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EFFECTS OF THE ORGANOPHOSPHOROUS MALATHION ON THE BRANCHIAL GILLS OF A FRESHWATER FISH *GLOSSOGOBIUS GIURIS* (HAM)

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ABSTRACT
Gills are vital structures for fish, since they are the main site for gaseous exchange as well as partially responsible for osmoregulation, acid-basic balance, excretion of nitrogenous compounds and taste. Chemicals in the water may alter the morphology of branchial cells of fish and are the useful model for environmental impact and ecotoxicology studies. In order to investigate the effects of an organophosphorous compound, malathion, on the gills of the fish, *Glossogobius giuris* were exposed to sublethal concentration (0.05, 0.25 and 0.5ppm) for 24, 48, 72 and 96 hr. Through light microscopy, shrinking of the branchial epithelium, followed by detachment and hyperplasia were observed. Externally, the branchial filaments presented the gradual disappearance of microridges. Even in higher sublethal concentration (0.5ppm), the organophosphorous pesticide reduced the health and fitness of the fish, as consequence of secondary effects derived from changes in the secondary epithelium, cellular size of chloride, mucous and pillar cells, cell diameters and significant shape and tissue damage was noticed.

KEY WORDS: Gills, *G.giuris*, Malathion, Toxicity, Morphology, Cytometry, Filaments.

INTRODUCTION

The effects of pollutants on aquatic animals can be assessed by population studies, in particular through the evaluation of the survival rates and reproductive success. Fish gills can be used as model for studies on environmental impact (McKim and Erickson, 1991). However, as in aquatic animals particularly fish gills are directly exposed to the environment, the fish gills may be used as indicators of water quality (Rankin *et al.*, 1982). Laurent and Perry (1991) consider the morphologic changes in gills, as a consequence of environmental changes and as adaptive attempts in conserving some physiological functions. There are reports on important organs of fish such as gills, being affected by organic pesticides (Gill *et al.,* 1988; Guimaraes *et al*., 2007; Matqueiro *et al.,* 2009). Organophosphates are considered highly toxic to many aquatic species (Qin and Dong, 2004 and Velmurugan *et al.,* 2009).

Although the use of many pesticides is heavily regulated in some parts of the world, studies to assess toxicity of organophosphate insecticides used in India are required to establish safety levels, as some of those are still licensed in this country and more stringent control is needed. Malathion is one of several OP pesticides developed to substitute organochlorides. OPs are less persistent in the atmosphere, being easily linked to organic matter, being adsorbed to sediments and particled material in suspension (EPA, 2000).The toxicity of malathion is the result of a metabolic conversion processed in the endoplasmic reticulum of the hepatocytes, where the group P=S is transformed into P=O. The inhibition of AChE is the most critical toxic effect because it results in the accumulation of the neurotransmitter acetylcholine in the synapsis, interrupting the neural transmission. Although substantial reductions in the activity of AChE of the brain of fish have not been fatal, the effect of this condition on activities as feeding, reproduction and relationships prey-predator is not known (Silva *et al.,* 1993). OP compounds are more biodegradable than the organochloride, and therefore, often used to combat insects. These compounds in aquatic bodies often affect non target organisms in general and fishes in particular. The present study, was, therefore undertaken to study the effect of lethal and sublethal concentration of malathion on the histopathology and cell diameters of branchial gills.

MATERIALS AND METHODS

Biological material *Glossogobius giuris* (Ham) was obtained in and around from Bangalore rural area by using gill net with the help of a fisherman. Fishes of total length 7.0 - 9.0 cm, mean weight of 35.0 g, were maintained in 50 litres aquaria, protected by green shields to avoid external stimuli of the fish (Fanta, 1995) and to improve their adaptation. The fishes were acclimatized for two weeks to laboratory conditions (temperature 27±°C; pH 7.0; photoperiod 12 hr light/12 hr dark). They were fed with commercial pellets (Toryo) and earthworm once a day, after one hour of the start of the light period.

Tested substance

Malathion is an insecticide. It is a colorless liquid, and has a strong scent. The product used in the experiments was supplied by the Rallis India limited Industry. The malathion [O, O, dimethyl, S-(1, 2, dicarbo ethoxyethyl) Phodophorodithiote] was dissolved in acetone and added to test water to obtain the desired concentration. The stock solution of 1 mg/ l was prepared separately and the dispersed degrees of concentration were prepared by adopting the dilution techniques as outlined in APHA (1971).

Toxicity tests

Experiments were carried out in 50 litres aquaria. Tests were carried out in four batches: (i) control - 10 fishes were maintained in water without malathion; (ii) (iii) and (iv) experimental - 10 fishes were kept in each of the aquaria contaminated different sub lethal concentration of malathion (0.05, 0.25 and 0.5 ppm) were lower than the LC_{50}(1ppm for 96 hr). Environmental conditions were similar in all the four aquaria. In 0.5 ppm concentration caused the smallest number of dead individuals (1 fish in 96 hours of exposition). The concentration of 0.05 and 0.25 ppm was chosen because these were generally the smallest concentration used in toxicity tests with *G. giuris*.

Behaviour

Observations were made regarding the behavioral changes of fishes with respect to opercular movement, respiration, balance, grouping and preferred region in the experimental aquaria.

Gills of *G. giuris* were sampled for histological investigation the branchial arch was dissected out from fishes supplemented with to 0.05, 0.25 and 0.5 ppm at 24, 48, 72 and 96 hr after exposure to malathion respectively, at the same time, gills from control fish were also dissected. Tissues were fixed in Bouin's solution for 24 hr, embedded in paraffin and stained with hematoxylin/eosin (HE) (Culling *et al.,* 1985), for light microscopic study.

RESULTS

Normal gills morphology

A gill of G.giuris has 5 pairs of cartilaginous branchial arches. Gill filaments or primary lamellae (p), were lined by a stratified squamous epithelium. At the epithelial surface, the polygonal cells showed concentric microridges. The apical portion of chloride cells(c) was rarely observed among the epithelial cells of the filament (Fig.1).The average cell diameter of chloride cells were 36.37 ± 0.61 µ. Mucous cells are oval or round in shape and are commonly located in the outermost layer of the filament epithelium and lamellae epithelium (arrow). The diameters of mucous cells are 45.64 ± 0.84µ. Respiratory or secondary lamellae(s) were leaf like structures in an oblique display to the primary lamellae. The surface of gill lamellae is covered with simple squamous epithelial cells and many capillaries separated by pillar cells (arrow head) run parallel along the surface and the cell diameter is 25.35 ± 0.40 µ (Table 1). They were lined by a smooth squamous epithelium, without microridges at the cell surfaces, and with a prominent nucleus. There was a clear transition from the branchial filament to the respiratory lamella. Respiratory lamellae had a thin epithelium, sustained by pillar cells that surround blood spaces.

TABLE 1. Mean values of pillar, chloride and mucous cell diameters (µ) of *Glossogobius giuris* after exposed to sub lethal concentrations of malathion

Duration of exposure	Malathion concentration (ppm)	Pillar cell diameter(µ)	Chloride cell diameter (µ)	Mucous cell diameter (µ)
Control	_____	25.35 ± 0.40	36.37 ±0.61	45.64± 0.84
	0.05	23.48 ± 0.80	33.40 ± 0.33	35.26 ± 0.73
24 h	0.25	20.63 ± 0.41	33.69 ± 0.71	28.52 ± 0.64
	0.5	19.74 ± 0.30	35.25 ± 0.55	33.21 ±0.45
	0.05	18.78 ± 0.43	31.54 ± 0.44	32.32 ±0.35
48 h	0.25	20.67 ± 0.90	33.78 ± 0.84	34.82 ± 0.28
	0.5	25.35 ± 0.71	30.46 ± 0.76	41.77± 0.46
	0.05	26.46 ± 0.52	37.35 ± 0.41	43.76 ± 0.67
72 h	0.25	26.22 ± 0.33	35.46 ± 0.80	44.88 ± 0.36
	0.5	25.88 ± 0.44	38.55 ± 0.35	43.75 ± 0.19
	0.05	25.56 ± 0.61	28.47 ± 0.65	43.89 ± 0.78
96 h	0.25	26.48 ± 0.84	32.56 ± 0.24	37.76 ± 0.51
	0.5	25.64 ± 0.95	31.78 ± 0.31	33.66 ± 0.86

Effects of malathion on the gills of *G. giuris*

The effect of malathion on the branchial epithelium was quite drastic: structural changes of the gill lamellae organization, epithelial detachment, necrosis, hyperplasia, loss of the microridges, altered cellular morphology and cell diameter were observed at different sub lethal concentrations.

Bioassay 0.05 and 0.25 ppm

Time of exposure – 24 and 48hr. Fish exposed to 0.05 ppm of malathion for 24 hr exhibits hypertrophy of mucous cells (Fig.2) did not present alterations in the branchial architecture but, fish exposed to 0.25 ppm of malathion for 48 hr showed hyperplasia in the primary and secondary lamellae and a marked swelling of blood sinuses in the

secondary lamellae (Fig.4) and some foci of sub-epithelial edema, lamellar fusion and foci of blood congestion. The pillar and chloride cell diameter (18.78 ± 0.43 and 31.54 ± 0.44 µ) were significant decreases at 0.05 ppm after 48 hr exposure in compared to control pillar and chloride cell (25.35 ± 0.40 and 36.37 ± 0.61 µ) respectively (Table 1).

Time of exposure -72hr. All branchial tissues were altered. The inter lamellar epithelium became irregular, the surface corrugated, mucous cells showed irregular shape, slight decrease the cell diameter (43.76 ± 0.67 µ) with flat nucleus compared to control. Hyperplasia was quite frequent, and cellular proliferation occurred mainly on the surface of the respiratory lamellae (0.05 ppm), that tended to fuse. Telangiectasia of primary lamellae followed by erythrocyte accumulation and shrinkage of secondary lamellae (Fig.5) and hypertrophy of pillar and chloride cells were observed. The cell diameter was 24.46 ± 0.52 and 37.35 ± 0.41 µ at 0.05 ppm of malathion exposure and 26.22 ± 0.33 and 35.46 ± 0.80 µ at 0.25 ppm of malathion after 72 hr exposure respectively (Table 1).

Time of exposure – 96 hr. Intense tissue degeneration and disorganization occurred, with consequent loss of blood spaces. Wrinkled, irregular secondary lamellae followed in decrease of chloride and mucous cell diameter (32.56 ± 0.24 and 37.76 ± 0.51 µ) respectively at 0.25 ppm of malathion exposure when compared to control (36.37 ± 0.61 and 45.64± 0.84 µ). The discontinuous, contrasted and oedematous primary lamellae (0.05ppm) and marked hyperplasia of the filament observed (Fig.7).

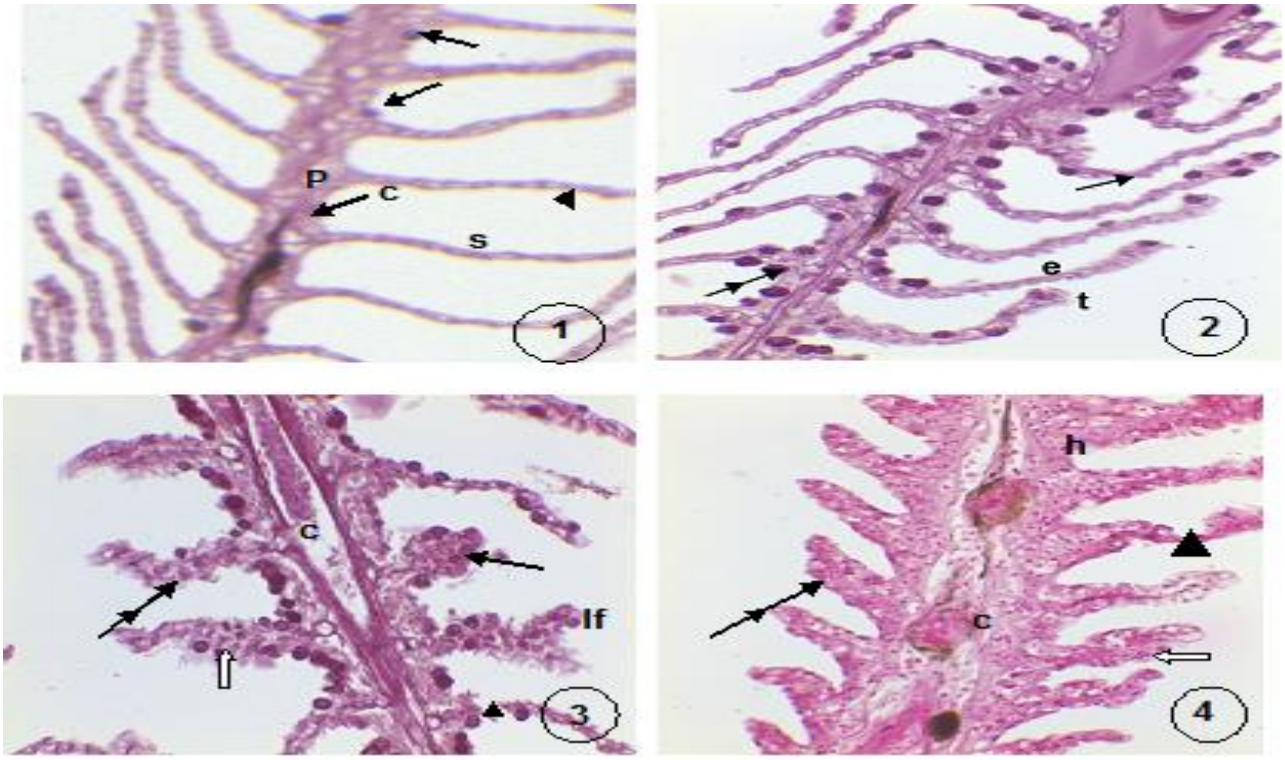

Figure 1. Branchial gills of *Glossogobius giuris* control fish. Primary (p) and Secondary lamellae (s), filament (f), chloride cell (c), mucous cells (arrows), pillar cell (arrow head), H&E x 250.

Figure 2. Malathion 0.5 ppm (24hr after exposure). Telangiectasia (t), sub- epithelial edema (e), hypertrophy of mucous cells (double headed arrow) and disorganization of secondary lamellae (arrow), H&E x 250.

Figure 3. Malathion 0.05 ppm (48 hr after exposure). Blood lamellar congestion (c), lamellar fusion (l f),Hyperplasia of secondary lamellae (arrow), disruption of structure of mucous and epithelial layer (white arrow) , indistinct filament layer and secondary lamellae (triangle), destruction of striated epithelium (double arrow), H&E x 250.

Figure 4.Malathion 0.5 ppm (48hr after exposure).Hypertrophy of filament (h), degeneration or disintegration of secondary lamellae (triangle), blood lamellar congestion (c), lamellar fusion (double arrow), shrinkage of epithelial cells (white arrow), H&E x 250.

Bioassay 0.5ppm

Time of exposure – 24 and 48 hr. Oedematous nature of mucous cells, indistinct filament layer and hyperplasia of gill epithelium (Fig.3). Decrease in the amount of microridges at the epithelial cells surface of the primary lamellae, as well as generalized wrinkling was observed. The marked structural variations of pillar, chloride and mucous cell diameter after exposure to 0.5 ppm of malathion for both 24 and 48 hr (Table 1).

Time of exposure – 72 hr. Epithelium detachment of respiratory lamellae was observed. However, the most evident alteration was observed at the surface of the filament epithelium, where microridges were substituted by cell membrane folds. Hyperplasia in the primary and secondary lamellae, marked swelling of blood sinusus (Fig.6) followed by the increase in the cellular hypertrophy of pillar and chloride cells (Table 1).

Time of exposure - 96 hr. Complete destruction of secondary lamellae and blood accumulation in primary lamellae and shows indistinct cell structure. At the surface of the filament cells showed necrosis, changes in the shape of the microridges that either became punctiform or disappeared in some areas and indistinct, irregular shape and sizes pillar, chloride and mucous cells were observed (Fig.8).

Some behavioral symptoms of *G.giuris* after contamination with OP

Bioassay 1ppm

The concentration of 1 ppm of malathion was lethal for *G.giuris*. 50% fish died after 96 hr of exposure, fish lost balance and intensified respiratory frequency. After 96 hr, the opercular and jaws movements connected to respiration stopped. Fishes kept the mouth constantly opened, and swam without rest, subsequently becoming lethargic and dying.

Bioassay 0.05, 0.25 and 0.5ppm

The sub-lethal concentration (0.05, 0.25 and 0.5ppm) of malathion exposure all the fishes were survived for more than 96 hr. However, some behavioral changes were observed in the first 24 hr. All fishes remained close to the water surface. They did not form groups and the decrease of activity was evident. The fishes were in rest most of the time, while their respiratory movements were accelerated.

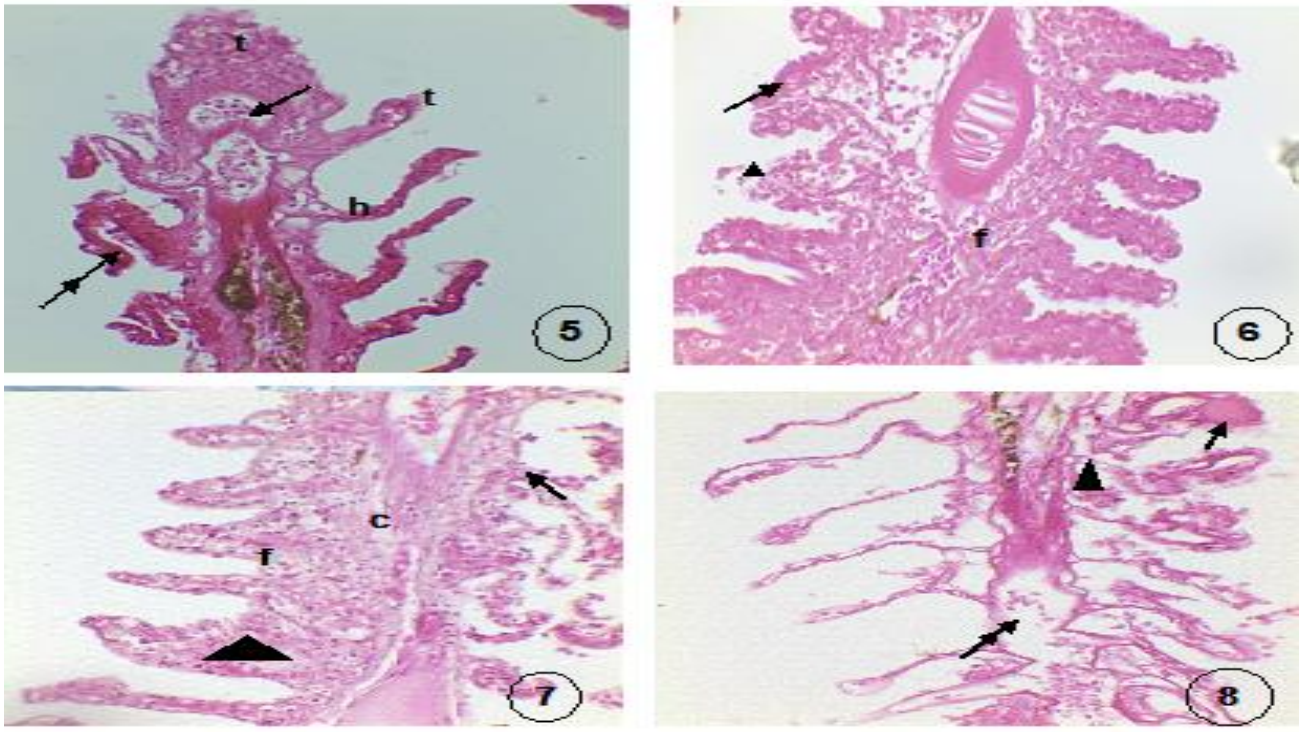

Figure 5. Malathion 0.05 ppm (72 hr after exposure). Telangiectasia (t) tip of primary and secondary lamellae, hypertrophy of filament (h), accumulation of erythrocytes (arrow), shrinkage of secondary lamellae (double arrow), H&E x 250.

Figure 6. Malathion 0.5 ppm (72 hr after exposure).Hyperplasia (h) in the primary and secondary lamellae, marked swelling of blood sinuses (telangiectasia) arrow, some foci of sub-epithelial edema (triangle) and foci of blood congestion (f) , H&E x 250.

Figure7. Malathion 0.05 ppm (96 hr after exposure). Hyperplasia of filament (f), necrotic epithelial cells (arrow), blood congestion (c), swelling of secondary lamellae (triangle) ,H&E x 250.

Figure8. Malathion 0.5 ppm (96 hr after exposure).Complete distruction of secondary lamellae and blood sinuses (arrow),necrosis of filament (triangle), telangiectasia (t), blood accumulation in primary lamellae, indistinct cell structure (double arrow),H&E x 250.

DISCUSSION

Fish gills were chosen for this investigation because they were in direct contact with the aquatic environment and, therefore, could be good indicators of water quality. Fish gills are responsible for pH and osmotic regulation, gas exchange and for the excretion of nitrogenous wastes. As

such, gills are in continuous contact with water and are directly affected by contaminants (Koca *et al*., 2008). In *G.giuris*, these changes appeared in the first hour after contamination with malathion. This indicates probably an initial loss of the osmotic balance in the cells. The severity of gill damage depends on the concentration of toxicants and on the time of exposure (Oliveira *et al.,* 1994 and Velmurugan *et al.,* 2009). Goss *et al.,* (1992) proposed that the squamous cells of the respiratory epithelium of freshwater fish took part in ionic regulation mechanisms. In fact, the ionic change Na^+/H^+, and the enzymes involved in this process were in the membrane of these cells. It was possible that a sub lethal dose of malathion changed the acid-base balance and the absorption of Na^+ in freshwater fish, *G.giuris*, since the wrinkling reached the cell membrane.

Epithelial detachment in *G.giuris* was observed in the sublethal concentration of malathion. In Mallatt's review (1985), this was the most common branchial change that occurred in freshwater fishes rather than in seawater fish. This could be due to the fact that the fishes were in hyper osmotic in relation to the environment, facilitating the influx of water through the epithelium lesion, increasing the volume in the edema, and consequently the detachment. Abel (1976) repeated this process as being a decrease of the superficial area of the gills what was necessary to maintain the internal osmotic surrounding regarding the functional loss of the epithelial cells. Nowak (1992) found that the respiratory epithelium detachment resulted in the increase of the diffusion distance, affecting the gaseous exchanges. This phenomenon has also been described in another type of environmental contamination, heavy metals (Oliveira et *al.,* 1994) and salinity (Fanta *et al.,* 1995).

In *G. giuris* the hyperplasia was subsequent to the process of epithelial detachment, which suggested that the former was a consequence of the latter. The hyperplasia was characterized by cellular proliferation in the interlamellar region of the respiratory lamellae, decreasing the surface area. Magor (1988) reported that these cells stemmed from the epithelium of the filament in the interlamellar space and could act as a barrier impeding the diffusion of harmful substances to the blood of the fish. The bronchial responses could be good to hinder the entrance of intoxicants in the blood flow, but they have the undesirable effect of decreasing the oxygenation of tissues, generating a barrier to gaseous exchanges (Mallatt, 1985). This breathing difficulty was confirmed by the behavioral response of *G. giuris*, because when exposed to the sub lethal dose, the animals had strong breathing difficulties. It is possible, therefore, to trace a parallel among the hyperplasia and the behavioral effects of the intoxicated animals. However, in *G.giuris* a different result was obtained after contamination with malathion the whole structure of the respiratory lamella including the shape of pillar, chloride and mucous cells, were altered. This collapse of the pillar cells - progressive along the 96 hours of the experiment - was followed by a loss of shape of the erythrocytes that can indicate osmotic and ionic alterations similar observation was also pointed out by Guimaraces *et al.,* 2007.

Morphologic alterations of the pillar cells can have several secondary consequences. These cells control the blood pressure of the fish, and changes in the blood pressure and flow can affect the number of irrigated lamellae, the distribution of the blood within the lamellae, the permeability of the bronchial epithelium and, as a consequence, the osmoregulatory and gaseous exchange mechanisms causing several physiological disorders (Randall, 1982 and Evans *et al.,* 2005). The deformation of erythrocytes was obvious at higher concentration for longer duration and has possibly reduced the capacity of oxygen transport, consequently causing a certain level of hypoxia. Consequently, the fish tries to compensate the lower levels of oxygen in its tissue by an increase of the respiratory frequency, as was observed in *G.giuris*. This is observed not only after intoxication with chemicals, but always when there is a change in the respiratory lamellae, caused by any environmental changes (Fanta *et al.,* 1989 and 1995 and 2003).

Fusion of the secondary lamellae could cause a decrease in free gas exchange, thus affecting the general health of the fish (Skidmore and Tovell, 1972). The presence of dilatation observed in the gill filaments may be considered as an ion trap to concentrate traces of metals from water favoring cell addition between neighboring secondary lamellae (Bhagwant and Elahee, 2002). This could serve to protect the epithelial against both mechanical abrasions and infection, as suggested by Olson and Fromm (1973). The presence of leucocytes clearly indicates an inflammatory reaction and cellular infiltration, thus reflecting on immunological response to environmental contaminants (Tao *et al*., 2000). Lifting and swelling could be related to a decrease in the Na^+ - and K^+- activated ATPase and / or a decline in blood Na^+ and Cl^- concentrations (Neiboer and Richardson, 1980). Lamellar fusion then results as a protective response on the gill surface of *G. giuris.*

Separation and dilatation of lamellar epithelium or hyperplasia of epithelium on the tip inflammation could be a defense response of circulatory system against the pollutants. Mallatt *et al.,* (1985) suggested that inflammatory changes tend to be largely nonspecific, simply reflecting a physiological adaptation to stress. It is evident that the separation of the secondary lamellar epithelium and decrease in oxygen consumption will cause abnormalities of the fish respiratory functions. Therefore, cell proliferation of secondary lamellar filaments and lamellar cell hypertrophy decreases the spaces between lamellae and causes its fusion. Such lesions would increase the thickness of the water – blood barrier and decrease the taking of oxygen. It has been argued that if the harmful agent is not removed, these lesions can cause capillary hemorrhage (Nowak, 1992 and Jiraungkoorskul *et al.,* 2002). In this study, ballooning dilation and the presence of erythrocytes from the capillaries are clear indicators of damage. Mallatt *et al.,* (1985) and Pugazhvendan et al., (2009) showed similar results when they investigated the way of action of a specific poison in the bronchial system of lamprey larvae and freshwater fish, *Ophiocephalus punctatus*. The present study the alterations

on the surface of the gills reflect the way of toxic action of the poison, one can assume that 1ppm malathion, even being a sublethal dose, was extremely toxic for *G.giuris*.

The lamellar epithelial surface has short and sturdy microridges commonly termed as microvilli. The filament consists of three types cells namely pillar cells arranged in a coddle stone fashion, the mucous and chloride cells. Out of these three types, the pillar cells are more abundant. The epithelial cells of gill filament have shrunken resulting in the thinning and degeneration of microridges and protrude from the centre of the cell surface, leading to lifting up or detachment of the epithelial layer thus causing inflammation in the pillar cells. The extreme form of degeneration of cell surface appears as bulbous distortion of membrane at the tip of the gill filament. As the tip of the gill filament is in direct contact with the flow of polluted water. Therefore it developed a globular type of alterations on the gill surface has been reported earlier due to various types of stress conditions such as heavy metals (Athikesavan *et al.,* 2006), Pesticides (Cengiz, 2006, Johal *et al*., 2007). The extreme deleterious effect is the hyperplasial condition found to be subsequent to the processes of epithelial detachment characterized by the cellular proliferation in the inter-lamellar region. The present studies have revealed that if the ambient water has very minute quantity of pesticide, it can lead to the collapse of respiratory system of the fish as low concentration of pesticide reduced the density of mucous cells, thus effecting the first line of defense of fishes immunity, hence the fishes living under these conditions are more prone to bacterial infection and acute condition of hyperplasia (Oliveira *et al.,* 1994).

The present studies reveal that the gills are susceptible to high degree of damage even to a very low amount of toxicant present in the water. As the low concentration of toxicants in the water may not be detectable by ordinary water analysis techniques and may be considered as within the safe limits, but in actual practice the vital organs especially the gills are adversely affected. The histological changes observed in fish used in this study show that pollution is affecting freshwater fish living in these aquatic bodies and that this is seriously threatening an agricultural region. Urgent measures must be taken to correct this situation before it becomes a critical issue for the region.

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