Bull. Environ. Contam. Toxicol. (1997) 59:619-625

Bulletin of Environmental Contamination and Toxicology

Toxicity Assessment of Pesticides Using the Microtox Test: Application to Environmental Samples

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Received: 5 March 1997/Accepted: 18 July 1997

To fully evaluate the environmental impact of pesticides, both physicochemical and toxicological analyses should be performed (EPA 1993). Physicochemical analyses do not provide information about the toxicity of environmental samples, for interactions of the complex mix of compounds can occur, so toxicity detection is crucial in assessing environmental contamination. If toxicity is not detected further and more expensive chemical analyses can be minimized. Microbial tests have been widely used in toxicity screening because of the similarity of complex biochemical functions with higher organisms, ease of handling, short exposure time, and reproducibility of the inter-laboratory results. Several microbioassays which provide standardized cultures of bacteria in freeze-dried form are now commercially available (Dutka et al. 1991). During the last 10 years, the luminiscent bacteria toxicity test distributed commercially as Microtox has been used increasingly to assess the toxicity of environmental samples like water or sediments (Hoke et al. 1993; Kaiser et al. 1988; Kwan and Dutka 1990; Tay et al. 1992), industrial waste samples (Din and Abu 1993; Lambolez et al. 1994) and several groups of individual organic compounds (Kaiser and Palabrica 1991; Kaiser and Ribo 1985; Somasundaram et al. 1990). The results of the Microtox assay have been compared to acute bioassays, with different organisms (bacteria, microcrustaceans, fishes) for a large number of pure compounds and complex mixtures. The sensitivity of Microtox compares favourably with other tests and good correlation has been found (Becerro et al. 1995; Din and Abu 1993; Kaiser 1993).

The validation process to establish the reliability of a test is of major interest, so the present work was undertaken to evaluate the Microtox test and its usefulness in generating precise and accurate toxicity data on pesticides. Estimated medium effective concentration (EC_{50}) values for bioluminescence reduction, evaluated using the *Photobacterium phosphoreum* bioassay, are reported for various categories of pesticides commonly used in agricultural production. The Microtox assay is also employed in evaluating the toxicity of environmental samples to assess the impact of pesticides used in agricultural production.

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MATERIALS AND METHODS

The Microtox Model 500 Toxicity Analyzer, freeze-dried bacteria (approx. 10^6 cells), reconstruction solution, diluent (2% NaCl) and adjustment solution (non-toxic 22% sodium chloride dilution) were obtained from Microbics corporation (Carlsbad, CA, USA).

The Microtox test is based on the measurement of light production inhibition by organisms. The procedures recommended in the instrument manual (basic or standard procedure and the 100% test) were used for the toxicity test (Microbics 1989). In the basic bioassay sample solutions can be tested up to a concentration of 45% and Microtox analyzes four decreasing concentrations and one control per sample. The 100% test procedure, however, has been developed for screening samples with an EC_{50} value >45 %. All samples were adjusted to 2 ± 0.2% NaCl and the tests were run at 15°C. Light measurements were taken at 0 and 5 or 15 min.

The EC_{50} values were calculated using the Microtox calculation program. Graphic plots of the Γ values (defined as the quotient between the lost light and the light remaining) as functions of the sample concentration were made by the program. The concentration of sample causing a 50% reduction in light was designated the EC_{50} value (expressed in µg/mL) for the sample. The difference between basic or 100% procedure is that in the 100% test for calculating the Γ value no correlation factor is employed and data obtained are less exact. So, the Microtox basic procedure is normally used to determine the toxicity of the samples.

The first test solution for each studied compound had a concentration near the upper limit of the compound's solubility in water. All test solutions were prepared using pesticide standards obtained from Promochem (Wesel, Germany). The purity of all the compounds ranged from 97 to 99.9%. A solution of the compounds was prepared in distilled water and, where necessary to solubilize the compounds, with addition of up to 5% methanol, provided by Romyl (Leics, UK).

RESULTS AND DISCUSSION

The chemical concentrations used in an acute toxicity test are routinely based on the results obtained from a “pretest”, since there are no standard guidelines for conducting these tests. The selection of pesticide concentrations was limited by the fact that the Microtox test requires that the compound be soluble and stable in 2% saline. The initial experiments determined the amount of bioluminescence inhibition caused by specific concentrations of the test chemicals. The initial concentrations of the pesticides were then modified in order to obtain concentrations in which, after the dilution steps indicated by the Microtox Basic procedure, the amount of pesticide which reduced the bioluminescence of *Photobacterium phosphoreum* by about 50-60% was in the intermediate zone of the graphic dose-response, where the confidence intervals at 95% of the EC_{50} values were minimum. These concentrations were used as standard concentrations for comparison between the different tested pesticides.

An important limitation of the Microtox system is that only the toxicity of aqueous solutions can be determined. Addition of organic solvents to increase the solubility of chemicals resulted in increased toxicity to *Photobacterium phosphoreum.* However, an organic solvent is normally used to dissolve substances with minimal water solubility.

Several, like ethanol (Tay et al. 1992) or dimethylsulfoxide (Dutka et al. 1991; Kwan and Dutka 1990), have been studied, but methanol is the most common solvent used in toxicity testing involving *Photobacterium phosphoreum* because its EC_{50} value is higher (approx. 25000 µg/mL) (Thomulka et al. 1993). According to the data obtained by different authors, the percentage of the solvent in the test solution should not be greater than 10%. Recommended percentages include 4% (Kwan and Dutka 1990), 5% (Kaiser and Palabrica 1991), 8% (Somasundaram et al. 1990) and 10% (Kaiser and Ribo 1985).

This preliminary investigation showed that concentrations of 5% methanol were not toxic to the bacteria. The toxicities of the most soluble pesticides included in the study did not differ significantly when the EC_{50} values, expressed in µg/mL, were determined at 5 and 15 min exposure, with individually prepared pesticide solutions in distilled water and with addition of 5% of methanol (see Table 1).

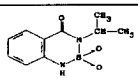
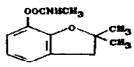
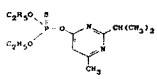
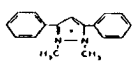
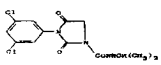
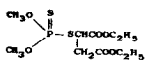
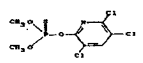
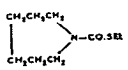
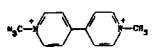
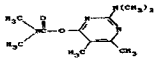
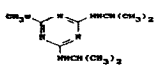
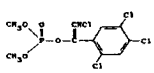
PESTICIDE	Conc.	EC_{50}(µg/mL) 5 min		EC_{50}(µg/mL) 15 min	
	(µg/mL)	H_2O	H_2O+ 5% metOH	H_2O	H_2O+ 5% metOH
Bentazone	180	28.92	30.3	28.6	33.3
Chlormequat	8000	3387.6	3576.9	1482	1505.9
Diquat	3000	508.7	500.1	71.4	92.7
Difenzoquat	400	49.9	43.2	25.3	26.5
Mepiquat	8000	>3600	>3600	>3600	>3600
Molinate	25	4.4	4.2	9.1	8.4
Paraquat	4500	2322.7	2015.2	349.7	358.6

metOH: Methanol

Finally, the acute toxicity of pesticides used in this study was evaluated by means of the Microtox test. Test solutions were prepared in distilled water or in a 5% methanol aqueous solution when it was necessary to solubilize the compounds. The results of the acute toxicity tests are reported as the EC_{50} values at 5 and 15 min exposure (Table 2). Each value was determined a minimum of three times with individually prepared test solutions, usually on three or more different days, and the mean of the values is given.

Reproducibility in evaluating toxicity measured as the relative standard deviation between experiments run with the same samples, using different freeze-dried bacteria vials and on different days, is also reported in Table 2. The test's basic procedure proved to be very precise, with relative standard deviation values ranging from 2.47 to 14.12 at 5 min exposure and from 2.51 to 14.99 at 15 min exposure. These values are similar to those obtained by other researchers, which range from 3 to 20 % (Microbics 1994) and can be attributed to the variability inherent in the biological processes.

Table 2. EC_{50} (μg/ml) and RSD % of pesticides.

PESTICIDE	Chemical structure	Concent. (μg/ml)	5 min		15 min	
			EC_{50}	RSD%	EC_{50}	RSD%
Bentazone*		180	28.92	11.3	28.58	14.99
Carbaryl		15	0.606	13.12	0.799	11.97
Carbofuran		150	27.46	13.75	23.61	16.82
Chlormequat*	$[ClCH_2CH_2N(CH_3)_3]^+$	8000	3387.6	6.34	1482	13.82
Diazinon		120	83.74	2.47	74.58	5.66
Diquat*		3000	508.73	5.12	71.4	7.47
Difenzoquat*		400	49.88	3.84	25.26	4.51
Iprodione		25	>11.25	--	>11.25	--
Malathion		150	33.57	8.84	33.73	14.65
Mepiquat*		8000	>3600	--	>3600	--
Methylchlorpyriphos		25	>11.25	--	>11.25	--
Molinate*		25	4.16	9.83	8.36	9.58
Paraquat*		4500	2322.7	13.67	349.65	11.19
Pirimicarb		50	>22.5	--	>22.5	--
Prometryn		50	>22.5	--	>22.5	--
Propanil		200	35.13	8.41	28.15	9.18
Tetrachlorvinphos		50	5.35	9.82	2.56	5.26

*Test solutions prepared in distilled water

Because of its sensitivity and high reproducibility the Microtox system has proven useful in estimating the relative toxicity of the selected pesticides. However, available information on the microbial toxicity of pesticides is limited, and no data were found for five of the 17 studied compounds, because saturated solutions of these compounds in the 5% methanol aqueous solution did not produce any measurable effect using the basic Microtox procedure. So, for evaluating the toxicities of these pesticides (iprodiona, mepiquat, methylchlorpyriphos, pirimicarb and prometryn) the 100% test method had been carried out and also none of their provided a measurable EC_{50} value. The rest of pesticides had a similar EC_{50} by the two methods, however values obtained by testing a sample with the 100% tests were less exact, with confidence intervals broader than in the basic test. The difficulty in determining the toxicity of less soluble compounds with the Microtox test has been observed by other investigators (Somasundaran et al. 1990).

Although the EC_{50} value used to classify a substance as toxic or non-toxic is arbitrary, and may ignore categorical degrees of toxicity, this value does provide a reference point for descriptively evaluating the data. Somasundaran et al (1990) determined the EC_{50} values of several pesticides and their metabolites and established that most of them were relatively toxic to *P. phosphoreum* with EC_{50} value < 21 µg/mL. However, Thomulka et al. (1993) defined the compounds with an EC_{50} value equal to or greater than 100 µg/mL as non-toxic to the bacteria.

In our study, most of the pesticides are considered relatively non-toxic because they are not usually found at this level in environmental samples. However, the insecticides carbaryl and tetrachlorvinphos and the herbicide molinate were the most toxic compounds to *P. phosphoreum* with EC_{50} values lower than 5.35µg/mL. Cationic herbicides are the least toxic to the bacteria, with EC_{50} values which were even higher than 3000 µg/mL. The toxicity values are consistent with those obtained by other authors for carbaryl, carbofuran, diazinon and malathion, but paraquat seems less toxic than the results reported in the literature [Kaiser and Palabrica 1991; Somasundaran et al. 1990). For most of the compounds tested in this study no data are available in the literature.

EC_{50} values obtained at 5 and 15 min exposure (see Table 2) also demonstrate the different behaviour of all the studied pesticides. Toxicity can not always be completed in 5 min measurement time. Sometimes different chemicals affected the bacteria in different ways and the toxicity was greater when the time of exposure was increased. There are pesticides like carbofuran, chlormequat, diazinon, diquat, difenzoquat, paraquat, propanil and tetrachlorvinphos, that have an EC_{50} value at 15 min that is smaller than the value obtained at 5 min. However, there is another group of pesticides which completed their toxic effect after 5 min exposure. Bentazon and malathion belong to this group. Carbaryl and molinate appeared to behave in an unusual way, that is, producing a major effect at a short exposure time. This is probably due to a slight stimulation of bacterial metabolism when the time of exposure increases.

Application of Microtox Test to Environmental Samples: Soil and surface waters are interesting matrices for toxicological studies in which the impact of the presence of pesticides in the environment needs to be evaluated.The application of an aquatic bioassay like Microtox to evaluate soil toxicity involves two independent steps: desorption of pesticides adsorbed to the soil particles in an aqueous phase (leaching test) and exposure of the test organisms to the chemicals dissolved in the leachate.

Three soils, all from an important agricultural area, were selected for this study. The leachate was obtained according to the EEC Directive 84/449. The toxicity of the leachates was expressed as Toxicity Units (TU) and calculated as $100/EC_{50}$. Toxicity values at 5 min exposure were 1.18, 1.52 and 1.78 TU respectively, with relative standard deviations (R.S.D.) which ranged from 5.58 to 13.06%. At 15 min exposure only one of the leachates showed major toxicity (4.44 TU with R.S.D. of 16.47%), thus suggesting that this sample contained compounds which do not complete its toxic effect at 5 min. The other two samples had a similar toxicity (1.35 and 1.16 TU with R.S.D. of 5.29 and 4.28%, respectively).

The toxicity of water samples was determined directly after filtering the water to remove plant materials. The samples were collected from four irrigation channels in the same agricultural area as the soil samples and stored at 4 °C until analysed. The Microtox analyzer is not able to determine the TU of surface water samples, that is, the maximum measurable concentration of the toxic compounds presented in the samples do not produce a 50 % reduction of bioluminiscence.

Toxicity values obtained with the Microtox bioassay in environmental samples demonstrated that soil samples were more contaminated than water samples and, in spite of the increased use of synthetic pesticides in this important agricultural area of Valencia, the distribution of their residues in the aquatic environment is reduced at the moment.

The Microtox test can be used to provide a direct assessment and to integrate the effects of the biologically active fraction of all of the toxic chemicals presented in environmental samples like soil leachates or surface waters. Comparison of the toxicity values with those obtained with pure compounds is difficult because potential interactions due to the presence of a complex chemical mixture in an environmental sample are not known. On the other hand, interactions between pesticides and different components of the sample can affect the availability of organic chemicals and also their potential toxicity to the bacteria.

The results demonstrate that the Microtox test was sensitive to several kinds of pesticides and permitted a quantitative EC_{50} interpretation of the results with a high degree of reproducibility. Other attractive features of this bioassay are low cost, rapid response to toxicants, modest laboratory equipment and small sample volume.

Quantifying toxicity is a common aim in contamination studies and may even serve as a routine procedure for monitoring environmental samples. The information obtained in a toxicological study provides a valuable supplement to the analytical data obtained in more traditional laboratory studies.

Acknowledgments. The authors wish to thank the "Fundación Caja Madrid" for the financial support of this project and for the grant received by M.J. Redondo, and the Science and Education Ministry for M.J. Ruiz's grant.

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