopropyl thiocarbamate), napropamide, nicosulfuron, norflurazon and primisulfuron. The DMSO does not appear to affect the apparent toxicity of the herbicides in the volumes used in these experiments. The other herbicides were dissolved in water.

Some of the herbicides were found to lose toxicity with time. Stock solutions were made at weekly intervals. Paraquat readily accepts electrons and becomes reduced to form the paraquat radical. This can reduce molecular oxygen to form the superoxide radical (Ahrens 1994). None of the herbicides affected the absorbancy of the dye in the absence of the bacteria. Solutions of some were not clear when dissolved in water. Trifluralin was bright yellow and sethoxydim was milky white. This was corrected by the measurements of absorbance at time = 0.

The herbicides were used exactly as obtained from the University farm. No effort was made to isolate the active agent in the herbicide. It is not known whether the inactive agents added to the herbicides to make them marketable contribute to the toxicity. We produced values representative of the toxicity of the herbicide available to the public. The common name, the manufacturer's name and the source for the herbicides are presented in Table 1. The herbicides can be found on the WEB under the common name or the manufacturers name

Table 1 Common name, manufacturers name and source for herbicides.

Common Name	Manufacturer's name	Source
2,4-D	Weedar 64	Union Carbide
Alachlor	Bronco	Monsanto
Bensulide	Prefar 4E	UHS, others
Bromoxyni	Buctril	Rhone-Poulen
Clomazone	Command 4EC	FMC Corporation
Cyanazine	Bladex 90DF	DuPont
DCPA	Dacthal	GFS Chemicals
Dicamba	Clarity	Sandoz Crop Protec.
Diuron	Karmex	Drexel Chemical Corp.
EPTC	Eptam	Syngenta
Ethalfluralin	Sonalan	Dow Elanco
Fluazifop-P	Fusilade DX	Zeneca
Glyphosate	Roundup	Monsanto
Imazapyr	Arsenal	American Cyanamid
Imazethapyr	Pursuit	American Cyanamid
Isoxaben	Gallery	Dow Elanco
Mepiquat chloride	Pix	BSAF
Metasulfuron	Ally	DuPont
Metribuzin	Lexone DF	Mobay Corporation
Naproamide	Dvrinol	United Phosphorous
Nicosulfuron	Accent	DuPont
Norflurazon	Zonal Rapid 80	Syngenta
Oxadiazon	Ronstar	Aventis Crop Protection
Paraquat	Gramoxone	Syngenta
Primisulfuron	Beacon	Ciba-Geigy
Qunichlorac	Facet 75 DF	BSAF
Sethoxydim	Poast	BSAF
Thiazopyr	Visor 2E	Rohm and Haas
Thifensulfuron	Pinnacle	DuPont
Trifluralin	Treflan EC	Dow Elanco

RESULTS AND DISCUSSION

The IC50 values determined for the 30 herbicide formulations tested are presented in Table 2.

Table 2. Herbicide toxicity (mg L)

	n	mean ± S.D.
2,4-D	9	347 ± 135
Alachlor	6	111 ± 8.88
Bensulide	7	53.7 ± 9.66
Bromoxynil	8	26.0 ± 4.94
Clomazone	15	23.8 ± 3.81
Cyanazine	13	781 ± 2.10
DCPA (Dacthal)	6	427 ± 148
Dicamba	6	>1200
Diuron	9	87.0 ± 13.1
EPTC	9	51.4 ± 3.08
Ethalfluralin	6	>1200
Fluazifop-P	6	>1200
Glyphosate	6	18.1 ± 2.17
Imazapyr	6	229 ± 2.29
Imazethapyr	10	353 ± 49.4
Isoxaben	16	365 ± 58.4
Mepiquat-Chloride	6	>1200
Metasulfuron	6	>1200
Metribuzin	6	>1200
Naproamide	10	289 ± 28.9
Nicosulfuron	10	267 ± 29.4
Norflurazon	8	182 ± 25.5
Oxadiazon	6	269 ± 43.0
Paraquat	7	48.9 ± 5.86
Primisulfuron	6	269 ± 159
Quinclorac	6	>1200
Sethoxydim	6	2.70 ± 0.46
Thiazopyr	6	43.2 ± 1.78
Thifensulfuron	7	928 ± 37.2
Trifluralin	7	10.3 ± 1.84

n = number of samples tested. S.D.= standard deviation.

The IC50 values obtained in this study were compared with the values reported by the manufacturers of the herbicides. These values are shown in Table 3. The protocol used to determine the toxicity by the manufacturers is not known.

Table 3. Comparison of assays for toxicity (mg /L.)

Herbicide	*Sinorhizobium meliloti*	Rat Assay	Rainbow Trout	*Daphnia magna*
2,4-D	347	639-764	377	25
Alachlor	111	930-1350	5.3	10
Bensulide	53.7	770	0.72	--
Bromoxynil	26.0	440	0.1	0.11
Clomazone	23.0	1369-2077	19	5.2
Cyanazine	781	182-334	9	49
DCPA	427	>10,000	--	--
Dicamba	>1200	1707	135	110
Diuron	86.8	3400	--	--
EPTC	51.4	1652	19	--
Ethalfluralin	>1200	--	--	--
Fluazifop-P	>1200	>5000	1.37	>10
Glyphosate	18.1	>5000	8.2 - 86	5.3 -780
Imazapyr	229	>5000	>100	>100
Imazethapyr	353	>5000	340	>1000
Isoxaben	365	>10,000	>1.1	>1.3
Metasulfuron	>1200	>5000	>150	>12.5
Metribuzin	>1200	1090-1206	76	4.5
Naproamide	289	>5000	16.6	14.3
Nicosulfuron	267	>5000	>1000	>1000
Norflurazon	182	9000	8.1	>15
Oxadiazon	269	>5000	1-9	0.5 - 8.0
Paraquat	47.9	112-150	32	--
Primisulfuron	269	>5050	210	260
Quinclorac	>1200	>2610	>10	--
Sethoxydim	2.70	4.1-500	32	--
Thiazopyr	150	>5000	3.4	5.9
Thifensulfuron	92	>5000	>250	470
Trifluralin	10.3	>5000	0.041	0.56

Toxicity for Sinorhizobium is expressed in ppm, the IC50 value. The toxicity for rats is reported as mg /kg body weight, the LD50. Toxicity for rainbow trout and for *Daphnia* reported as the LC50, the concentration of toxin in the water (mg/l) resulting in death of half the animals. Values for tests run with animals are from the MSDS provided by the manufacturers of the herbicide.

The statistical significance of the values is uncertain. Values for mallard ducks and for quail were comparable to values for the rat. These values are simply presented without comment in the MSDS for the herbicide. Presumably values for the rat are

for toxins administered orally in the conventional fashion (Rodericks, 1992). These values can be obtained from the WEB.

Typically, chemicals were more toxic when measured with tests run with *Daphnia magna* (and related organisms as *Ceriodaphnia)* and with fish than with rats. In rodent tests, the toxic chemicals were force fed to the animals so they pass through the acidic stomach. This can degrade some toxins (Rodericks, 1992). In the tests with fish and with *Daphnia magna*, the toxins were added to the water provided to the animals. Similarly, the bacteria were simply immersed in the dilute toxin.

The bacterial test was not as sensitive to most of the herbicides than the tests with *Daphnia magna* and the trout fingerlings. However, the bacterial test was more sensitive to thifensulfuron and nicosulfuron than the other tests.

Five herbicides were tested for their ability to inhibit growth of *S. meliloti:* bromoxynil, glyphosate, paraquat, ethoxydim and trifluralin. Cells were plated on the medium containing the IC50 for the herbicide. Cell numbers were compared with control plates receiving no herbicide. Only paraquat was found to inhibit growth of the bacteria. This herbicide generates free superoxide radicals and did not permit growth of the bacteria presumably because of this.

Glyphosate provides a good example of a compound that has different effects on different organisms. The LD50 for rats is greater than 5000 ppm. This same compound was found to be toxic for *S. meliloti* at 18.1 ppm, comparable to the toxicity measured with the rainbow trout fingerlings, with *Daphnia* (Table 2) and Microtox™ (Kaiser and Palabrica 1991). Glyphosate inhibits 5-enolpyruvil-shikimate-3-phosphate synthase (EPSP) in the synthesis of aromatic amino acids (Amhrein et al. 1980). Glyphosate is thought to be toxic for plants because of this effect on aromatic amino acid synthesis. However, some plants do not survive exposure to glyphosate even when provided with aromatic amino acids exogenously (Lee 1980). Animals do not synthesize aromatic amino acids so glyphosate should not be toxic. Trout fingerlings and *Daphnia magna* are animals that do not make aromatic amino acids. Yet glyphosate is toxic for these organisms. Glyphosate obviously has more activities in cells than inhibition of a reaction in the synthesis of aromatic amino acids. If a compound is toxic for one indicator organism, it could well be toxic for human beings.

The Microtox™ assay has been used to estimate the half life of herbicides in soil (Lu et al. 1993). Similarly, the *S. meliloti* test was used to estimate the half life of 3 herbicides in soil. Bromoxynil, glyphosate and sethoxydim were tested in 3 different soils. These soils included soil from a cotton field which receives a variety of agricultural chemicals during the growing season; soil from an alfalfa field which receives no chemicals; and from uncultivated desert soil. The bacterial counts were comparable in the three soils, about 1×10^7 cells gm^{-1}. The herbicides were

dissolved in water and added to the soils. Analysis showed that all the soils contained calcium. It is known that calcium inhibits the reduction of MTT by *S. meliloti.* The addition of 2.5 μmoles EDTA to each assay eliminates the inhibition by calcium but permits the toxicity of nearly all organic compounds to be measured (Botsford 2000).

Bromoxynil proved to be most stable, and was present after 15 days in all three soils. Glyphosate disappeared in 2-8 days suggesting it is broken down by the soil microflora. Sethoxydim disappeared in a few hours even in autoclaved soil suggesting it is tied up on soil particles and is no longer soluble (Lichtenstein 1980). It was anticipated that the microflora in soil from cotton fields would degrade herbicides most readily since this soil is exposed to a variety of agricultural chemicals each year. Microflora able to deal with organic chemicals should be selected. However, glyphosate was degraded somewhat more rapidly in the soil from an alfalfa field. These experiments need to be repeated with different amounts of the herbicide added to soil samples. A quantitative relationship between the herbicide and its degradation must be shown. But this work showed that this approach could be used to determine rates of degradation of herbicides in the soil.

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