

Tolerance of Two Ciliates, *Stylonychia mytilus* and *Paramecium caudatum*, Isolated from Industrial Effluents to an Organophosphate, Endosulfan and their Potential use in Bioremediation of Insecticide-contaminated Wastewater

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Abstract. Two ciliate protozoans, *Stylonychia mytilus* and *Paramecium caudatum* isolated from tannery effluent and cultured in a medium containing inorganic salts showed tolerance against endosulfan (1.8 µg/ml). The protozoans uptake the insecticide from the medium for utilization. *Stylonychia mytilus* reduced 21% endosulfan from the medium within 48 hours of inoculation of insecticide containing medium with the protozoan cells; at the end of 8 days of culturing, the reduction of endosulfan in insecticide containing medium was 78%. The *Paramecium caudatum* on the other hand reduced concentration of endosulfan in medium by 11% after 2 days of inoculation, whereas after 8 days of culturing, the reduction was 72%. The insecticide utilization by protozoa as a carbon source for their growth can be exploited for insecticide removal and environmental clean-up operations.

Key words: Endosulfan, bioremediation, *Paramecium caudatum*, *Stylonychia mytilus*.

INTRODUCTION

I ncreasing environmental pollution and the continuous development of new chemicals and drugs has led to ever growing concern about the potential effects of these compounds directly or indirectly on human health. Endosulfan is a commonly used cyclodiene insecticide applied to protect cotton, tobacco, coffee, cereal, fruit, and vegetable crops against a wide array of insect pests. The U.S. Geological Survey estimated that a total of 812,787 kg of endosulfan was applied in the United States during 1992 (Thelin and Gianessi, 2000). Ontario, Canada, used 25,000 kg of endosulfan in 1992 on fruit and vegetable crops alone (Harris *et al.*, 2000). Agricultural runoff of rain and irrigation water introduces endosulfan into the aquatic environment, where it poses significant toxicological risks to resident organisms (Scott *et al.*, 1990).

This chlorinated compound exists in two

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stereoisomeric forms, endosulfan I and endosulfan II. The molecular weight of endosulfan is 406.9 and its solubility in water is approximately 0.32 mg/l (Kidd and James, 1991). Endosulfan sulfate can persist in natural water for months and can be as toxic to aquatic organisms as the parent compound (Wan *et al.*, 1995). It is registered fish toxicant (Paul and Raut, 1987) also equally toxic to aquatic invertebrates and has been implicated increasingly in mammalian gonadal toxicity (Sinha *et al.*, 1997; Turner *et al.*, 1997), genotoxicity (Chaudhuri *et al.*, 1999) and neurotoxicity (Paul and Balasubramanian, 1997).

The desorbed pesticides and xenobiotics dispersed in soil aqueous phase are assumed to be available as substrates for competent microorganisms (Burns *et al.*, 1996). Microbial population has a direct correlation with the amount of organochlorinated insecticides (Edwards *et al.*, 1992; Sethnathan *et al.*, 2004), which are used as a sole energy source by microorganisms (Mohn and Tiedje, 1990; Madsen, 1991; Cabras *et al.*, 1995; Lee *et al.*, 2006; Kumar *et al.*, 2008). Pesticide degradative genes are found to be located on

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0030-9923/2008/0004-0255 \$ 8.00/0

plasmids, transposons and chromosomes in microbial system (Kumar *et al.*, 1996).

Microbial xenobiotics removal has received much attention during the past few years due to the potential use of microorganisms for cleaning anthropogenically polluted water (Ledin, 2000). Microorganisms act on the insecticides by metabolizing and /or transporting these chemicals through successive trophic levels in food chains. Uptake of insecticides by various groups of algae, fungi and bacteria has been extensively studied (Lal and Saxena, 1982; Lal *et al.*, 1987a; DeLorenzo *et al.*, 2002). Protozoans have also been reported to be present in and metabolizing industrial effluents contaminated by toxic metal ions such as Cu^{2+} , Hg^{2+} , Ni^{2+} , Pb^{2+} , Zn^{2+} and Cd^{2+} and other toxic compounds (Shakoori *et al.*, 2004; Haq *et al.*, 1998; Madoni *et al.*, 1996).

Compared to other groups, data regarding the toxic effects of endosulfan on protozoans are, however, limited. In addition, information is lacking regarding the potential for protozoa to process endosulfan. The objectives of this study was to evaluate the survival of protozoa in media containing endosulfan and determine the uptake or processing of insecticide by these organisms.

MATERIALS AND METHODS

Sample collection

Wastewater samples from a tannery effluent were collected in screw capped sterile bottles from Kasur (Pakistan). Some physicochemical parameters of wastewater *viz.* temperature ($^{\circ}\text{C}$), pH, dissolved oxygen (mg/l), chromium ($\mu\text{g/ml}$), cadmium ($\mu\text{g/ml}$), and lead ($\mu\text{g/ml}$) were measured. The samples were inoculated in Bold-basal salt medium in 100 ml conical flasks (Haq *et al.*, 1998). A large number of bacteria, yeast, algae, rotifers, and various protozoa were present in the original wastewater sample.

Isolation and culturing of protozoa

For isolation of protozoa, antibiotics, *i.e.*, ampicillin (25 $\mu\text{g/ml}$), chloramphenicol (35 $\mu\text{g/ml}$) and gentamicin (25 $\mu\text{g/ml}$), were used to prevent growth of bacteria. Algae were excluded by keeping the culture in semidarkness. Yeast was excluded by

absence of any organic substance in the medium. Axenic culture of protozoa was made according to Shakoori *et al.* (2004). One hundred milliliter of Bold-basal salt medium with 8 boiled wheat grains in 250 mL conical flask was inoculated under aseptic conditions with 10 μL of inoculum containing 40-50 ciliates. The culture was maintained in the laboratory for one week at room temperature ($25\text{-}27^{\circ}\text{C}$) and at pH 7.5. The growth of ciliates was observed in the cultures by counting the number of ciliates in a haemocytometer at regular intervals.

Insecticide used

In the present study technical grade endosulfan (98.6%) was obtained from AgrEvo Pakistan Private Limited. An endosulfan stock solution was prepared in 100% acetone, and the doses were administered to obtain a final acetone concentration of 0.1% in each treatment. The other concentrations of endosulfan tested were 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, and 1.8 $\mu\text{g/ml}$. Control cultures without ciliates contained an equivalent amount of insecticide. Three replicate cultural flasks were used for each insecticide concentration.

Growth pattern of protozoa

The growth curves of *Stylonychia* and *Paramecium* were determined in Bold-basal salt medium containing 8 boiled wheat grains and without and with endosulfan at a concentration of 0.1 $\mu\text{g/ml}$ 1day for 12 days. The pH of medium was adjusted at 7.5 and at temperature of $25\pm 2^{\circ}\text{C}$. The growth of culture was checked by counting number of protozoan cells in the medium as described earlier (Haq *et al.*, 1998). Every time three readings were taken, their mean and standard error of the mean were calculated.

Determination of level of insecticide tolerance

Resistance of ciliates to insecticide was checked by addition of the respective endosulfan doses in Bold-basal salt medium. The concentration of endosulfan in the medium on the first day was 0.2 $\mu\text{g/ml}$ with an increase of 0.2 $\mu\text{g/ml}$ every two days for 18 days. Although protozoans would lyse at certain concentrations of insecticides the movements were taken as signs of life. When the

protozoans became inactive, no more endosulfan was added. The effect of endosulfan was checked on the growth of ciliates by counting number of protozoan cells every day in the medium containing endosulfan. At least three counts were taken to get a mean of every reading. The growth was compared with that of the control culture, which had no insecticide. The activity, shape and size of the protozoans were also noted. The size was measured with an ocular micrometer after restricting the movement of ciliates on a glass slide by methylcellulose and staining with 1% neutral red. All the experiments were done in triplicate.

Insecticide uptake and processing by the protozoans

The endosulfan uptake or processing ability of *Stylonychia* and *Paramecium* was checked by adding endosulfan at a concentration of 1.0 µg/ml in 50 ml of Bold-basal medium inoculated with 10µl of original laboratory culture (42±3 cells) at 25±2°C. In addition three controls of culture medium, two for the two ciliates alone, and one for the insecticide alone containing endosulfan at a concentration of 1.0 µg/ml but was without the ciliates, were used. The cultures were incubated for 8 days and from each medium (control and treated) 5 ml culture was taken out under sterilized conditions after 0, 2, 4, 6 and 8 days, respectively. The cultures were weighed and spun down at 3000 rpm for 5 minutes, the supernatant was discarded and the weight of the cell pellet was determined on a sensitive balance. Also the weight of controls (insecticide alone and ciliates alone processed the same way) was taken and excluded from that of the culturing medium. The weight of insecticide was taken into consideration, as it would settle down at the bottom of the tube along with the cells. The insecticide was measured as follows:

$$\begin{array}{ll} T_P - C_P = I_{UN}, & C_I - I_{UN} = I_{UT} \\ T_S - C_S = I_{UN}, & C_I - I_{UN} = I_{UT} \end{array}$$

Where T_P is *Paramecium* treated with insecticide; C_P is *Paramecium* growing with out insecticide in the medium; T_S is *Stylonychia* treated with insecticide; C_S is *Stylonychia* growing with out insecticide in the medium; I_{UN} is unutilized insecticide in the medium in which ciliate was growing, and I_{UT} is insecticide

utilized by the ciliate. The reduction in the weight of insecticide in culturing medium containing test organisms was considered as insecticide uptake ability of the isolates. A graph was plotted between % reduction in weight of pellet containing insecticide (Y-axis) and time (X-axis).

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken; their mean, and standard error of the mean were calculated and student's 't' test of significance was applied to determine the level of deviation between the two values.

RESULTS

Some physicochemical characteristics of industrial wastewater of five different ponds, from where the ciliates were isolated, were recorded. The temperature of ponds harboring the ciliates ranged between 17.66°C and 23°C, pH between 7.46 and 8.93, and dissolved oxygen between 0.36±0.01 and 1.77±0.03 mg/ml. These ponds had Cr^{6+} ranging between 0.30±0.04 and 2.10±0.08 µg/ml, $Cd^{□2+}$ ranging between 0.03±0.04 and 1.76±0.004 µg/ml, $Pb^{□2+}$ ranging between 0.01 ±0.04 and 0.20 ±0.004 µg/ml and $Cu^{□2+}$ ranging between 0.03 ±0.04 and 0.70 ±0.004 µg/ml.

The growth curve pattern of *Stylonychia* and *Paramecium* was obtained by counting the number of cells in the culture every day for 12 days. There was a gradual increase in the number of cells in culturing medium. The number of *Stylonychia* cells increased from 433 to 5866 cells/ml (13.55 fold increase) in 12 days in control medium, whereas from 300 to 1200 cells/ml (4 fold increase) in medium containing insecticide. The number of *Paramecium* cells increased from 133 to 6500 cells/ml (49 fold increase) in 12 days in control medium, whereas from 100 to 1833 cells/ml (18.33 fold increase) in medium containing insecticide. The growth curves are shown in Figure 1.

The *Stylonychia* and *Paramecium* were found to be resistant to endosulfan at a concentration of 1.8 µg/ml. There was apparently no reduction in the size

of ciliate cells. Movement, which is a vital sign of life, was taken as a parameter of insecticide toxicity. A slight change was observed in the movement of ciliates in the presence of an insecticide at a concentration of 1.8 µg/ml.

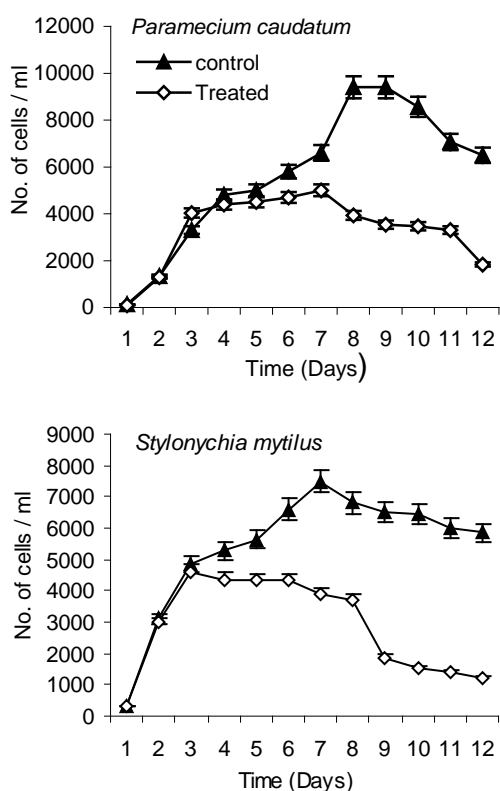


Fig.1. Growth curves of protozoans, *Paramecium caudatum* and *Stylonychia mytilus* in the presence of endosulfan. Controls did not contain insecticide.

Mitotic activity, which is indicated by cell population, has been adversely affected by the presence of insecticide in culture medium. The control culture for *Stylonychia* contained 1.00×10^2 cells/ml on day 1, which increased to 1.866×10^3 cells/ml after 18 days. However, when endosulfan (1.8 µg/ml) was added the number of *Stylonychia* increased from 1.00×10^2 to 1.133×10^3 cells/ml in 18 days. The control culture for *Paramecium* contained 1.00×10^2 cells/ml on day 1, which increased to 1.166×10^3 cells/ml after 18 days. In the presence of endosulfan (1.8 µg/ml) the number of *Paramecium* cells increased from 1.00×10^2 to

3.66×10^2 after 18 days in culturing medium. The increase in the cell population in the presence of endosulfan was 3.6% (*Paramecium*) and 11.3% (*Stylonychia*), respectively.

Paramecium could decline 11% endosulfan after 2 days, 39% after 4 days, 50% after 6 days and 72% after 8 days from the medium containing endosulfan at a concentration of 1.0 µg/ml (Table I). *Stylonychia* could also efficiently process endosulfan and could decrease 21% of endosulfan after 2 days, 44% after 4 days, 56% after 6 days and 78% after 8 days, respectively (Table II) from the medium containing endosulfan at a concentration of 1.0 µg/ml (Fig. 2).

Table I.- Weight of pellet containing endosulfan measured from ciliate, *Paramecium*, at different time period (dosed at 1.0 µg/ml).

Time (Days)	T _P -C _P : I _{UN} Pellet (mg)	C _I -I _{UN} : I _{UT} Pellet (mg)	% removal
2	0.615±0.024*-	0.019±0.002-	11
	0.596±0.003: 0.017	0.017±0.001: 0.002	
4	0.75±0.057-	0.018±0.003-	39
	0.739±0.002: 0.011	0.011±0.002: 0.007	
6	1.17±0.022-	0.018±0.003-	50
	1.161±0.001: 0.009	0.009±0.003: 0.009	
8	1.43±0.022-	0.018±0.003-	72
	1.425±0.002: 0.005	0.005±0.001: 0.013	

*Means ± standard deviation.

DISCUSSION

Nature has demonstrated some subtle and intricate mechanisms for selectively controlling the mobility of toxic pollutants in the environment. The sharp increase in the level of environmental pollution by inorganic and organic pollutants resulted in the development of resistance to these pollutants in these organisms. Bonnet *et al.* (1999) studied the potential biodegradation of whey by a protozoan ciliate, *Tetrahymena pyriformis* that could reduce the pollutant load of whey.

It is well recognized that microorganisms have a high affinity for metals and toxic xenobiotics and can accumulate both heavy metals and toxic

xenobiotics by a variety of mechanisms (Pas *et al.*, 2004; Yilmaz, 2003). These have been used to

Table II.- Weight of pellet containing endosulfan measured from ciliate, *Stylonychia*, at different time period (dosed at 1.0 µg/ml).

Time (Days)	T _S -C _S : I _{UN} Pellet (mg)	C _I -I _{UN} : I _{UT} Pellet (mg)	% removal
2	0.50±0.004* 0.485±0.002: 0.015	0.019±0.002- 0.015±0.001: 0.004	21
4	0.83±0.028- 0.820±0.009: 0.010	0.018±0.003- 0.01±0.002: 0.008	44
6	1.23±0.045- 1.222±0.001: 0.008	0.018±0.003- 0.008±0.028: 0.01	56
8	1.40±0.022- 1.396±0.003: 0.004	0.018±0.003- 0.004±0.024: 0.014	78

*Means ± standard deviation.

remove pollutants from contaminated sites on a large scale. Microorganisms have a high surface area-to-volume ratio because of their small size and therefore provide a large contact area that can interact with pollutants in the surrounding environment (Ledin, 2000). Microbiological detoxification of polluted water is economical, safe, and sustainable (Eccles, 1995).

An endosulfan degrading mixed bacterial culture was enriched by Sutherland *et al.* (2000) from a soil with a history of endosulfan exposure. Siddiqui *et al.* (2003) also isolated bacterial strains from soil samples having history of pesticide application capable of utilizing endosulfan as a source of energy. Lee *et al.* (2006) reported the degradation ratios for endosulfan or endosulfan sulfate in minimal medium containing endosulfan (23.5 µg/ml) or endosulfan sulfate (23.5 µg/ml) by *Pseudomonas sp.* were 52% and 71%, respectively.

The bioconcentration potential for endosulfan in ciliates may depend on the life stage of the organism. Liu *et al.* (1996) reported correlation of bioconcentration factor (BCF) of the fungicide multi-effect triazole (MET) in *Daphnia magna* closely with lipid content. As growth progressed, lipid content increased and thus increased the bioconcentration of MET in *D. magna* tissue. Lipid

content may also be important in estimating pesticide bioconcentration potential in ciliates.

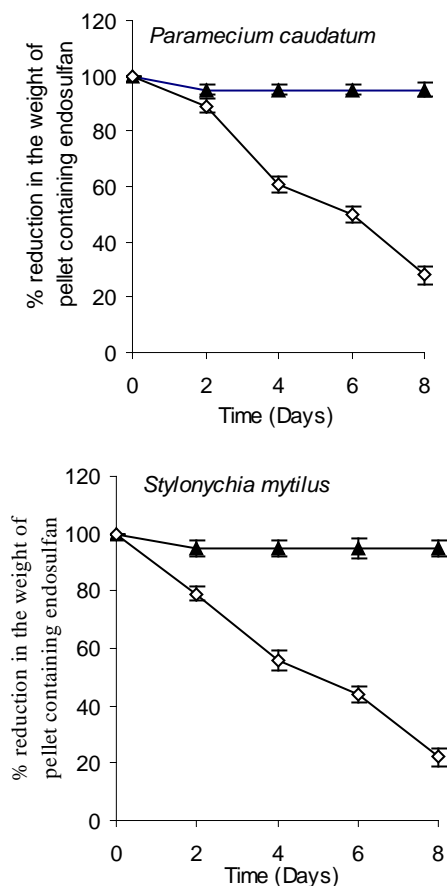


Fig. 2. Uptake of endosulfan by *Paramaecium caudatum* and *Stylonychia mytilus* in insecticide containing medium. The control did not contain protozoan cells.

Boucard *et al.* (2004) reported that species possessing resistant cysts are more likely to survive the presence of sheep dip formulations. The scale of the contamination is dependent upon microbial number and survival in the soil and water environments. The presence of sheep dip formulations potentially impacts on both these factors by depleting protozoan populations. There are reports that fenitrothion, chlorpyrifos, malathion and endosulfan were accumulated but not metabolized by *Tetrahymena pyriformis* (Lal *et al.*, 1987b).

The rate of accumulation was not as high as

usually observed in other microorganisms during the initial stages. Such as accumulation of insecticides is mainly attributed to adsorption and it depends also upon the physiological state of cells (Hansen, 1979). *Paramecium caudatum* and *Stylonychia mytilus* were less sensitive to endosulfan than other freshwater species tested. For example, 96-h LC₅₀ values for rainbow trout, fathed minnow, channel catfish, and bluegill sunfish range from 1.2 to 1.5 µg/l (Johnson and Finley, 1980).

Ciliates, essential components of nearly all ecosystems, are attractive models for toxicological and ecotoxicological studies, due to their relative ease of culturing, short life cycle, cosmopolitan distribution, and sensitivity to environmental changes. These microorganisms actively contribute to the amelioration of the effluent quality, since the majority of them feed upon dispersed bacteria (Madoni, 2000). In this study we have reported the isolation of *Stylonychia mytilus* and *Paramecium caudatum*, which are resistant to endosulfan and have the capacity to utilize it as a carbon source. This capability of these ciliates can be exploited for bioremediation of toxic xenobiotics.

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(Received 17 August 2007, revised 10 January 2008)