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Abstract

A simple method to measure toxic chemicals has been used. This assay uses the bacterium *Rhizobium meliloti* as the indicator organism. This assay was used to measure the toxicity of 30 herbicides used on the New Mexico State University farms. One of the herbicides, Poast (sethoxydim), was found to be toxic at less than 10 ppm (mg l^{-1}). Nine were found to be toxic at less than 100 ppm, 13 to be toxic at 100 to 1000 ppm and 7 were found to be toxic at concentrations greater than 1000 ppm. The toxicity using this test was compared with values from the manufacturers using animal tests and *Daphnia* tests. Three herbicides were tested for their stability when mixed with three soil types. Soil was taken from a cotton field, from an alfalfa field and from an uncultivated desert soil. Sethomydim was found to have a half life (that is the toxicity decreased by 50%) of 8.8 to 21.1 hours in the three soils. Glyphosate was found to have a half life of 2.4 to 7.8 days. Bromxylnil was found to be stable for at least 15 days, the toxicity decreased less than 50% in this interval. The assay can be used to determine the toxicity of herbicides and to follow the fate of herbicides in the soil.

Keywords: toxic herbicides, bacteria, measurements, persistence

Introduction

The purpose of this study was to demonstrate the utility of a new assay for toxic chemicals. The toxicity of 30 commercial herbicides used on the Lyendecker Plant Science Research Center farm at New Mexico State University was determined. The breakdown of three herbicides in three different soils was followed using this assay for toxic chemicals.

Because of the soil dynamics encountered , the persistence of herbicide toxicity in soils can not be predicted (Cousins and Mortimer, 1995; Lichtenstein, 1980). Agronomists are interested in monitoring the fate of herbicides. A simple, inexpensive test for herbicides could influence how the herbicides were applied (Hague and Freed, 1975, Hasenbuiller, 1978, Kotoula-Syka et al., 1993). Some herbicides could pose health hazards if they persist long enough to contaminate ground water (Kolpin et al., 1995). Herbicides also pose a problem with ornamental plants (Keese et al., 1994). Waste water from nurseries can pose a problem from high concentrations of herbicides.

Many factors influence the fate of herbicides in the soil. Herbicides can be leached away from the site of application with precipitation and irrigation (Haque and Freed, 1975; Jager, 1983, Keese et al., 1994). These chemicals can accumulate in ground and surface water where they have the potential for affecting plants and animals. Herbicides can be degraded by the soil microflora and other biotic factors. Herbicides can be lost by volatilization (Cousens and Mortimer, 1995) and photodegradation (Lu et al., 1993). They can also be adsorbed irreversibly to soil particles (Hasenbuiller, 1978; Jager, 1983). The extent of contamination by pesticides is significant. Agricultural pesticides contaminate 24% of 68,824 wells tested in 45 states by the Environmental Protection Agency (1993). In the United State 165,000 km of rivers and 830,000 ha of lakes are contaminated by pesticides (Environmental Protection Agency, 1992).

This assay for toxic chemicals is simple, is fast, is inexpensive. The data can be analyzed with a pocket calculator. It could be used in a third world situation. This work suggests it can be used to follow the fate of herbicides in soil. There are other methods to measure toxic chemicals using bacteria (Bitton and Dutka, 1986). One of

these, Mixrotox™ uses a bioluminescent marine bacterium, *Photobacterium phosphoreum* as the indicator organism. Toxic chemicals inhibit the production of light. The assay requires a luminometer and a refrigerated water batch and the data analysis requires a computer. Another, Polytox™ follows the effect of toxic chemicals on oxidation by a consortium of bacteria isolated from sewage sludge. This assay requires respiromneter. Again, the data analysis requires a computer. Either of these assays requires that $25-30,000 be available.

The assay is based on the ability of *Rhizobium meliloti* to reduce a tetrazolium dye, 3-[4,5-Dimethyl-thiazol-2,3-yl}2,5-diphenyltetrazolium bromide, MTT. The bacterium appears to use the dye as a terminal electron acceptor. Normally this bacterium reduces this dye to an intense blue color very readily. It was found that toxic chemicals, chemicals that damage living organisms, pentachlorophenol, tetrachloro-ethylene, chlorobenzene, inhibited the reduction. The intensity of the blue color can be determined with a spectrophotometer and is proportional to the concentration of the toxic chemical present. We have tested 10 bacteria and find that many can reduce the dye but toxic chemicals don't seem to inhibit the reduction. *R. meliloti* appears to be unique in that it can reduce the dye readily and this reduction is inhibited by toxic chemicals.

Materials and Methods

Pepraration of bacterial cells. *R. meliloti* 102f34 was originally obtained from Dr. Gary Ditta, University of California, San Diego. Other strains of *R. meliloti* have been tested and have been found to work comparably. The bacterium was grown in CDM medium (Gonzalez-Gonzalez et al., 1990) supplemented with 0.1 % acid hydrolyze casein. An inoculum was grown in this medium. CDM medium was inoculated with a 1% inoculum that was no more than 24 hours old. Cells were grown overnight. The cells were harvested by centrifugation in a preparative centrifuge for 10 minutes at 10,000 x g. The cells were washed once in 0.01 M KPO_4 buffer, (pH 7.5). The cells were then diluted to an absorbance at 550 nm of 0.3. Mannitol, 0.1%,

was added and the cells were stored in an ice bath.

The Assay. Reactions were run in 13 x 100 mm disposable test tubes. 1 ml Tris buffer, 0.1 M (pH 7.5); the toxic sample and water in 1.2 ml and 1.0 of the diluted cells were combined. The absorbance was measured at 550 nm. 0.1 ml of a tetrazolium dye, 3.2 mM was added and the tubes were placed in 30^{o} water bath and incubated 20 minutes. Tubes were removed, chilled in the ice bath, and the absorbance at 550 nm measured. The difference in absorbance, the value measured at 20 minutes less the value measured at 0 minutes, was used in subsequent determinations.

The data were plotted as the absorbance (on the Y axis) vrs. the concentration of toxic chemical (on the X axis). A regression line was fitted. If the R^2 for the regression was less than 0.8, the data were discarded. When $R^2 = 0.8$, $R = 0.894$. The $p = 0.01$ value for a regression when $v = 5$ (v = number of samples) is 0.874. Any data from plots with an R^2 of at least 0.80 is significant at the 0.01 level (Rolf and Sokal, 1981). From the regression line, the value for the toxic chemical, x, resulting in 50% inhibition of reduction of the dye could be calculated using the equation:

$$x = \frac{Y/2 - B}{m} \qquad \text{(Eqn. 1)}$$

x is the concentration of the chemical. Y is the absorbance of controls without a toxic chemical. B is the Y intercept from the regression line. m is the slope of the regression line. Assays were repeated several times with different preparations of the cells. The average value from all the assays was determined and the standard deviation calculated. The assay has been described in detail (Botsford, 1997).

Normally stocks were made up as 3300 mg l^{-1}. A value expressed as μl of the stock was obtained. This could be converted to ppm with this calculation.

$$\mu l \text{ IC50} \times \frac{1.0 \text{ L}}{10^6 \mu l} \times \frac{3300 \text{ mg}}{1.0 \text{ L}} \times 10^3 \mu g \times \frac{1 \text{ tube}}{3.3 \text{ ml}} = \mu g \text{ ml}^{-1} = \text{mg L}^{-1} = \text{ppm} \qquad \text{(Eqn.2)}$$

Soil Samples

Three soils were collected. Soil was taken from an alfalfa field with a USDA classification of clay loam (Glendale). Soil was taken from a cotton field with a USDA classification of very fine sandy loam, thick surface (Brazito). Soil was taken from the

desert with a USDA classification of very gravelly sandy loam (Terino-Casito association). Information of the soils was taken from a soil survey of Doña Ana County, New Mexico (US Department of Agriculture, Soil Conservation Service, 1980). Properties of these soils are presented in Table 1.

Herbicide Degradation in Soil

Three herbicides with low IC50 values (that is herbicides that are highly toxic) were chosen for soil studies: bromoxyynil, glyphosate, and sethoxydim. Soil studies were performed in small plastic cups. Holes were punched in the bottom of the cups to permit drainage. The cups were filled with soil. The soil was wetted thoroughly. The herbicide was added in 1 ml increments. The amount of fluid that could be added without drainage loss was determined prior to the experiment with another soil sample. The bromoxynil was added to a final concentration of 60.1 mg gm^{-1} soil. Glyphosate was added to a final concentration of 7.8 mg gm^{-1} soil. Sethoxydim was added to a final concentration of 1.44 mg gm^{-1} soil.

At each sampling, 1 g samples were taken by scraping soil from the upper layer of the soil. Samples were diluted in sterile distilled water. A 5 ml sub sample from each was taken and the soil removed by centrifugation in a clinical centrifuge 5 minutes at 2000 rpm. The resulting supernatants of these subsamples were assayed for toxicity. From this the toxicity, the μl of sample inhibiting the reduction of the dye 50% was determined.

Calcium was found to inhibit the reduction of the dye at very low concentration, 5.65 ppm (51 uM). This inhibition is eliminated by the addition of 2.5 μmoles EDTA (ethylene diamino tetracetic acid). This much EDTA eliminates inhibition by as much as 100 ppm calcium. EDTA was routinely added to samples and to controls.

A minimum of 5 volumes of each sample and two controls were run routinely. Trials were performed several times with different preparations of cells.

The values from these samples was plotted as the percentage initial toxicity (sample taken within 25 minutes of the addition of the herbicide to the soil) vs. time. The toxicity of the samples taken later was determined and the IC50 plotted. With time, the toxicity, the inhibition of the reaction was reduced. The time taken to reduce the toxicity by 50% was determined by inspection of the plots.

Results

The IC50 values determined for the 30 herbicides tested are presented in Table 2. Low IC50 values correlate with high toxicity, low values mean less toxin is required to inhibit reduction of the dye. 1 herbicide, sethoxydim, was found to be toxic at less than 10 ppm, 9 herbicides were found to be toxic at less than 100 ppm, 13 were toxic at less than 1000 ppm and 7 were found to be toxic at levels greater than 1000 ppm.

Some of the herbicides were found to lose toxicity with time. Routinely stock solutions were made at weekly intervals. The toxicity of paraquat was not linear when diluted. Paraquat readily accepts electrons and becomes reduced to form the paraquat radical which can reduce molecular oxygen to from the superoxide radical (Ahems, 1994). The damage to the mechanism responsible for reduction of the dye may be in part due to the superoxide radicals generated rather than an undefined interaction between the organic chemical and the mechanism responsible for reduction of the dye.

All the herbicides were tested for their effect on reduction of the dye without bacteria. None of the herbicides reduced the dye in the absence of the bacteria. Solutions of some herbicides were not clear when dissolved in water. Trifluran was bright yellow and sethoxydim was milky white. This could be corrected by the time = 0 measurement of the absorbance in the test tubes.

Seven of the herbicides were dissolved in DMSO: Cyanazine, diuron, EPTC, napropamide, nicosulfuron, norflurazon and primisulfuron. DMSO inhibits the reduction of the dye 50% at 467 μl. The DMSO would have affected the apparent toxicity of these compounds. It was assumed that the toxicity of the herbicide and DMSO were additive. The portion of the toxicity due to DMSO was calculated and subtracted from the toxicity observed.

Comparison with data from the manufacturer

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

Loss of Toxicity of Herbicides in the Soil

The half life determined for the herbicides in soil is presented in Table 4. It is significant that soil receiving Sethoxydim was no longer toxic after only a few hours. Glyphosate was no longer toxic after a few days. Bromoxynil was stable for at least 15 days in all soil types.

Discussion

It has been assumed that the dye is reduced by the electron transport system in the bacteria. In procaryotes, the electron transport system is intimately associated with the cytoplasmic membrane. Compounds that affect the cytoplasmic membrane would affect electron transport. However, there is no experimental evidence to support this assumption. The dye can be reduced by a variety of reductases (Biitton and Dutka, 1986). We have followed the growth of the bacterium with 4 toxic chemicals at the concentration inhibiting reduction of the dye by 50%. We find the bacteria grow approximately half as fast as the control cultures. This bacterium is obligately aerobic. Presumably growth is reduced because electron transport is inhibited by the toxic chemical. We have isolated 5 mutants unable to reduce the dye. We plan to clone and to sequence the gene(s) coding for the functions responsible for the reduction.

The values obtained with this assay are much lower than those obtained with animals but are comparable to those obtained with rainbow trout and are somewhat higher than values obtained with the sand flea, *Daphnia* (Nestrud and Anderson, 1994). The Microtox and Biotox Assays for toxic chemicals uses the bioluminescent marine bacterium *Photobacterium phosphoreum* as the indicator organism (Bitton and Dutka, 1986; Kahru, 1993). With this assay, the IC50 for 2,4-D was found to be 74.0, for glyphosate to be 7.73, and for paraquat to be 777 ppm (Kaiser and Palabrica, 1991). Some manufacturers also included data from work with quail and ducks. When the results using this assay are compared with values for 15 organic chemicals using the fat head minnow assay, the values are comparable (Botsford, 1997). The Rhizobium assay is comparable to legally accepted methods for determining the toxicity of toxic chemicals.

Data for toxicity with rats does not correlate well with any other biological endpoint or physicochemical parameters, such as the n-octanol water partition coefficient. The latter has been recognized as a most important bulk parameter which

provides a good measure for the solute interaction with cell membranes (Kaiser and Palabrica, 1991). In these animal tests, the toxic chemical is forced into the animal's stomach from a flexible tube. The toxic chemical can be broken down by the acidity in the stomach and by the intestinal microflora. The toxic chemical must kill the animal to be significant. These chemicals may not be lethal but they could make the animal very ill. This can not be determined with animals.

Glyphosate provides a good example of a compound that has different effects on different organisms. The LD50 for rats was found to be >5000 ppm and to be >4640 for the quail. This same compound was found to be toxic for *R. meliloti* as measured by this assay at 18.1 ppm, in the same range as was found for rainbow trout and *Daphnia* (Table 3) . Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) in the shikimate pathway found in synthesis of aromatic amino acids (Amrhein et al., 1980; Duke and Hoagland, 1978; Thelen et al., 1995). Animals do not synthesize any of the aromatic amino acids so glyphosate is considered to be harmless for animals. In contrast, plants do synthesize aromatic amino acids. Glyphosate is assumed to be toxic for plants because it inhibits synthesis of these amino acids. However, some plants treated with glyphosate did not survive even when provided exogenously with aromatic amino acids suggesting there may be additional mechanisms of toxicity for this compound (Lee, 1980).

The significance of the apparent breakdown of the herbicides in soil is uncertain. They were added to the soil in much higher concentration than would ever accumulate in soil. The sethoxydim was inactivated as rapidly in soil that had been autoclaved as in the control soils. This suggests that the compound was not degraded by the soil microflora. The herbicide may be irreversibly sorbed onto soil particles (Lichtenstein, 1980). The toxicity of glyphosate was reduced at a rate appropriate for microbial decomposition of the herbicide. Bromoxynil appears to be a recalcitrant molecule. However, it should be noted that was added to the soilat a high concentration and this may have inhibited its breakdown. This must be rexamined. The loss of toxicity is uncertain. The total numbers of culturable bacteria were determined in the three soils by plating dilutions onto R_2A medium. Comparable counts were observed in all the soil (Table 1).

This method does appear to provide a method to follow the disappearance of herbicides in soil, the decrease in toxicity can be followed. However, the protocols used must be refined. The rate of application of the herbicides was much higher than would be encountered in a real situation. The herbicides used in these studies may have sorbed to the upper layers of soil particles causing a stratified distribution of the herbicide in the sample. A better soil sampling method would be to add the herbicide to the soil and then to mix the soil thoroughly before placing it into the container. Thus the herbicide would be uniformly distributed in the soil.

Further studies in this area should include a comparison between formulated herbicides used in the field and the active ingredients in the herbicide. The compounds used in the formula could contribute to the toxicity. We hope to run experiments to correlate disappearance of the toxicity with disappearance of the chemicals measured with analytical methods. This work simply shows that this assay can be used to follow the disappearance of toxicity in soil. This approach has been used with more complex methods to follow degradation of a few herbicides (Cousens and Mortimer, 1995; Gaggi et al., 1995, Greene et al, 1985; Somasundaram et al., 1990).

Table 1. The characteristics of the soils tested[a].

Soil Properties	Cotton field soil	Alfalfa field soil	Desert soil
gravel	--	--	23-63%
medium sand	--	20-45%	25-63%
very fine sand	50-70%	--	--
silt	25-45%	40-50%	25-45%
clay	15-20%	28-40%	15-20%
organic matter	2.91%	6.08%	2.22%
calcium	0.20%	0.52%	0.06%
pH	8.1	7.8	8.3
viable plate counts	2.85×10^6	1.70×10^7	9.35×10^6

[a]The physical properties of the soils were taken from the 1980 Soil Survey of Doña Ana County, New Mexico. The calcium in the soils and the pH was measured in the soil testing laboratory at New Mexico State University. The viable plate counts were determined by the authors using R2A medium.

Table 2

Toxicity of the herbicides[a]

Common name	Manufacturer's name	n	ave	sd
2,4-D	Weedar 64	9	347	39.4
Alachlor	Bronco	6	111	9.24
Bensulide	Prefar 4E	7	53.7	9.80
Bromoxynil	Buctril	8	26.0	4.80
Clomazone	Command 4CE	15	23.8	3.71
Cyanazine[a]	Bladex 90DF	8	245	15.6
DCPA	DACTHAL	6	427	73.4
Dicamba	Clarity	6	>1200	
Diruona	Karmex	9	87	16.4
EPTC[a]	Eptam	9	51.4	5.49
Ethalfuralin	Sonalan	6	>1200	
Fluazifop-p	Fusilade DX	6	>1200	
Glyposate	Roundup	6	18.1	2.26
Imazapyr	Arsenal	6	229	3.06
Imazethapyr	Pursuit	10	353	49.3
Isoxaben	Gallery	16	365	53.35
Mepiquat-Chloride	Pix	6	>1200	
Metasulfuron	Ally	6	>1200	
Metribuzin	Lexone DF	6	>1200	
Naproamide[a]	Devrinol 2E	10	289	42.5

Nicsulfuron[a]	Accent	10	267	42.6
Norflurazon[a]	Zorial Rapid 80	8	182	37.01
Oxadiazon	Ronstar	6	269	42.8
Paraquat	Gramoxone	7	48.9	5.67
Primisulfurona	Beacon	6	269	58.5
Qunclorac	Facet 75 DF	6	>1200	
Sethoxydim	Poast	6	2.70	0.44
Thiazopyr	Visor	6	43.2	1.79
Thifensulfuron	Pinnacle	7	928	40.8
Trifluralin	Treflan EC	7	10.3	1.83

[a]DMSO used as solvent. The other herbicides were dissolved in water.

All the assays were performed at least three times with at least three different batches of bacterial cells.

Table 3

Comparison of assays for toxicity

Common name	Rhizobium Assay[a]	Rat Assay[b]	Rainbow Trout[c]	*Daphnia*[d]
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[a]	245	182334	9	49
DCPA	427	>10,000[e]		
Dicamba	>1200	1707	135	110
Diruon[a]	86.8	3400		
EPTC[a]	51.4	1652	19	
Ethalfuralin	>1200			
Fluazifop-p[3]	>1200	>5000	1.37	>10
Glyposate	18.1	>5000	8.2	5.3
Imazapyr	229	>5000	>100	>100
Imazethapyr	353	>5000	340	>1000
Isoxaben	365	>10,000	>1.1	>1.3
Mepiquat-Chloride	>1200			
Metasulfuron	>1200	>5000	>150	>12.5
Metribuzin	>1200	1090-1206	76	4.5

Naproamide[a]	289	>5000	16.6	14.3
Nicsulfuron[a]	267	>5000	>1000	>1000
Norflurazon[a]	182	9000	8.1	>15
Oxadiazon	269	>5000	1-9	0.5-8.0
Paraquat	47.9	112-150[f]	32	
Primisulfuron[a]	269	>5050	210	260
Qunclorac	>1200	>2610	>100	
Sethoxydim	2.70	4.1	32	
Thiazopyr	150	>5000	3.4	5.9
Thifensulfuron	928	>5000	>250	470
Trifluralin	10.3	>5000	0.041	0.56

[a]Toxicity reported as ppm from Table 2.

toxicity reported as LD50 (value resulting in death of half the animals) as mg/kg body weight (ppm).

[c]Toxicity reported as the LC50, the value resulting in death of half the animals after a 96 hour exposure. Reported as mg toxin per L of water.

[d]Toxicity reported as the LC50, the value resulting in death of half the animals after a 48 hour exposure. Reported as mg toxin per L of water.

eThe toxicity of DCPA was found to be 1270 in the Bobwhite quail.

[f]The toxicity of paraquat was found to be 4048 ppm in Mallard ducks.

Table 4.

Half life of herbicides in soil[a]

	Soil Type		
Herbicide	Cotton Field	Alfalfa Field	Desert
Sethoydim	19.1 (hr)	8.76 (hr)	21.1 (hr)
Glyphosate	187 hr (7.8 da)	58.9 hr (2.4 da)	76.2 hr (2.8 da)
Bromoxynil	>15 da	>15 da	>15 da

[a]The toxicity of water drained from each sample was determined. The values for the toxicity were plotted vs. time. When the toxicity had decreased 50%, the time was noted.

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