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Full Length Research Paper

Cytotoxic effects of delfin insecticide (*Bacillus thuringiensis*) on cell behaviour, phagocytosis, contractile vacuole activity and macronucleus in a protozoan ciliate *Paramecium caudatum*

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The freshwater protozoan ciliate *Paramecium caudatum* was used to assess the potential cytotoxic effects and functional activities of biological insecticide delfin. Delfin® WG **is a biological insecticide based on the SA-11 strain of *Bacillus thuringiensis* subspecies *kurstaki* serotype 3a, 3b. In acute toxicity studies, cell motility was affected slightly at lower concentrations but at higher concentrations cells exhibited rocking movements, later on cells became motionless. LC_{50} for 3 h exposure was found to be 250.17 ± 15.33 ppm. In acute exposure cells showed deformities such as swelling of cells, oval shaped deformity, and at higher concentrations shortening of longitudinal axis in the body size with blackening of cytoplasm occurred. Leaking of cytoplasmic contents was also observed. A significant depletion of phagocytosis was observed on exposure to 100 ppm of delfin for 30 min to 1 h duration and was time dependent. Changes in pulsatory vacuole activity were observed on exposure 25, 50 and 100 ppm for 20 min. The macronuclear aberrations increased with increasing concentrations of delfin up to 100 ppm. Macronuclear aberrations such as rod shaped macronucleus, marginalization of macronucleus, fragmentation, vacuolization and complete diffusion of macronucleus were observed and were dose dependent. Our findings on phagocytosis, contractile vacuole activity and macronuclear changes indicate a potential physiological and cytogenetic effect of delfin on *P. caudatum*. The simplicity in handling, faster generation time, easy maintenance in the laboratory, rapid performance and high reproducibility makes ciliates as suitable tools and tags for physiological, genotoxicity studies and risk assessment.**

Key words: Delfin, *Paramecium caudatum,* macronucleus, phagocytosis, contractile vacuole activity.

INTRODUCTION

Monitoring of aquatic pollution represents one of the major activities involved in measurement aimed at environmental protection. Usage of non-target organisms in environmental toxicology is needed to understand wide range of toxic effects caused by the pesticides and other pollutants on different organisms. Attempts have been made to replace fish and other aquatic biota commonly used as bioindicators of organic pollutants in recent years successfully by ciliates. Free living protozoa are often used as bioindicators of chemical pollution, especially in aqueous environment (Venkateswara Rao et al., 2006; Masood, 2006a).

One important contribution of microorganisms in the present era is their usage as gauging agents of toxicosis stress, bioremediation and as biomonitors in the aquatic bodies which are getting polluted by pesticide residues, domestic sewage and industrial effluents. The potential of ciliates as test biota have been reviewed recently by Sauvant et al. (2000) and Niculescu et al. (2000), but most of these assays concerned essentially *Tetrahymena pyriformis* and *Colpidium campylum*. The selection of a

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species as a suitable model was based on its ubiquitous distribution, large size for a unicellular organism among other ciliates which facilitates observations of behavior and physiological changes in the presence of toxicants, easy maintenance in a laboratory under normal conditions and survival capacity in a confined environment for several days. Thus their use in microbiotests is possible in any laboratory. Until now, only descriptive and ecological studies of these protozoans have been published. Mortality, behavioral and morphological changes have been used for assessing changes in water quality. Mortality rate and population growth impairment is often used sub-lethal toxic endpoint for organic (Larsen et al., 1997) and inorganic (Sauvant et al., 2000) compounds. Other parameters such as cell motility (Darcy et al., 2002) and swimming patterns (Noever et al., 1994), cytoskeleton analysis (Dias et al., 2003; Casalou et al., 2001), phagocytosis (Nilsson, 2003a; Masood, 2006b), can be assessed and have been proposed to determine the physiological and energetic state of ciliates when in contact with pollutants. The aim of the present study was to present a new, rapid, simple, sensitive and low cost microbiotest based on freshwater ciliate *Paramecium caudatum.* This is one of the most commonly used ciliated protozoa for laboratory research.

In the present work, *P. caudatum* was used as model organism to assess the effect of delfin through an *in vitro* series of cytotoxicity tests. Delfin is a water dispersible granule for the control of a wide range of caterpillars in vegetables, cabbage moth, cabbage white butterfly, potato moth and ornamentals. The bioassays performed in this study include simple and fast acute test that give information about the locomotor behaviour, morphology, phagocytosis, contractile vacuole activity of the cells when exposed to different concentrations of delfin. Genotoxicity of the pesticide was determined using macronuclear aberrations as an end point.

MATERIALS AND METHODS

Test compound

Commercial grade of delfin insecticide used in this study was manufactured by CERTIS LLC, Columbia, USA, and imported and packed by Margo biocontroles (P) Ltd, Bangalore, India.

Test material selected for study

P. caudatum being cosmopolitan in nature is found in ponds, streams and lakes, was selected as test species for present studies. It was collected from fresh water pond within the vicinity of Osmania University, Hyderabad, India. The organisms were cultured in sterilized hay infusion medium at room temperature in the laboratory to obtain a pure line culture. 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 and preserved for further use. Sterile precautions were maintained throughout the study. Hay infusion medium has been widely used as a basic and most appropriate culture medium for ciliates. It gives the ciliates an environment more close to their own habitat.

Culture of the experimental organisms

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. 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 6^{th} day.

Acute toxicity studies

Stock solution and experimental concentrations of delfin were prepared as recommended by APHA (1995). Stock solution of 1000 ppm of delfin was prepared using 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. The test procedures adopted were those suggested by Apostol (1973) and Jaleel (2002). Acute toxicity test was conducted for 3 h duration. In acute experiments 0.5 ml of pesticide solution was added to 4.5 ml of culture medium to achieve desired concentration of each pesticide. 50 organisms were introduced in each cavity block. Triplicates were maintained for all test concentrations. Each cavity block, after adding pesticide was placed under binocular microscope and counting was done at 10 min interval during first one hour and thereafter 20 min interval during the next 2 h. LC_{50} value and lethal concentration were calculated against the mortality curve for three hours duration. Controls devoid of pesticide, with same number of organisms were run simultaneously.

Phagocytosis

Phagocytosis was studied after exposure to sub-lethal concentration (1/3 of LC_{50} value) of delfin. The test organisms were exposed to 100 ppm concentration of delfin for 30 min and 1 h duration. Twenty five treated cells from the same concentration after 30 min and 1 h exposure time in the toxicant were picked with the help of micropipette, mixed with one molar carmine suspension and kept for 10 min. Ten organisms from the two exposed groups (30 and 60 min) were taken, immobilized on protamine coated slides and the number of food vacuoles formed was counted. Similarly control paramecium without pesticide mixed with carmine suspension, immobilized and counting of food vacuoles formed were done. Preparation of carmine suspension and counting of food vacuole was done by the method suggested by Nilsson (2003a).

Contractile vacuole activity

Contractile vacuole activity was measured in the cells after being immobilized by protamine coated slides as per the method suggested by Marsot and Couillard (1973). From the cells exposed to sub-lethal concentration for 10 and 20 min, single individual cell normal in every visible respect was picked with the help of micropipette and the rate of pulsation of one vacuole i.e. the time required for one complete pulsation was determined (from the beginning of one contraction to the beginning of next). Observations were made on the cells in each concentration and equal number of observations was done in controls at room temperature.

Genotoxicity studies

Cytochemical studies were conducted to demonstrate and study the macronuclear morphology of *P. caudatum* on exposure to sub-

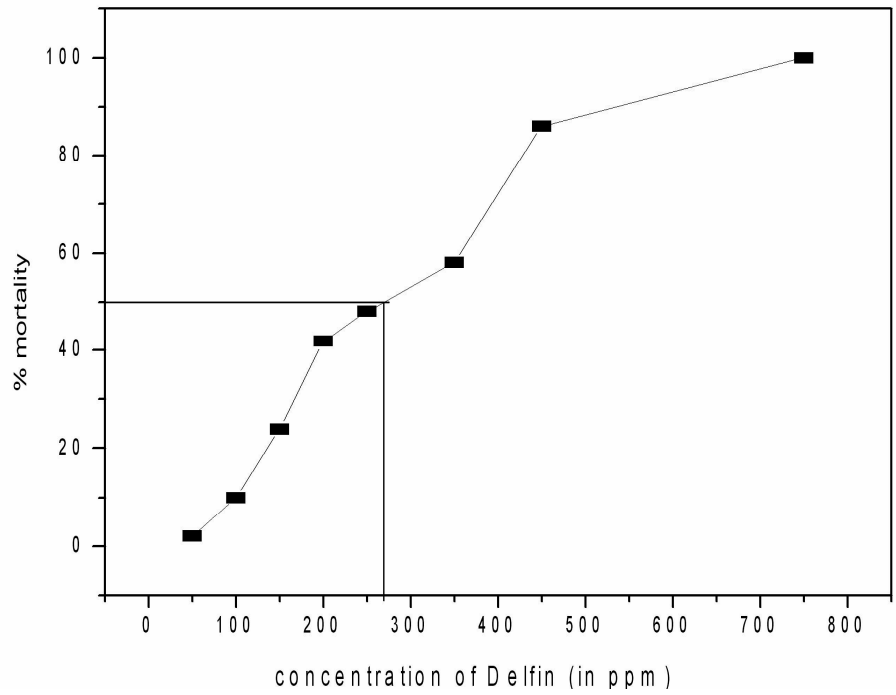

Figure 1. Acute toxicity (LC_{50} value) of delfin against *P. caudatum.*

lethal concentrations of delfin. Nuclear staining was done by Feulgen fast green technique and it was found to be the most suitable technique for the demonstration of nuclear apparatus in ciliates 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 min. Hydrolysis followed by transferring the slides to Schiff's reagent and incubated for 1 h. Then the cells were immersed in three changes of sulphurous acid salt solution for 5 to 6 min, again rinsed in distilled water, dehydrated in graded alcohols, cleared in xylene and mounted in DPX.

All the results were presented with suitable statistical interpretation such as test of significance and SD.

RESULTS AND DISCUSSION

A lethal concentration of 750 ppm of delfin caused immediate cell death with complete blockage of ciliary movements, contractile vacuole activity and food vacuole activity. The calculated LC_{50} value for 3 h exposure was 250.17±15.33 ppm (Figure 1). At the concentration of 200 ppm ciliates showed abnormal and disturbed movements. Shortening of longitudinal axis, vacuolization of cytoplasm, blackening of cytoplasm and enlargement of contractile vacuole were observed on exposure to higher concentrations of 300 and 400 ppm. No visible and morphological cytotoxic effects were observed at 100 ppm concentration for 180 min exposure (Figure 2). Mahboob et al. (1998) reported lethal dose (LD_{50}) of vepacide *Azadirachta indica* was 1566.85 ±134.06 mg/kg in rat by the oral route. The rats showed symptoms such as dullness, irritation, lacrimation and diarrhea. It also altered food intake, body weight, and hematological and biochemical parameters in rats (Rahman and Siddiqui, 2004). First visible changes to occur at lower concentrations of our test organism were irregularities in ciliary beating, that is, short interruptions and spinning movements, which often resulted in swimming away of the cell. At higher concentrations, the movement of the cilia became weaker and irregular after a while deformity in the cell occurred and finally the organism died. Under pesticidal stress, many cells could not move normally in a straight line but were spinning around themselves. Stress egestion of food vacuoles was also observed. Boenigk et al. (2005) reported similar results on flagellates Ochromonas and *Bodo saltans*. They noticed irregularities in flagella beating and stress egestion of food vacuoles after ten minutes exposure to heavy metal mercury. Nilsson (2005) reported loss of cell shape and enlargement of contractile vacuole in *Tetrahymena* on exposure to higher ethanol concentration. The cells were mostly without visible oral structure or food vacuoles. Nilsson further observed that in moderate concentrations (1 mM of Zn) mobility of ciliates became more leisurely and some cells became immobile. However, at higher concentration (1.5 to 2 mM) a high fraction of cells (up to 80%) became motionless and a few cells had enlarged contractile va-

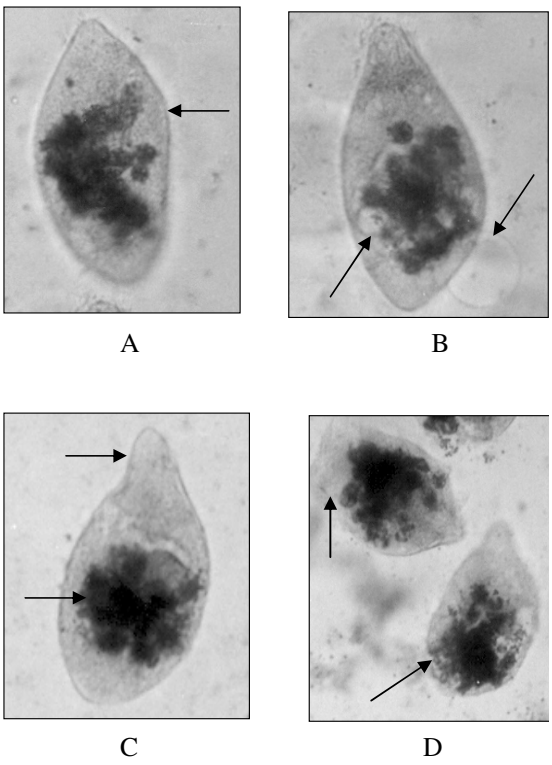

Figure 2. Delfin induced morphological deformities in Paramecia. **A**: Swelling and shortening of longitudinal axis (200X). **B**: Vacuolization of cytoplasm and blebbing (200X). **C**: Blackening of cytoplasm and narrowing of anterior (200X). **D**: Oval shape deformity and rupturing of cell membrane (200X).

cuoles and were spherical in shape (Nilsson, 2003a).

Inhibition in food vacuole activity was observed on exposure to 100 ppm and the experimental ciliates showed 5.5 and 4.3 mean number of food vacuoles respectively in 30 and 60 min exposure. The 30 min exposure of 100 ppm concentration exerted less inhibiting effect (36.05% reduction) than the 1 h exposure of 100 ppm concentration which caused 50% reduction in phagocytosis as represented in Figure 3. Formation and movement of the food vacuoles depend on ciliary motility because cilia are responsible for both locomotion and moving food vacuoles towards the cytostome. Factors effecting the ciliary action should also affect the rate at which a food vacuole is formed. Balf (2005) reported changes in the phagocytosis of Tetrahymena on exposure to cigarette extracts. Antibiotics caused inhibition of phagocytic activity in *Tetrahymena pyriformis* (Rebendal and Karpinska, 1981). Hussain et al. (2008) and Jaleel. (2002) reported the inhibition of phagocytosis in *P. caudatum* when exposed to different concentrations of carbuforan and DDVP (Dichlorvos), respectively.

Efficiency of the phagocytic activity and its inhibition by the pharmacological compounds *in Paramecium aurelia* was well demonstrated and studied by Surmacz et al. (2001). Fok et al. (1985) reported that Trifluoperzine completely blocked digestive vacuole formation in *P. caudatum.* The effect of Trifluoperzine (TFP) may be due to the calcium/calmodulin signal regulating formation of the food vacuoles. Actin may be a primary target of pharmacological agents used to inhibit Paramecium phagocytosis. Ethanol, in higher concentrations, affected both phagocytosis and cell proliferation dose and time dependently (Nilsson, 2005). Nilsson (2003b) reported a close relationship between the motility of cell and capacity for phagocytosis. When cell motility was disturbed or cells exposed to high concentration of heavy metals, the phagocytic activity was very much reduced and the phagocytic activity of organisms was closely related to the composition of culture medium and the presence of organic matter in the medium.

Delfin caused a sharp and high cut off in the number of food vacuoles formation after one hour exposure. This observation suggested that there could be a severe injury upon the cell membrane and the internal contents of the animals. It is likely that delfin inhibits cytoplasmic contractions, which are necessary for separation of the food vacuoles. This is also clear from the present studies that the ciliates do not loose the capacity for suspension intake but it is suppressed to certain extent. This effect was time dependent. Any addition of pesticides to the cell's external environment lead to changes in the pH of the media, and subsequently changes in food vacuole formation.

Paramecium, a favourite organism for the study of contractile vacuole structure and function, has been investigated by earlier authors and those works have examined for many years. Kitching (1956) in his pioneering studies on the physiology of contractile vacuole pointed out that a pressure on or a tension at the vacuole membrane could produce the pressure required to discharge the fluid contents of the vacuole through the vacuole pore. Delfin had maximum inhibitory effect on the contractile vacuole activity of *P. caudatum* at 50 ppm concentration when exposed for 20 min, where the average time for one pulsation was 10.60 s as compared to control value of 6.66 s. In all the concentrations (100, 50 and 25 ppm) tested, the vacuolar activity was clearly diminished. In 100 ppm concentration after 20 min exposure, the vacuolar activity recorded as 9.00 s; comparable to control value of 6.66 s (Table 1 and Figure 4).

In the higher concentrations of delfin, on shorter exposure time, ciliates showed decreased vacuolar activity, which must be due to sudden response to changes in external osmolarity of the medium and also disruption of the vacuolar apparatus. Therefore the pulsation rate of the contractile vacuole changed immediately. However after some time, it recovered to certain degrees to show the pulsation rate near control value. Toxicity of a chemi-

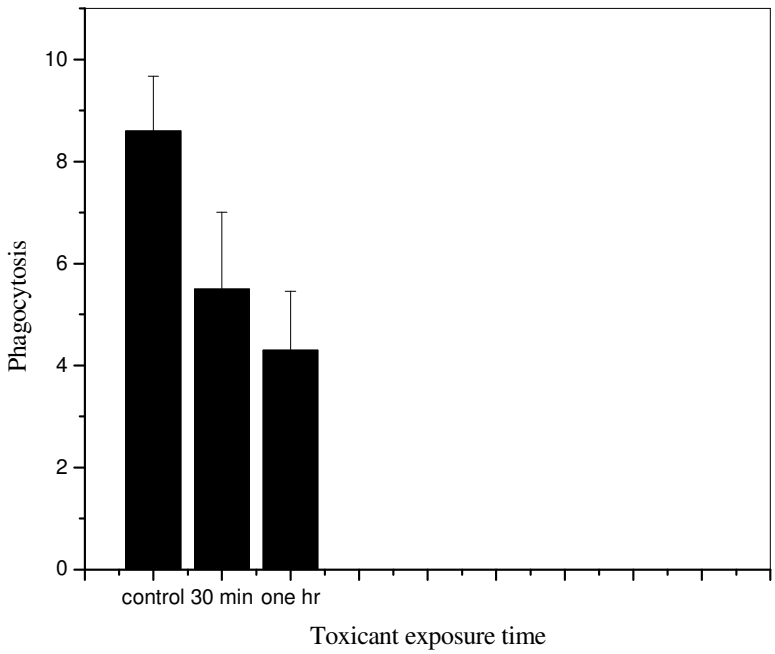

Figure 3. Food vacuole activity in control and delfin (100 ppm) treated Paramecium.The values represented are Mean ± S.D (n = 5).

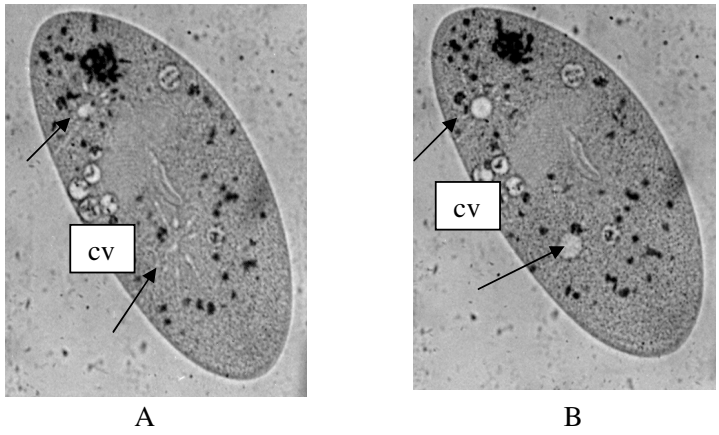

Figure 4. Contractile vacuole activity in *P. caudatum*. **A**: Paramecium showing radiating canals of contractile vacuoles (400X). **B**: Paramecium showing fully formed contractile vacuoles (400X).

Table 1. Contractile vacuole activity in *P. caudatum* exposed to delfin.

Concentration (ppm)	Exposure time (min)	Average time for one pulsation (s)	Pulsations per min
100	10	8.57	7.00
	20	9.00	6.66
	10	9.47	6.33
50	20	10.60	5.66
	10	7.20	8.33
25	20	8.57	7.00
Control		6.66	9.00

Table 2. Macronuclear aberrations in *P. caudatum* exposed to delfin for one hour. Values are significant at $p < 0.05$ level; n = 5.

Concentration (ppm)	Percent abnormal forms	Various abnormal forms				
		Unevenly divided	Vacuolated	Fragmented	Rod shaped	Other deformities
100	71%	17	13	19	14	8
50	56%	14	7	13	12	10
25	43%	8	4	9	13	9
10	35%	6	2	3	21	3

cal in aquatic organisms depends on various factors such as the developmental stage of organism, composition of the medium, pH of external environment and oxygen content. The pulsatory vacuole expultion frequency can be modified by other external factors (Patterson, 1980). Stock et al. (2002) found that the ionic composition of both the cytosol and the contractile vacuole fluid change in response to changes in the ionic environment of the cells. They concluded that osmolarity of the contractile vacuole fluid was always hypertonic to the cytosol, and also osmolarity of cytosol is hypertonic to the external osmolarity. The water segregation rate was strongly reduced as the external osmolarity was increased (Toumani et al., 2002). Observations of contractile vacuoles in ciliates have shown that the contractile vacuole complex is dynamic and highly responsive to changes in the cell's environment.

Several workers suggested the usefulness of nuclear study as model system for screening the environmental mutagenicity in ecosystem bioindicators. Consequently in order to evaluate the quality of aquatic environments, this test has been carried out in several organisms, such as mussels, fishes etc (Klobucar et al., 2003; Minissi et al., 1996). It represents an important environmental tool for the diagnosis of areas under human influence, by detecting mutagenic agents responsible for genetic instability. The data illustrates the presence of large number of fragmented and unevenly divided macronuclear forms when exposed to different concentrations of delfin. The highest total abnormalities (71%) were recorded when *P. caudatum* was exposed to 100 ppm concentration of delfin for an hour. In concentrations of 100, 50, 25 and 10 ppm, the percent abnormal forms recorded were 71, 56, 43 and 35% respectively. The vacuolization of macronucleus among 13% was recorded at 100 ppm concentration of delfin. The vacuolization represents the sites of vanished macronuclear part. The lowest abnormalities recorded were 35%, at 10 ppm of delfin for an hour (Table 2).

Ruiz and Marzin (1997) demonstrated the mutagenicity of captofol and captan on *Salmonella typhimurium* and *Escherichia coli*. The mutagenic activity of captafol and captan to these strains gave positive results. Tatiana and Fontanetti (2006) reported many nuclear abnormalities, such as vacuole nuclei, nuclear fragmentation, nuclear retraction and kariolysis and they also found binucleated erythrocytes and polynucleated erythrocytes in *Oreochromis niloticus* exposed to waters affected by refinery effluent. Fenech and Crott (2002) have shown the induction of nuclear abnormalities such as nuclear buds and nucleoplasmic bridges in human lymphocytes, under conditions of folic acid deficiency. Yokote (1982) reported the presence of bilobulated and fragmented nuclei in fish erythrocytes. Induction of nuclear abnormalities in haemocytes of the mussel, *Mytilus galloprovincialis,* exposed to benzo[a]pyrene, was shown by Venier et al. (1997). The data from the literature demonstrated a direct relationship between genomic instability and some abnormalities, such as nuclear fragmentation, vacuolization, nuclear buds, nucleoplasmic bridges, unevenly divided and karyolysis (Fenech and Crott, 2002; Serrano-Garcia and Montero-Montaya 2001). These nuclear alterations were considered to be the consequences of genotoxic events. So that 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 comprehensive understanding about the toxic effects of delfin on ciliated model *P. caudatum*, its suitability for evaluation in toxicant influx and their possible role as cheap and simple model in toxicity evaluation. It is further concluded that such studies could serve towards a better understanding of ecosystem viability and potential role of ciliates as bioindicators in water quality and stress recovery.

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