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Environmental Contamination and Toxicology

4-Chloro-2-Methylphenoxyacetic Acid Containing Compounds. Genotoxicity Evaluation by Mutatox® Assay and Comparison with Acute (Microtox®) and Embryo (FETAX) Toxicities

C. Vismara, A. Garavaglia

Department of Biology, University of Milan, 26 Via Celoria, 1-20133 Milano, Italy

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Since the environment is constantly exposed to a continuously increasing number of xenobiotics, there is a need for a rapid and sensitive battery of bioassays for biological monitoring assessment, that should evaluate toxicity, genotoxicity and embryotoxicity.

In the 80's a new genotoxicity test using a bacterial system and bioluminescent technique (BLT) was developed to determine the capability of tested compounds to restore luminescence by inducing mutation on dark mutants bacteria (Ulitzur *et al.* 1980; Ulitzur 1986). This technique was further developed by Microbics Corporation setting up the Mutatox Genotoxicity Test System, a good alternative to the Ames method (Johnson 1992). The rapid and relatively low cost Mutatox test, is a bacterial bioluminescence assay (BBA) that detects genotoxic activity in test compounds by measuring the light emissions produced by special dark mutant strain (Ml 69) of *V. fischeri* (Rowe *et al.* 1994).

In the current study we have investigated the genotoxicity of the herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA) using Mutatox. We have evaluated the purified MCPA Na salt, the commercial formulation (Erbitox E30), the technical grade MCPA Na salt (96% of purity) and its two synthesis intermediates, phenol and chlorocresol. Each was tested with and without S9 enzyme system activation. Moreover, we have compared the Mutatox data with those obtained using Microtox (acute toxicity test) and FETAX (Frog Embryo Teratogenesis Assay- *Xenopus),* previously performed in our laboratory (Vismara *et al.* 1996; Bernardini *et al. 1996).*

Correspondence to: C. Vismara

MATERIALS AND METHODS

Light production from luminescent bacteria was measured in a Microbics Model 500 Analyzer, a temperature controlled photometer, using the guidelines given by Microbics Corporation (1993). All compounds were tested for their ability to induce light production in the dark mutant of *Vibrio fischeri* (Ml69). The freeze-dried bacteria, Mutatox reconstitution solution (ultra pure water) and test medium (a solution containing 3% NaCl plus appropriate nutrients to support the necessary cell growth and light production) were supplied by Microbics Corporation (Carlsbad, California).

The commercial formulation of MCPA (Erbitox E30) was purchased from Società Italoamericana Prodotti Antiparassitari, Roma, Italy; technical grade MCPA Na salt (96% of purity), phenol and chlorocresol were from Sigma Chemical Co., St. Louis, Missouri, USA. The MCPA Na salt was purified by crystallization and acid-base purification starting from 97% MCPA (Fluka Chemie AG, Buchs, Switzerland), and its purity concerning phenol and chlorocresol presence was checked by HPLC. Purified MCPA Na salt contained less than 0.04% (w/w) of phenol and less than 0.004% (w/w) of chlorocresol (Bernardini *et al.* 1996).

The initial concentrations used for Mutatox, diluted following the Mutatox manual, were determined with a range finding test carried out on the basis of our previous investigations with Microtox. They were obtained from stock solutions prepared fresh for each experiment. Aliquots from the rehydrated bacteria were incubated with eight different dilutions of test sample in growth medium without (direct assay) and with a rat hepatic fraction for exogenous metabolic activation of promutagens (S9 assay). Light emitted from the bacteria was measured after 16, 20 and 24 hours of incubation at 27°C for medium without S9. Enzyme activation of compounds required 45 minutes of preincubation at 35°C. Phenol (a mutagen) and pyrene (a promutagen) were positive controls.

The results were expressed as Maximum Peak Concentration (MPC) reached during the test, correlated to the value of Light Units (LU). The LU value is the ratio of the light emitted by the treated bacteria (T) to the average blank value (B).

The genotoxic response was assumed as positive by increased light levels of at least twice the average control readings in at least two different sample dilutions. Test chemical concentrations ranged from 22.65 to 2900 mg/L for Erbitox E30, from 23.44 to 3000 mg/L for purified MCPA Na salt, from 11.72 to 1500 mg/L for technical grade MCPA Na salt, from 0.6 to 38.4 mg/L for chlorocresol and from 2.34 to 300 mg/L for phenol. Each compound was tested in duplicate in each Mutatox test and its response was validated with three tests repeated on different days. The data were compared with the EC_{50} of Microtox and with the LC_{50}, TC_{50} and MCGI of FETAX.

RESULTS AND DISCUSSION

Up to now, the Mutatox bioassay was mainly used for monitoring environmental genotoxic pollutants as well as for the detection of the potential genotoxicity of sediments (Johnson 1992; Ho *et al* 1994; Hoke *et al* 1994). In this study we have evaluated the herbicide MCPA in its formulations, including its two synthesis intermediates, phenol and chlorocresol, to evaluate their mutagenic potential as environmental pollutants. The mutagenicity values after 16, 20 and 24 hours of incubation are reported, for all tested compounds, in Table 1. The results showed that Erbitox E30, with MPC and LU values similar after 16 and 20 hours of incubation, was the least mutagenic of the tested compounds. This tendency is emphasized at 24 hours when the MPC increased two times with a drastic reduction of bioluminescence. In the case of Erbitox E30, we must consider that only 28% of the photoactive compound, with a purity of 87%, is present while the remaining is made of an unknown mixture of additives required for the commercial product.

Of more interest were the results obtained from purified MCPA Na and from MCPA Na technical grade. They showed the same value of MPC (187.5 mg/L) after 20 hours of incubation, but LU of the technical grade was about 10 times higher than the purified MCPA Na salt. This could be ascribed to the presence in the technical grade of two contaminants, phenol and chlorocresol. Phenol is well known to be a highly mutagenic compound, explaining its use as the positive control in the non-activation test. For phenol the MPC value was 150 mg/L, which is similar to previous results (Mutatox manual 1993). Chlorocresol was more mutagenic than phenol, with an MPC at

Table 1. Mutagenicity of tested compounds. MPC and LU values obtained after 16 (A), 20 (B) and 24 (C) hours of incubation with a direct assay (i.e., without S9 enzyme system activation).

A

Test compound	MPC (mg/L)	LU (T/B*)
Erbitox E30	725	163.2
purified MCPA Na	187.5	27.8
technical grade MCPA Na	93.75	15.8
phenol	75	3921.6
chlorocresol	9.6	3511.2

B

Test compound	MPC (mg/L)	LU (T/B*)
Erbitox E30	725	142.3
purified MCPA Na	187.5	38.7
technical grade MCPA Na	187.5	313.7
phenol	150	4010.7
chlorocresol	9.6	1765.6

C

Test compound	MPC (mg/L)	LU (T/B*)
Erbitox E30	1450	12.8
purified MCPA Na	187.5	52.2
technical grade MCPA Na	187.5	174.7
phenol	150	1559.3
chlorocresol	9.6	419.6

T/B*= ratio of the light emitted by the treated bacteria (T) to the average blank value (B).

9.6 mg/L. At this concentration the phenol was non-mutagenetic. Therefore chlorocresol proves to be the most mutagenic of the tested compounds.

With Mutatox a dose-response curve can be obtained, as shown for chlorocresol (Fig 1). It is a typical dose-response Gaussian curve, as the mutagenic activity increases as a function of concentration to the MPC value and then decreases because the toxic effects prevail on the mutagenic one.

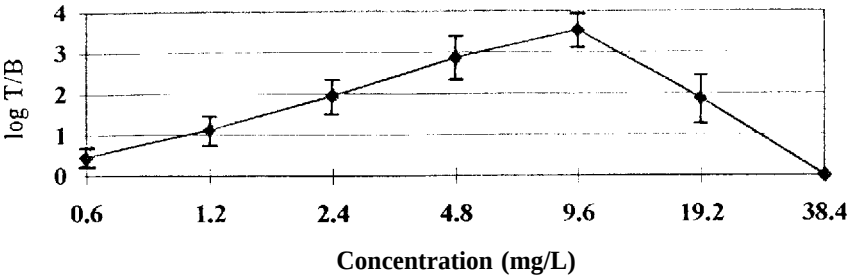

Figure 1. Dose-response results of chlorocresol directed assay, after 16 hours of incubation.

All tested compounds, including phenol and chlorocresol, did not show any signs of mutagenetic effects after treatment with exogenous metabolic activation system (S9) as mammalian liver detoxification nullifies the mutagenic activity of these compounds.

Lastly, we have compared the Mutatox results with those from Microtox and FETAX tests. The Microtox assay was developed to assess the toxic effects of industrial effluents (Bulich 1984) and aquatic pollutants (Ribo and Kaiser 1987). The toxicity endpoint is the median effective concentration (EC_{50}) for inhibition of *Photobacterium phosphoreum* luminescence. FETAX, with its three endpoints, mortality (LC_{50}), teratogenicity (TC_{50}) and growth inhibition (MCGI), is a reliable bioassay that makes use of *Xenopus* embryos (Dumont *et al.* 1983; Bernardino *et al.* 1996). FETAX results may be useful in comparison with Mutatox results as the processes which ensure the stability of genetic material are basic to enable organisms to proliferate in their environment. Unfortunately, widespread use of man-made chemical formulations could interfere with these genetic processes increasing the frequency of genetic diseases. The mutagenic activity of chemicals seems to be correlated to their embryotoxicity (Malling and Wassom 1977); consistently our data show that chlorocresol, the most mutagenic compound among those we have tested, resulted also to be the most embryotoxic one. The results obtained by Mutatox, Microtox (Vismara *et al.* 1996) and FETAX (Bernardino *et al.* 1996) for all the tested compounds are compared in Table 2. One should keep in mind that the bacterial strains of Microtox and Mutatox are different and that the respective mechanisms of action are quite different. In all considered tests the chlorocresol is the most toxic, mutagenic and

Table 2. Mutatox, Microtox and FETAX results (mg/L).

	Mutatox	Microtox	FETAX		
Test Compound	MPC	EC_{50}	LC_{50}	TC_{50}	MCGI
Erbitox E30	725	270			
purified MCPA Na	187.5	248	3607	2691	>1000 <2000
technical grade MCPA Na	187.5	121			
phenol	150	25	178	141	<25
chlorocresol	9.6	1.8	13.4	12.1	<2.5

embryotoxic compound and it is a possible step of the environmental degradative process of MCPA (Sattar 1982). Therefore, chlorocresol is to be considered a dangerous pollutant.

In conclusion, for an evaluation of the ecotoxicological effects of a pollutant it is necessary to use a battery of tests that takes into account not only the toxic effects but mutagenicity and embryotoxicity as well.

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