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Scanning electron microscopy (SEM) of scale of freshwater exotic fish, *Ctenopharyngodon idella* exposed to organochlorine pesticide, endosulfan

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Abstract
The effect of pesticide on scales *Ctenopharyngodon idella* has been studied using scanning electron microscopy. The test fish was exposed to different sublethal concentrations (0.0007mg/L and 0.001mg/L) of organochlorine pesticide, endosulfan for 15, 30 and 45 days. The scales of exposed fish showed significant alterations such as damaged radii, broken circuli, structural loss in the focal region, broken, uprooted lepidonts/tubercles and even their sloughing off in rows from their point of attachment to the circuli. The intensity of damage to scale was found to be dose and exposure period dependent. It can thus be suggested that this noninvasive technique is very helpful for evaluating the health status of an aquatic body and could be incorporated into water monitoring surveillance.

Keywords: Sublethal concentrations, *Ctenopharyngodon idella*, endosulfan, scanning electron microscopy, lepidonts, circuli, tubercles

1. Introduction
The deliberate discharge or accidental release of harmful chemical substances into the environment has the potential to disrupt the structure and function of every natural ecosystem but most sensitive and evident crack is on water ecology because water bodies are the ultimate recipients for multitude of suspected pollutants. Fishes are considered as the most useful organisms to evaluate the health status of any aquatic body as they cannot escape from effects of pollutants present therein. Fishes form an important link in the food chains and show sensitivity to various pollutants. Several techniques such as biochemical assays, haematological profile, micronucleus tests etc. are used to monitor the environmental contamination by chemicals involve killing of fishes. The reduction in stocks of most of fish species due to anthropogenic activities demands the development of non-invasive technique for such assessments especially the one which is equally effective in study of live as well as dead fish as information collected in case of mass mortality of fish due to any reason will be of great significance. Under these circumstances, lepidological study is the best alternative.
The body of vast majority of fishes except members of order Siluriformes remains covered with scales which constitute their exoskeleton. The anterior part of scale is overlapped by the posterior part of preceding scale which is important from mechanical point of view for smooth flow of water over the body during locomotion. Detailed study of structure of fish scale has been employed for various purposes: to study phylogeny and evolution of fishes [1-2], in identification, classification, age determination, growth studies [3-12], as evidence of species specificity [13], as well as for understanding of fish population [14]. As the fish grows, concentric lines of growth called circuli are added on surface of scale due to deposition of calcium salts secreted by underlying skin [15]. Formation of circuli and other structures associated with them has been found to alter by feeding conditions, physico-chemical changes in environment etc [16]. Being present on the outer surface, scales remain in continuous and direct contact with pollutants and exhibit stress induced alterations in their morphology and mineralization [17-18] and that also much before fish exhibit many other visible symptoms of toxicity [19]. Above all, the scales can be easily removed from the fish's body without killing the fish or even from the dead fish.
Ctenopharyngodon idella (Cuv. and Val.) selected as experimental model, for the present investigations is an exotic freshwater, herbivorous fish and is easily available in Barnala,

Punjab, India as it is being used by farmers for effective weed control in fish ponds and also for being source of high quality protein. The pesticide Endosulfan (6, 7, 8, 9, 10, 10 hexachloro-1-5, 5a, 6, 9, 9a hexahydro, 6, 9, methano-2, 4, 3-benzodioxathiepine 3-oxide) selected as test chemical, no doubt has been banned by Supreme Court of India, is still being used in Punjab, India for controlling pests of cotton due to its broad spectrum action. Moreover, in environment several transformation products of endosulfan such as endosulfan sulphate, endosulfan diol, endosulfan lactone, endosulfan ether, endosulfan alcohol, endosulfan α-hydroxy ether have been identified [21]. Alterations in serum glucose, serum proteins, serum cholesterol [22] and certain morphometric characters of *Ctenopharyngodon idella* (Cuv. and Val.) [23]. on exposure to endosulfan have been observed. Keeping in mind the above facts, an attempt has been made to study the ultrastructural changes induced by endosulfan in the scales of *Ctenopharyngodon idella*, employing scanning electron microscopy. The scanning electron microscopic (SEM) study of scales to know about environmental conditions provides valuable information of morphological alterations which are not clear under light microscope.

2. Materials and Methods

2.1 Experimental animal and chemical

The present investigations have been carried out with the fingerlings of *Ctenopharyngodon idella* (12.56 ± 1.52 cm and 25.40 ± 2.53 gm) procured from local fish farm at Barnala (29.30 ºN-32.32 ºN latitude and 73.55 ^{0}E-76.55 ºE longitude) where farmers have put this fish to their ponds to control unwanted vegetation as the cost of stocking phytophagous fish is lower than the cost of herbicidal control. After giving a dip in 0.1% $KMnO_4$ for 2-3 minutes, fish were acclimatized to laboratory conditions in dechlorinated tap water for seven days. During acclimatization, the fish were fed with grass, berseem, banana leaves. Endosulfan 35 EC (commercial name), manufactured by Excel industries limited, India purchased from local market, Barnala, India was used for the present investigation.

2.2 Short term and chronic exposures

To determine LC_{50} value of endosulfan, the short term toxicity tests (96 hours) were carried out by exposing the test fish to wide range of endosulfan concentrations prepared in dechlorinated water taken in Syntex, white coloured plastic (non-poisonous) tanks of 25 litres capacity fitted with filters and aerators. Ten fishes were exposed to each concentration of pesticide in dechlorinated water and two replicates were used for each concentration. Appropriate controls in the endosulfan free water were maintained simultaneously. Short term exposure to endosulfan gave 96 hour LC_{50} values, to be 0.0052 mg/L. For chronic toxicity tests, three experimental groups of fish (8 fish in each group) were exposed to two sublethal concentrations, 0.00075 mg/L (20% of 96 hour LC_{50}) (Group-I) and 0.001 mg/L (10% of 96 hour LC_{50}) (Group-II) for 15days, 30days, and 45days in plastic tanks fitted with filters and aerators. A parallel control set (Group-III) was run simultaneously in toxicant free tap water. The tanks were cleaned, water was changed and aliquots of stock solution were added to each experimental tank (non-poisonous, aerated) every alternate day so as to bring the endosulfan concentration to the desired levels.

After 15, 30, and 45 days of exposure, the scales of the fish were removed from all the three groups (control and two pesticide concentrations) with the help of tweezers from the second row above the lateral line and below the dorsal fin. After removal, these scales were cleaned in distilled water with the help of fine brush and were subjected to sonication for 5 minutes. The scales were dried on filter paper and were placed between glass slides for 2-3 days. Cleaned and dried scales were then mounted on the aluminium stubs by double adhesive tape with dorsal surface upward and the ventral surface sticking to the tape. These scales were then coated with a thin layer (thickness = 100Aº) of gold palladium alloy in JEOL FINE COAT ION SPOTTED JFC-1100 sputted coater to make the surface of scale conductive in gold coating unit. The scales were viewed under vacuum in a JEOL, JSM-6100, scanning Electron Microscope at an accelerating voltage of 15-20 kv and low probe current at Central Instrumentation Laboratory, Panjab University, Chandigarh. Clear view of the scale was visible on the fluorescent screen of SEM.

3. Results and Discussion

The ultrastructural studies made on the scales of *Ctenopharyngodon idella* (Cuv. and Val.) revealed that cycloid type scale of this fish is roughly circular in shape. Its ventral side which remains attached to the skin is smooth and shiny. A distinct focus (F) has been seen in the anterior half of the scale. Distinct anterior, posterior and lateral fields have been observed (Fig.1a). Around the focus clear concentric rings called *circuli* are present. These circuli are not exactly circular in outline in the anterior field; are arranged in somewhat vertical position in lateral fields; are crowded near the anterior margins because of the anterior location of focus (Fig. 1a); irregular, widely spaced in post-focus region (Fig. 1b) and end abruptly (Fig. 1c) near the posterior margins of the scale.

Under X1200 magnification, the anterior circuli of scale have been found to bear rows of small projections called *lepidonts* (Fig. 4) which are triangular in shape and hence called *tubercles.* Each tubercle has been found to be lodged in a cavity in circuli. Its broad base gives firm attachment in the cavity of circuli. They are of different sizes. From the structure of tubercles/lepidonts it can be assumed that the circuli provide anchoring mechanism to the scale.

The radii have been observed to lie vertically and cut the circuli at right angles (Fig. 1b, 2a, 2b). No annulus formation has been observed as the fish under investigation is less than one year old.

3.1 Alterations due to Toxicity

The initial apparent effect of endosulfan toxicity was the loosening of the scales on the body of fish in both the concentrations. This effect has been due to extensive damage to the calcareous structures called lepidonts/ tubercles present on the anterior part of the scale (Fig. and is in conformity with Johal and Dua (1994) [17].

In both the concentrations viz. 0.00075 mg/L and 0.001 mg/L on exposure of 15 days, most of the circuli have been observed to become irregular and develop cracks (Figs. 2c, d), thus weakening the strength of circuli which support the lepidonts/tubercles. Cracks and breaks have not been seen in the circuli of scales present in the control set (Figs. 2a, b).

On prolonged exposures of 30 days and 45 days to 0.00075 mg/L of concentration, the circuli have been found to be broken (Figs. 2e, g) but increase in concentration to 0.001 mg/L has resulted in more irregularities, higher breakages and even disappearance of segments of circuli (Figs. 2f, h).

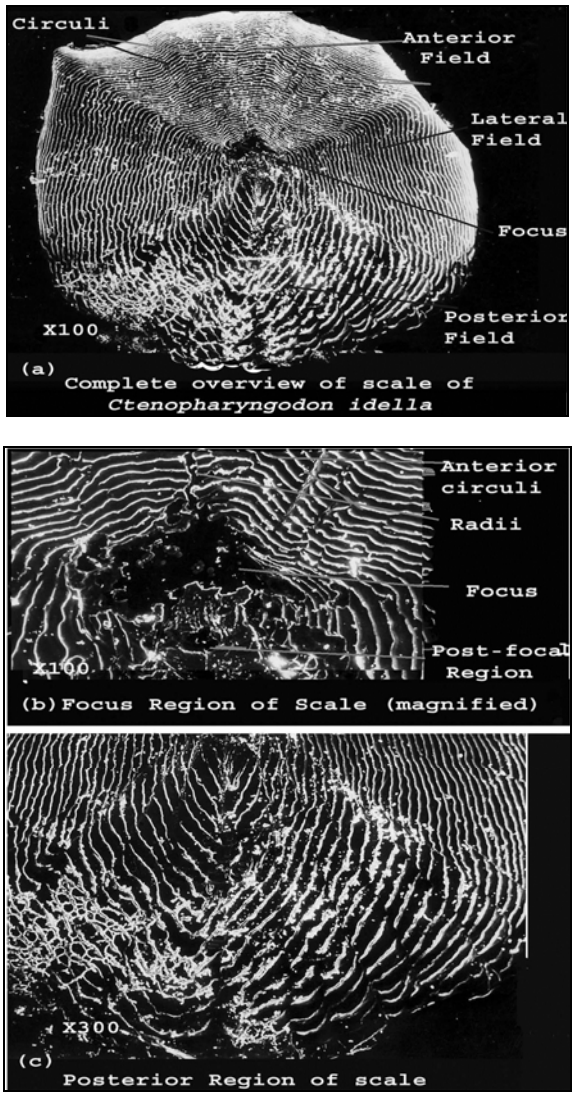

Fig 1(a-c): Scanning Electron micrographs of scale of *Ctenopharyngodon idella* (Cuv. and Val.) to show its structure.

In treated fishes, the focus part developed mesh like irregular structures and appeared to be diffused. The density of formation of mesh has been found to be concentration and duration dependent as reticulation of mesh increased with increase in concentration of endosulfan from 0.00075 mg/L to 0.001 mg/L and increase in duration from 15 days to 30 days when compared to control fish (Figs.3a-3f). After exposure of 45 days to 0.00075 mg/L of endosulfan, diffused focus was there (Fig. 3g) but in scales exposed to 0.001 mg/L for same duration focus has been found to have lost its structure (Fig. 3h). Redii, annuli in *Oreochromis mossambica* on exposure to fly ash were found destroyed [24].

In test fish exposed to 0.00075 mg/L of endosulfan for 15 days, individual breaks have been observed in few lepidonts/ tubercles (Fig. 4c). With increase in duration of exposure to 30 days in 0.00075 mg/L concentration, the damage in the form of uprooting of whole tubercles has been found to be more pronounced (Fig. 4e) than in fishes exposed to higher concentration of 0.001 mg/L for 15 days (Fig. 4d) but has been no doubt less than that in fishes exposed to higher concentration 0.001 mg/L for 30 days (Fig. 4f). On prolonged exposure of 45 days in both the concentrations (0.00075 mg/L and 0.001 mg/L), tubercles have been observed to lose their shape and undergo extensive damage involving sloughing off lepidonts/tubercles in rows from their point of attachment to the circuli (Fig. 4g, h). The results mentioned above are in agreement with many workers [25-30] who have reported disruption of ciculi, damaged lepidonts, and disorganized margins and disorganisation of calcareous material. All of them have proposed the use of fish scales as pollution indicators.

In present investigation also in the fishes exposed to lower concentration, disintegration of lepidonts/ tubercles is less than in those exposed to higher concentration. The scales especially the lepidonts/tubercles present on the circuli of scales could be employed for evaluation of the toxicological effects of chemicals on the fish without killing it and hence help in the diagnosis of the pollution much before the fish exhibit many other visible symptoms of toxicity.

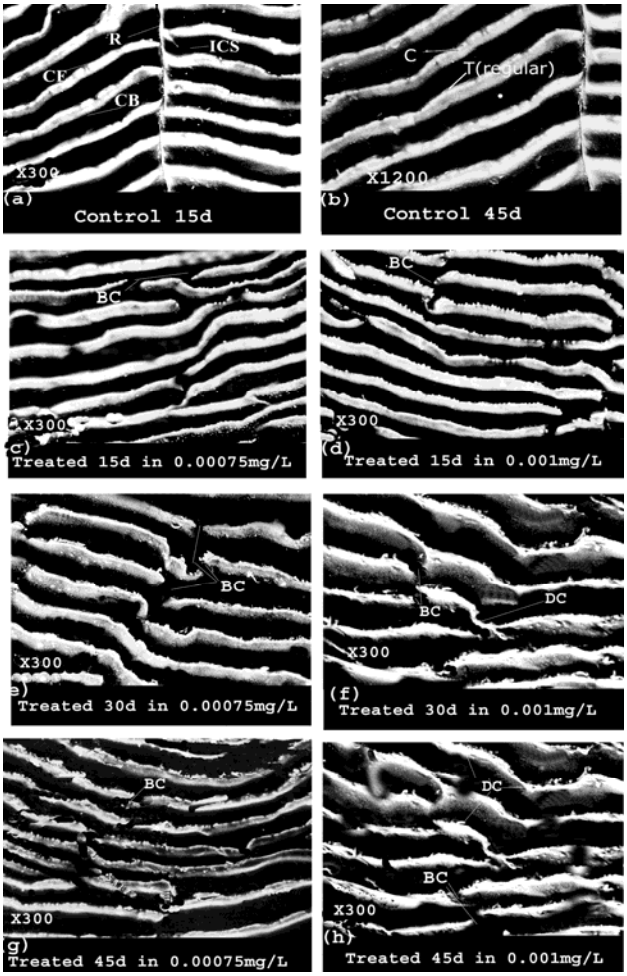

Fig 2 (a-h): Scanning electron micrographs of scales (small portion) of *Ctenopharyngodon idella* (Cuv. and Val.) showing effect of endosulfan on circuli of anterior field.
(C-circuli; CB-circuli base; CE-circuli edge; R-redii; BC-broken circuli; DC-Damaged circuli; ICS- intercirculi space; T-tubercle)

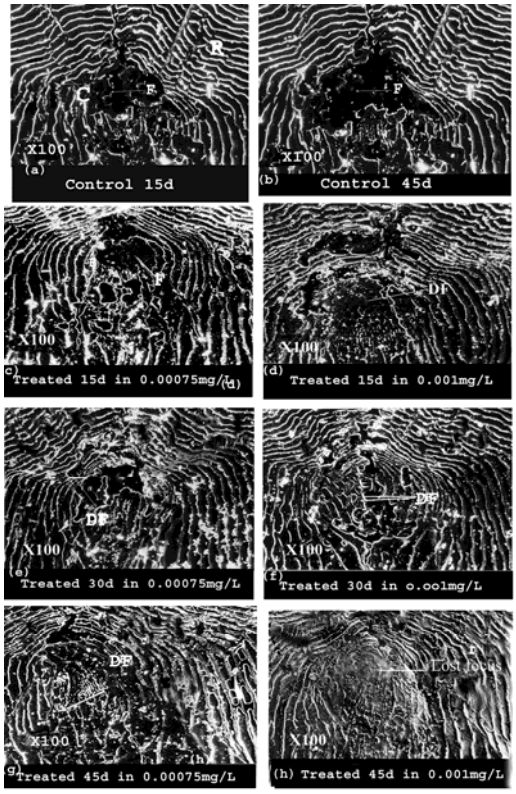

Fig 3 (a-h): Scanning electron micrographs of scales (small portion) of *Ctenopharyngodon idella* (Cuv. and Val.) showing effect of endosulfan on focus region. (C-circuli; F-focus; R-redii; DF-diffused focus)

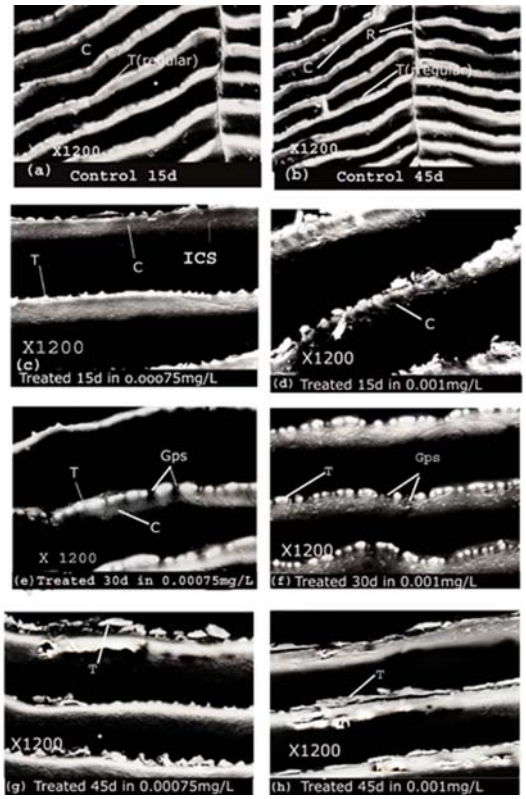

Fig 4 (a-h): Scanning electron micrographs of scales (small portion) of *Ctenopharyngodon idella* (Cuv. and Val.) showing effect of endosulfan on lepidonts/tubercles. (C-circuli; Gps-gaps; F-focus; R-redii; ICS- intercirculi space; T-tubercle)

4. Conclusions

Ultrastructural alterations in scale morphology could be attributed to endosulfan induced toxicity. The functional aspect of tubercles and circuli present on the scale is to anchor the scales firmly to the body of the fish. *Disintegration and sloughing off of the tubercles in rows from their point of attachment to the circuli, thence breaking of circuli in response to toxicant is responsible for loosening of scale on the body of fish in the present investigations.* So, alterations in the ultra-morphology of scales (especially the lepidonts/tubercles) of *Ctenopharyngodon idella*, a fish which flourishes successfully in different areas can act as an early indicator of the stress of very minute doses of pesticide and could be incorporated into water monitoring surveillance.

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