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Application of the Microtox System to Assess the Toxicity of Pesticides and Their Hydrolysis Metabolites

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The soil microorganisms, bacteria in particular, play an important role in the environmental fate of soil-applied pesticides. The ability of bacteria to metabolize pesticides and/or their metabolites for their benefit has been well documented (Sudhakar-Barik et al. 1979; Racke and Coats 1987). One result of this catabolism is the failure of some pesticides to adequately control the target pests because of decreased persistence (Felsot 1989). One of the properties of pesticides and their metabolites that may influence the induction or inhibition of enhanced microbial degradation is their toxicity to the soil microbes that are responsible for degradation.

Inasmuch as it would be inhibitively expensive and time consuming to determine the toxicities of pesticides and their metabolites to all possible soil bacteria involved in the enhanced degradation process, we used the Beckman Microtox system to assess their relative toxicities to a model bacterium. This system utilizes *Photobacterium phosphoreum*, a marine bacterium that is phylogenetically related to several genera of bacteria important in soil. The Microtox system was originally developed to assess the toxic effects of complex industrial effluents (Bulich 1984). Since its introduction in 1979, the application of the Microtox system has been extended to determine the toxicity of aquatic pollutants, wastewaters, fossil-fuel process waters, mycotoxins, and numerous other chemicals (Ribo and Kaiser 1987). Some state, provincial, and federal regulatory agencies employ the Microtox system in screening tests to monitor environmental pollutants.

In this study, we used the Microtox analyzer to determine the relative microbial toxicities of some pesticides and their metabolites. Pesticides known to be susceptible to enhanced degradation and others that have apparent resistence to enhanced degradation were included in this study. Because hydrolysis is a significant step in the chemical and microbial degradation of pesticides in soil (Matsumura 1980), the principal focus of this investigation was on hydrolytic metabolites.

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MATERIALS AND METHODS

Twenty-one chemicals (7 insecticides, 2 herbicides, and 12 of their hydrolysis metabolites) were used in this study. The chemicals obtained from their respective sources were: 1-naphthol, 4-nitrophenol, thiophenol, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenol, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2,4,5-trichlorophenol, Aldrich Chemical Company Inc., Milwaukee, WI; carbaryl, fonofos, and parathion, U.S. Environmental Protection Agency, Research Triangle Park, NC; methyl phenyl sulfone, Stauffer Chemical Co., Mountain View, CA; carbofuran and carbofuran phenol, FMC Corp., Princeton, NJ; methylamine, and sodium salicylate, Fisher Scientific Co., Itasca, IL; chlorpyrifos and 3,5,6-trichloro-2-pyridinol, Dow Chemical Co., Midland, MI; isofenphos and isopropyl salicylate, Mobay Chemical Corp., Kansas City, MO; diazinon and 2-isopropyl-4-methyl-6-hydroxypyrimidine, Ciba-Geigy Co., Greensboro, NC.

The Microtox test was performed using the Beckman Model 2055 Microtox Toxicity Analyzer and by following the procedures detailed in Beckman's operating manual (Anonymous 1979). The freeze-dried bacteria, reconstitution solution (organic-free distilled water), and diluent (a solution containing 20 g L^{-1} NaCl to provide osmotic stability for the marine bacterium) were purchased from Microbics Corporation, Carlsbad, CA.

The Microtox system measures the light emitted from bioluminescent *Photobacterium phosphoreum* that have been exposed to a chemical dissolved in the diluent. The details of theory and operation of the Microtox analyzer and of reagents used have been described (Anonymous 1979; Bulich et al. 1981). The incubator block and the chamber in which light production is measured were maintained at a temperature of 15 $\pm$ 0.1°C. The toxicity end-point is the 5-min median effective concentration (EC_{50}), which is the concentration that causes a 50% reduction in light output. The toxicities were determined according to the procedure described by Bulich et al. (1981).

The chemicals were dissolved in diluent, and a dilution series consisting of 5 concentrations were prepared for each chemical. Each of these dilution sets included one concentration that caused a 40 to 60% light reduction. Less-water-soluble compounds were first dissolved in methanol or DMSO and then serially diluted with the diluent. The concentration of the organic solvents did not exceed 8% (vol/vol) in the test samples. Our preliminary investigations showed that concentrations of solvent vehicles exceeding 8% were toxic to the bacteria. Each test was carried out twice for all chemicals, and in some cases 3 to 4 times until the EC_{50} values differed by not more than 20% from each other.

RESULTS AND DISCUSSION

The toxicities of the pesticides and their metabolites used in this study are expressed as EC_{50} (μg/ml) values in Table 1. The

available information on the microbial toxicity of pesticides and their metabolites is limited, and no prior data were found for 12 of the 21 chemicals studied. The concentration of pesticide/metabolite in soil usually does not exceed 10 to 30 ppm under normal use conditions. Some of the chemicals investigated were toxic at this concentration range, whereas others were not toxic to *P. phosphoreum* at this level.

Table 1. The toxicity of pesticides and their metabolites as determined with the Microtox system

Pesticide	EC_{50}	Hydrolysis metabolite	EC_{50}
2,4-D	100.7[a]	2,4-dichlorophenol	5.0[bd]
2,4,5-T	51.7[c]	2,4,5-trichlorophenol	1.8[d]
carbaryl	5.0[bd]	1-naphthol	3.7[d]
fonofos	5.2[bd]	methyl phenyl sulfone	3.2[d]
		thiophenol	4.8[bd]
parathion	8.5[be]	4-nitrophenol	13.7[f]
chlorpyrifos	46.3[g]	3,5,6-trichloro-2-pyridinol	18.6[h]
carbofuran	20.5[h]	carbofuran phenol	60.9[i]
		methylamine	34.6[j]
isofenphos	97.8[a]	salicylic acid	213.9[k]
		isopropyl salicylate	5.6[bd]
diazinon	10.3[ef]	hydroxypyrimidine	886.4[l]

[a-l]Means with the same letter are not significantly different at 5% level (Duncan's multiple range test).

Despite their structural similarity, 2,4-D and 2,4,5-T differed greatly in their microbial toxicity. Higher EC_{50} values indicate low toxicity, and the EC_{50} values for 2,4-D and its hydrolysis metabolite were 2- and 2.5-fold higher than those of 2,4,5-T and its hydrolysis metabolite. Similar observations of increased toxicity of 2,4,5-T and its phenolic metabolite have been reported (Ribo and Kaiser 1983). Ruckdeschel et al. (1987) found 2,4,5-trichlorophenol 2- to 12-fold more toxic than 2,4-dichlorophenol to 28 of 30 bacterial strains studied.

The increased toxicity of 2,4,5-T and its phenolic metabolite can be attributed to the presence of a third chlorine at the number 5-position of the benzene ring. Similar observations relating toxicity to the number and position of chlorine substitutions have been reported for other chlorophenols (Milner and Goulder 1986) and chlorobenzenes (Ribo and Kaiser 1983).

Except for chlorpyrifos and isofenphos, among the 7 insecticides studied all were relatively toxic to *P. phosphoreum* (EC_{50} < 21 ppm). Because most of the pesticides are degraded into breakdown products in soil and because some of these products are more persistent than the parent compounds (Somasundaram et al. 1987), the microbial toxicity of metabolites is of practical significance.

Some of the metabolites studied (3,5,6-trichloro-2-pyridinol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, and isopropyl salicylate) were more toxic to *P. phosphoreum* than their respective parent compounds. The EC_{50} values of these metabolites ranged from 1.8 to 18.6 ppm. The toxicity of 1-naphthol to *Pseudomonas* sp., *Nitrosomonas* sp., and *Nitrobacter* sp. has been reported (Ramakrishna and Sethunathan 1983). 1-Naphthol does not seem to serve as an energy source to microorganisms. This is evident from its accumulation in rice soils after repeated applications (Rajagopal et al. 1986). Repeated applications of 3,5,6-trichloro-2-pyridinol to soil resulted in less mineralization and increased persistence of chlorpyrifos (Somasundaram et al. 1989). This may be because of the toxicity of the pyridinol metabolite to soil microorganisms. 2,4-Dichlorophenol was 20-fold more toxic than its parent compound to *P. phosphoreum*. Tyler and Finn (1974) found that 2,4-dichlorophenol inhibited the growth of *Pseudomonas* sp. at 25 ppm, whereas 2,4-D had no effect on growth at concentrations up to 2000 ppm. Although 2,4-dichlorophenol was more toxic in these studies, recent investigations in our laboratory have indicated the nutritive value of 2,4-dichlorophenol and its ability to induce rapid degradation of 2,4-D (Somasundaram et al. 1989). Similarly, 4-nitrophenol, which has a low EC_{50} value (13.7 ppm) was utilized as an energy source by microorganisms, and its application resulted in an increase in the population of parathion-hydrolyzing microbes (Sudhakar-Barik et al. 1979).

Salicylic acid and 2-isopropyl-4-methyl-6-hydroxypyrimidine yielded higher EC_{50} values, reflecting their low toxicity to bacteria. Some soil bacteria carry degradative plasmids for salicylic acid metabolism (Chakrabarty 1972), and repeated applications of salicylic acid induced the enhanced degradation of its parent organophosphorus compound, isofenphos (Somasundaram et al. 1989). The highest EC_{50} value among all the chemicals studied was observed for the hydrolysis metabolite of diazinon (2-isopropyl-4-methyl-6-hydroxypyrimidine). The ability of microorganisms to mineralize this metabolite to CO_2 has been demonstrated previously (Sethunathan and Pathak 1972).

The Microtox test seems to be quicker than other methods in estimating the toxicity of chemical compounds to bacteria. The Microtox test was also more sensitive compared with other bacterial bioassays (Dutka and Kwan 1981; Ribo and Kaiser 1983). For example in Dutka and Kwan's study, the EC_{50} value for 1-naphthol was 3.80 ppm in the Microtox test as compared with > 100 ppm in a *Pseudomonas fluorescens* bioassay. Because of this increased sensitivity, some researchers are of the opinion that Microtox data should be restricted to rank chemicals according to their comparative toxicity and not for predictive purposes (King 1984).

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 *P. phosphoreum*. In this study, we could not determine the EC_{50} values for s-triazine metabolites (hydroxyprometryn and hydroxyatrazine) inasmuch as these compounds did not dissolve in the diluent, 8% methanol, or 8% DMSO (the solvents that do not interfere with toxicity determination). s-Triazine metabolites dissolve only in acidic solutions or other organic solvents such as chloroform (which are toxic to *P. phosphoreum*). Saturated solutions of these s-triazine metabolites in the diluent or methanol or DMSO did not produce any measurable effect. The difficulty in determining the toxicity of less-soluble compounds in Microtox tests has been observed by many other investigators (Qureshi et al. 1984; Ribo and Kaiser 1987).

In summary, the toxicity data generated in this study provide an indication of the potential toxicity of pesticide metabolites to bacteria. The EC_{50} values for 3,5,6-trichloro-2-pyridinol and salicylic acid and the metabolism studies of these chemicals conducted in our laboratory indicate that metabolites may play an important role in the induction or inhibition of enhanced microbial degradation of some pesticides. Some hydrolysis products that have been reported to serve as energy or nutrient sources for soil microbes displayed low EC_{50} values in this study. The toxicity of metabolites to *P. phosphoreum* may not correspond to toxicity to soil bacteria in all cases. No attempts have been made to relate the results of Microtox studies to the effect of pesticides/metabolites on microbes under actual field conditions. The Microtox system is not suitable for assessing the toxicity of less-water-soluble compounds, and in general, the data should be interpreted with caution because of the system's high sensitivity and differences in susceptibility to chemicals among the different genera of bacteria.

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