Journal of Environmental Biology
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September 2010, 31(5) 603-607 (2010)
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Cytotoxicity assessment of monocrotophos in *Paramecium caudatum* and *Oxytricha fallax*

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(Received: March 31, 2009; Revised received: June 29, 2009; Accepted: August 05, 2009)

Abstract: *Experiments were conducted to evaluate the toxic effects of monocrotophos in ciliate models Paramecium caudatum and Oxytricha fallax. In acute toxicity studies higher concentrations of monocrotophos caused marked increase in mobility of cells exhibiting rocking movements within two mins of exposure but were decreased after 30 mins. LC_{50} value by mortality curve for 3 hr acute toxicity test of Oxytricha fallax and Paramecium caudatum was found 307.744±33.27mg l^{-1} and 332.284±57.52 mg l^{-1} respectively. Oxytricha fallax was found sensitive than Paramecium caudatum to monocrotophos. In acute exposure cells showed deformities such as swelling, oval shaped deformity and in higher concentrations shortening of longitudinal axis with blackening of cytoplasm occurred. The length of paramecia was reduced prominently. Similarly, enlargement of contractile vacuole and stress egestion of food vacuoles was also observed. The morphological studies showed the changes in shape, size, colour and width of Paramecia and Oxytricha. Frequencies of macronuclear aberrations were significant showing deformities such as rod shaped, elongation, fragmentation, diffusion and total absence of nucleus and were concentration dependent. The data provided in the present study, on interaction of pesticides with nuclear structure can be of immense value because most of these pesticides have been reported to have carcinogenic, mutagenic and teratogenic properties.*

Key words: *Monocrotophos, Paramecium caudatum, Oxytricha fallax, Acute toxicity, Behavioural responses, Macronuclear aberrations*
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Introduction

Interest in the toxicity of pesticides, such as organophosphate (OP) compounds, has increased in the recent past as they enter waterways from agricultural and urban runoffs and may end up in aquatic environments and bioaccumulate in the food chain. Thus, pesticides contribute greatly to freshwater environment pollution, potentially causing harm to a large variety of non-target organisms (Madoni *et al.,* 1994; Walker *et al.,* 2001; Miyoshi *et al.,* 2003; Amanchi and Masood, 2007; Ramaneswari and Rao, 2008). It is generally agreed that in the field of environmental biomonitoring, toxicological bioassays can provide useful information for identifying those situations requiring a close investigation at an early stage (Hall and Giddings, 2000; Guti´errez *et al.,* 2003). From this point of view, it is of increasing interest to identify a panel of organisms displaying direct and sensitive responses to environmental perturbations. Assays with protists are regarded as valuable bioassays to be exploited in standardized laboratory procedures for evaluating the toxicity of chemical compounds or polluted waters (Narasimhan, 1999; Ujwala *et al.,* 2007; Fawole *et al.,* 2008).

Due to their small size, protists generally multiply through short cell cycles, thus making it possible to study the effects of pollutants on a large and genetically homogeneous cell population over a short period of time. Moreover, several species of protists can be cultured in the laboratory under seemingly natural conditions, so that their biological responses are more reliable. Numerous species feed on bacteria and are prey of higher organisms. As a consequence, contaminants can be potentially transferred along food chains and affect organisms at higher trophic levels, eventually leading to adverse effects on human health. On the other hand, an alteration in the protist component of microbial communities caused by the lethal effects of toxicants can alter the trophic chain and significantly affect the environmental balance (Yu *et al.,* 1999; Noboru *et al.,* 2002; Delmonte *et al.,* 2005; Trielli *et al.,* 2007; Kosuke *et al.,* 2008). The main objectives of present study were to determine LC_{50} and lethal concentration of monocrotophos, to investigate the cytotoxic effects under acute exposure in *Paramecium caudatum* and *Oxytricha fallax* and an attempt has been made to look into macronuclear deformities caused by monocrotophos.

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Materials and Methods

Test compound: The commercial grade sample of monocrotophos was supplied by Hyderabad chemical suppliers Ltd., Hyderabad. Monocrotophos (3-hydroxyl-*N*-methyl-*cis*-crotanamide dimethyl phosphate) ($C_7H_{14}NO_5P$), an organophosphorus compound, is a broad spectrum systemic insecticide and acaricide.

Test material and experimental organisms: *Paramecium caudatum* and *Oxytricha fallax* selected as test species for present studies were collected from fresh water pond within the vicinity of Osmania University, Hyderabad. The organisms were cultured in sterilized hay infusion medium at room temperature in the laboratory to obtain a pure line stock culture (Shiny *et al.,* 2005). The log phase

cultures were used for the present studies. Six grams of dried hay was boiled in one liter distilled water, cooled and filtered. Then, it was sterilized in an autoclave for 15 min at 15 pounds pressure and preserved for further use. Sterile precautions were maintained throughout the study. For culturing the organisms, hay infusion medium was diluted with distilled water in the ratio of 1:1 and was poured into different cavity blocks. Meat extract was supplemented to boost bacterial multiplication. Ciliates were inoculated into it under sterile conditions to obtain a pure line stock culture. The ciliates were added to the culture fluid in each cavity block and were covered by a lid to prevent the possibility of contamination and evaporation but at the same time to allow gaseous exchange between air and culture medium and sub-cultured on every 6th day.

Acute toxicity and behavioural studies: The toxicity to *Paramecium caudatum* and *Oxytricha fallax* of monocrotophos at different concentrations was determined and the possibility of utilizing these organisms for the pre-chemical screening under anthroponic pressure was examined. The toxicity effect at lower concentrations has been evaluated on the cell behaviour, cell morphology and cell viability. Stock solution and experimental concentrations of monocrotophos were prepared as recommended by APHA (2005). Stock solution of 1000 mg l^{-1} of monocrotophos was prepared using double distilled water as aqueous diluent. After preliminary rough dose finding experiments, the appropriate stock solutions and the test concentrations were selected, prepared a fresh and used for the toxicity studies as suggested by Apostol (1973). Acute toxicity test was conducted for 3 hr. In acute experiments 0.5 ml of pesticide solution was added to 4.5 ml of culture medium to achieve desired concentration of pesticide. Triplicates were maintained for all test concentrations. 50 organisms were introduced in each cavity block. Each cavity block, after adding pesticide was placed under binocular microscope and counting was done at 10 mins interval during first 1hr and thereafter 20 mins interval during the next 2 hr. LC_{50} value and lethal concentration were calculated against the mortality curve for 3 hr. Controls devoid of pesticide, with same number of organisms were run simultaneously.

Macronuclear morphology: Macronuclear morphology of *Paramecium caudatum* and *Oxytricha fallax* on exposure to sub-lethal concentrations of monocrotophos for 72 hr were studied. Nuclear staining was done by Feulgen fast green technique and it was found to be the most suitable technique as suggested by Rizzo and Nooden (1973). Schiff's reagent was prepared as suggested by De Tomasi (1936). Fixation of cells was done by Carnoy's fixative (Ethyl alcohol, Acetic acid and Methyl alcohol in the ration of 3:1:1 respectively). The cells were hydrolyzed first briefly in 1 N HCl maintained at room temperature and then at 60°C for exactly 8 mins. Hydrolysis followed by transferring the slides to Schiff's reagent and incubated for 1 hr. Then the cells were immersed in three changes of sulphurous acid salt solution for 5 to 6 mins, again rinsed in distilled water, dehydrated in graded alcohols, cleared in xylene and mounted in DPX.

Statistical analysis: All the results were presented with suitable statistical interpretation such as test of significance, Mean and SD using origin 6.1.

Results and Discussion

Acute toxicity and behavioural studies: The acute effects can be mortality, immobilization, reduced cell count or behavioral observations and it also includes immediate cytopathological responses manifested in the form of changes in body size and shape, ultra structural deformities as reported earlier (Rouabhi *et al.,* 2008; Uma *et al.,* 2008; Amanchi and Mohan Bhagavathi, 2009). Higher concentrations of monocrotophos (1000 mg l^{-1}) increased cellular activity within a minute of exposure due to cell lysis. LC_{50} value by mortality curve for 3 hr acute toxicity test of *Oxytricha fallax* and *Paramecium caudatum* was found 307.744±33.27 mg l^{-1} and 332.284±57.52 mg l^{-1} respectively (Fig. 1a,b). At 200 and 250 mg l^{-1} concentration cells became irritated within minutes of exposure and started swimming away. The cells were without visible oral structure, food vacuoles, only macronucleus and contractile vacuole were visible at this concentration. At moderate concentrations Paramecia and Oxytricha showed vacuolization, formation of trichocysts and morphological deformities such as spheroid shape, swollen body shape and shortened body length through anterior posterior axis. No visible cytopathological changes were observed at lower concentrations such as 50 mg l^{-1}. Movement, which is a vital sigh of life, was taken as a parameter in the present investigation. Abnormal behaviours such as restlessness, sudden and quick movements, swimming on the back and stress egestion of food vacuoles at higher concentrations were observed. Loss of movement coordination and orientation was observed at 350 mg l^{-1} concentration. The sensitivity and survival ability of Paramecia and Oxytricha in the present experimental conditions could be exploited for the biomonitoring

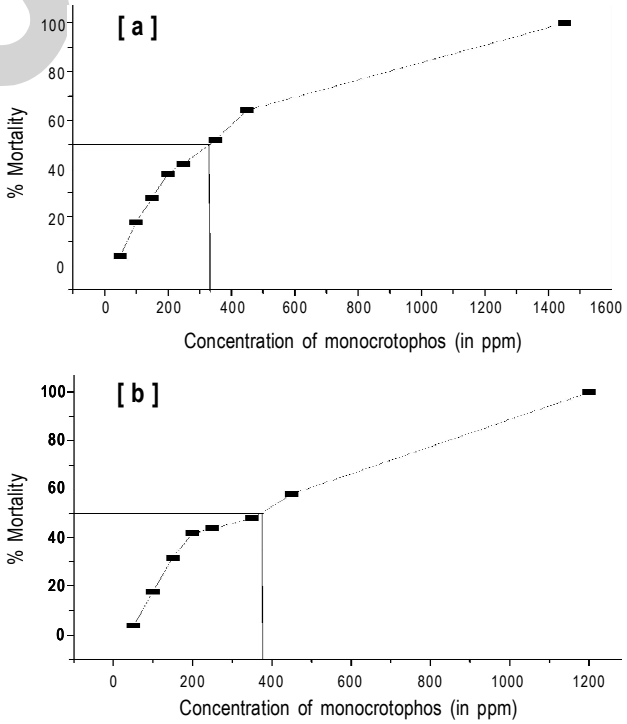

Fig. 1: Calibration curve showing lethal concentration and LC_{50} values of monocrotophos to (a) *Oxytricha fallax* and (b) *Paramecium caudatum*

Table - 1: Monocrotophos induced macronuclear aberrations (%) in *Paramecium caudatum* exposed for 72 hr

Concentration mg l^{-1}	Total abnormalities	Unevenly divided	Fragmentation	Rod shaped	Vacuolated	Other deformities
100	65.3±2.45	17.2±1.31	8.2±1.13	14.3±2.16	13.3±1.41	14.4±1.17
50	55.3±2.49	15.8±1.22	7.2±1.31	13.9±1.197	9.4±1.95	9.2±1.31
10	51.6±2.95	14.2±1.31	4.1±1.19	12.2±1.03	10.2±1.54	12.4±1.50
5	30.2±1.31	7.8±0.78	2.2±1.03	10.2±1.61	6.2±1.39	4.3±1.15
Control	0.18±0.23	- - - -	- - - -	- - - -	- - - -	0.18±0.23

Mean and SD values are significant at $p<0.05$ (n=5)

Table - 2: Monocrotophos induced macronuclear aberrations (%) in *Oxytricha fallax* exposed for 72 hr

Concentration mg l^{-1}	Total abnormalities	Diffusion	Disappeared	Rod shaped	Fragmented	Other deformities
100	56.1±1.66	14.8±1.31	10±1.82	9.2±1.93	11.9±2.13	9.9±1.66
50	46.9±1.66	12.9±2.68	8.7±1.7	8±1.15	6±1.49	11.2±1.33
10	40±1.88	11.9±1.91	6.2±1.98	6.3±.63	8.1±1.15	8.1±1.37
5	21.3±1.82	6.2±1.68	3.1±1.66	3.1±0.87	5.1±1.91	4.8±1.68
Control	0.36±0.18	- - - -	- - - -	- - - -	- - - -	0.36±0.18

Mean and SD values are significant at $p<0.05$ (n=5)

studies and also pesticide remediation from the polluted waters. Venkateswara Rao (2004) reported LC_{50} value of monocrotophos and its analogs 2-butenoic acid-3- methyl ester and 2-butenoic acid-3- ethyl ester on *Oreochromis mossambicus* at 96 hr. Exposed fish exhibited abnormal behaviour, which includes erratic swimming, loss in equilibrium and loss of mucus on gills.

In vitro observations of Nilsson (2003) revealed that at higher concentration of zinc (1.5 to 2 mM) about 80% of cells became motionless and a few cells had enlarged contractile vacuole and were spherical of shape. Nilsson (2005) reported on Tetrahymena, at lower concentrations of ethanol revealed normal cell behaviour, whereas at higher concentration, some cells lost their shape initially and became spherical with an enlarged contractile vacuole. In chronic toxicity after 24 hrs two distinct populations were seen, one of normal sized cells and another of small, mostly spherical cells. Golam *et al*., (2005) reported that, the cell shape may depend on the cytoskeletal structures such as microtubules and actin filaments in *Paramecium bursaria*. The effects of heavy metal ions on cytoskeletal structures, such as shortening of the microtubule length or depolymerization of actin microfilaments will disturb the shape of *Paramecium bursaria.* The toxic effects of basudin, AFD25, mercury and different mixtures of these three compounds were tested on *Euplotes crassus*. Exposure to these toxicants significantly affected cell viability, mean fission rate and lysosomal membrane stability (Trielli *et al.,* 2007). The correct size, shape and basic morphology of all ciliates depend on the correct structure and function of the cell membrane, which were being violated after adding this pesticide. The complete lysis of cells at a particular concentration might be due to the natural response to adverse environment.

Morphological changes in macronucleus: The types of macronuclear aberrations found in the present work were vacuolization of nucleus, elongation, karyolysis, fragmentation of nucleus, diffusion of nucleus, unevenly divided forms *etc.* The 100 mg l^{-1} concentration induced 65.3±2.45 and 56.1±1.66 deformities ranging from fragmented nuclei, rod shaped deformity and vacuolated macronucleus in *Paramecium caudatum* and *Oxytricha fallax* respectively (Table 1, 2). All tested concentrations caused various aberrations in the nuclear design in concentration dependant manner. The highest unevenly divided forms of nucleus were observed in 17.2±1.31 cells at 100 mg l^{-1} concentration in Paramecia. The rod shaped nucleus was observed in 14.3±2.16 cells at 100 mg l^{-1} concentration, which is highest recorded rod shaped deformity against monocrotophos. The nuclear aberrations are also observed in control because of abnormal growth and physiological status of a very few organisms in log phase culture. The formation of these abnormalities is related to cell division failures and cell death processes. It is evident from findings that monocrotophos acts as potential genotoxic substance at certain concentrations to ciliate models.

Kasturi bai and Dilli (1974) reported the toxicity of Folidol on the morphology and nucleus of 5 fresh water ciliates namely *Spirostomum ambiguum major, Spirostomum amgiuum minor, Blepharisma intermedium, Blepharisma seshachari* and *Frontonia leucas.* All the test concentrations of Folidol namely 50 µ, 40 µ and 1 µ caused toxic effects on the nuclear apparatus and morphology bringing about changes such as reduction in size, distortions to double macronuclei. Lal and Saxena (1980) observed that the *Stylonchia notophora* when treated with 100 ppm of DDT showed number of deformities in the nuclear morphology such as deep incisions and fragmented nuclei. Similarly, Dias *et al.* (2003) reported cytoskeletal alterations in *Tetrahymena pyriformis* when treated with Triton X 100. They observed that after 1 hr of treatment a large unstained area in the central part of the cytoplasm and the cell started to become round and the nucleus started moving to the cell periphery. Zhong-Dong Xu *et al.,* (2008) reported Zn (II)-L and Co (II)-L induced DNA damage in *Tetrahymena thermophila* by comet assay.

After treatment with the 0.05, 0.25 and 0.50 mg ml^{-1} Co, the tail length of the cells increased longer than those treated with the same dose of Zn significantly. Mohd Masood *et al.* (2008) found that when *Paramecium caudatum* was exposed to 280 ppm concentration of carbofuran, it caused 47% abnormalities in macronucleus, which included deformities such as fragmentation, uneven division and vacuolization. In related experiments, Amanchi and Hussain (2008) demonstrated cytotoxic effects of delfin insecticide (*Bacillus thuringiesis*) on cell behavior, phagocytosis, contractile vacuole activity and macronucleus in *Paramecium caudatum*. The macronuclear aberrations like marginalization, fragmentation, vacuolization and complete diffusion of macronucleus increased with increasing concentrations of delfin up to 100 ppm.

Venkateswara Rao *et al.* (2007) have noticed monocrotophos exhibiting a different mode of action in the non-target organism, *Paramecium caudatum* causing unusual changes in cell membrane which altered the morphology and locomotor behaviour. In the present study a total of 1000 cells both in control and treated were examined. Nuclear alterations were considered to be the consequence of genotoxic events and mutagenicity. A cell to perform its function, normal structure of nucleus is essential. All the metabolic activities depend on the correct structure and function of nucleus, which were being violated after pesticide exposure. In conclusion, data on interaction of pesticides on the nuclear structure can be of immense value because most of these pesticides have been reported to have carcinogenic, mutagenic and teratogenic properties.

The present study gave us clear understanding about the cytotoxic effects of monocrotophos on *Paramecium caudatum* and *Oxytricha fallax* and their sensitivity and survival ability has made them as cheap, simple and idle candidates for toxicity assessment of pesticides. It is further concluded that monocrotophos under the experimental conditions induced macronuclear deformities suggesting its mutagenic potential.

Acknowledgments

The authors are thankful to Prof. K. Rudrama Devi, Head, Department of Zoology, University College of Science, Osmania University for extending lab facilities and for her encouragement.

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