Observations on the effect of glyphosate based herbicide on ultra structure (SEM) and enzymatic activity in different regions of alimentary canal and gill of *Channa punctatus* (Bloch)

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ABSTRACT

Glyphosate is the isopropyl amine salt of N- (Phosphonomethyl)-glycine, a broad-spectrum nonselective herbicide, which has been extensively used to control annual and perennial weeds in agricultural, forest and aquatic systems. The ultrastructural changes in different regions of alimentary canal and gill were observed by Scanning Electron Microscopic study on a non-target aquatic teleostea fish, Channa punctatus. Fishes were exposed to herbicide at a dose of 4 mgl^{-1} generally used by farmers to control weeds in water bodies for a period of 45 days in laboratory condition with a control. Severe damage, shrinkage and degeneration of pentagonal cellular contour of stratified epithelial cells (SEC) were observed in gill. Shrinkage of SEC resulting in degeneration of microridges was observed in buccopharynx. Slight necrosed and distorted SEC was observed in oesophagus. Severe mucus secretion was observed in stomach. Erosion on the apical surface of mucosal folds and columnar epithelial cells (CEC) and necrosis of CEC was also noticed in stomach. Obliteration of CEC along its entire length from basement membrane was observed in the intestinal portion. After 45 days treatment by glyphosate protease activity was slightly reduced in stomach and intestine in comparison to control fish. Amylase activity reduced in oesophagus and intestine in treated condition. Lipase activity was also reduced slightly in stomach and intestine of glyphosate treated fish.

Key words: Amylase, alimentary canal, glyphosate, gill, lipase and protease.

Herbicides are widely used to control weeds in agriculture fields and aquatic systems. Constant flow of agricultural run off into aquatic systems can also leads to contamination of pesticides, herbicides in aquatic systems. The direct application of herbicides in aquatic system leads to loss of macrophytes. Non-target organisms such as fish may also be affected through loss of habitat and food supply (Ervnest, 2004). Glyphosate (*N–Phosphonomethyl-glycine*.)is used as a non-selective herbicide and for control of a great variety of annual, biennial, and perennial grasses, sedges, broad-leaved weeds, and woody shrubs. In aquaculture glyphosate is used for controlling aquatic weeds. It is perhaps the most important herbicide ever developed (WHO, 1994).

Because of its low persistence, repeated applications of this herbicide are practiced for the control of weeds in agriculture fields and thereby, large quantities of them find their way into the water bodies. However, only a few reports are available on the effect of Glyphosate on freshwater fish (Mitchell *et al*., 1987; Servizi *et al*., 1989). *Channa punctatus* (Bloch) is a bottom dweller carnivorous fish and is easy to culture in laboratory condition., The objective of this study is to determine the toxic effects of glyphosate on a nontarget organism, *Channa punctatus* with special emphasis on ultra structural changes (SEM study) in gill and different parts of alimentary canal and consequent enzymological changes, in view of the poor knowledge on the side effects of this agrochemical

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MATERIALS AND METHODS

Some live specimens of *Channa punctatus* were collected from local pond (date and place of collection to be mentioned), these specimens were then kept in aquarium for ten days for acclimatization. Live food (*Tubifex* sp) was given on daily basis. After acclimatization, the specimens were treated with glyphosate herbicide at a dose of 4-mg/l for 45 days. The treatment was given every alternate day maintaining a control side by side. Commercial name of this glyphosate based herbicide is 'Excel Mera-71'. Water of the aquarium was changed on every alternate day . Physico-chemical qualities of aquarium water such as temperature, pH, conductivity, hardness, chloride, alkalinity, dissolved oxygen, Na, were also measured as per APHA, 1998. For Scanning Electron Microscope (SEM) study following procedure was followed: The sample fish, *Channa punctatus*, was anesthetized with tricaine methonesulphonate (MS 222) and the required tissues such as Gill and the representative portions of alimentary canal *viz*., buccopharynx, oesophagus, stomach, and intestine were removed immediately and the luminal surface was exposed, through longitudinal incision. Then the mucosal surface of the incised tissues except gill were spread out and pinned with luminal surface uppermost on the cork sheets. The adhering mucous of the luminal surface was removed by rinsing in heparinized saline. After rinsing in 0.1M cacodylate buffer pH 7.5, the tissues were infiltered with 2.5% glutaraldehyde for 24 hr at 4^0C. After fixation the tissues were rinsed in buffer, trimmed in to 8 mm squares and subjected to post

fixation in 1% OsO_4 in 0.1M cacocodylate buffer, pH 7.5 for 2 hours then dehydrated through graded acetone. Subsequently, acetone was removed by amyl acetate and subjected to critical point drying (CPD) method with liquid carbon dioxide. The mucosal surface of each tissue was mounted on metal stubs, coated with gold with thickness of approximately 20 nm, and scanned in Hitachi, S-530 SEM.

For enzymological analysis the following methods were followed, *viz.*, for Protease activity (Snell and Snell, 1971), Amylase activity (Bernfeld, 1955), Lipase activity (Cherry and Crandell, 1932) and total protein content by Lowry (1951) Observations:

RESULTS AND DISCUSSION

Ultra Structural Changes (Sem Study)

Gill: Each gill filament bears many subdivisions or lamellae (Figs.1.1, 1.2, and 1.3) that are the main site of gaseous exchange. The free edges of the lamellae are extremely thin, covered with stratified epithelium (Fig.1.2), and contain a vast network of capillaries supported by pilaster cells. The gill consists of horizontal flat filaments. On the filaments there are found the secondary lamellae (Fig. 1.1). The outer stratified epithelial cells have microridges as viewed through SEM (Fig. 1.2).

Ultra structural changes of gill occured due to exposure of glyphosate herbicide and its surfactants present in the medium. The gill lesions included necrosis, hyperplasia, inflammation, epithelial cell lifting, cell swelling (Figs.1.3 & 1.4), and hypersecretion of mucus. Clubbing of the ends of the lamellae and a tendency of fusion of adjacent lamellae were the general effects of gills (Fig. 1.3). There was a severe loss of normal patterns of microridges on the stratified epithelium (Fig. 1.4).

Buccopharynx: In *C.punctatus* the mucosal surface exhibited irregular but prominent mucosal folds and the buccopharyngeal epithelium appeared in the form of pentagonal and/or hexagonal stratified epithelial cells. In *C.punctatus* the stratified epithelial cells are provided with labyrinth pattern of arrangement but regularly spaced microridges measuring about 1-10 µm in length. The outermost microridge of a particular cell fused with the same of the neighbouring cell forming a doubleridged structure (Figs. 2.1 & 2.2). A few taste buds surrounded by epithelial cells and a few wart-like structures are located at cell junction and probably represent the opening of mucous cells. Major changes following the treatment with glyphosate were wrinkling and shrinkage of the stratified epithelial cells, loss of lateral contacts between neighbouring epithelial cells and disorganization of the normal arrangement of microridges of the epithelial cells. Furthermore secretion of mucus was also noticed. (Figs. 2.3 & 2.4).

Oesophagus: The mucosal surface of the esophagus is typified by a series of regularly spaced round or oval stratified epithelial cells (Fig. 3.1), the plasma membrane of which consists of thick and linearly arranged microridges (Figs. 3.2 & 3.3). The microridges appear to be regularly spaced, leaving long and deep furrow or channels in between the stratified epithelial cells.

In treated fish slight necrosis was observed in the stratified epithelial cells (Figs. 3.3 & 3.4). In some places the rupture of the stratified epithelial cells culminated in the formation of an even cell sheet; as a result, labyrinth-patterned microridges were seen on the distorted epithelial cells showing an inconspicuous microridged structure on the epithelial cells (Fig. 3.4).

Stomach: In *C.punctatus* the mucosal folds anastomose with each other to form deep, empty and rectangular shaped concavities. The mucosal surface of this region is divided into oval, round, elevations corresponding to the surface of columnar epithelial cells which are densely packed with short but stubby microvilli (Fig. 4.1). Prominent gastric pits surrounded by the epithelial cells have also been detected in this region (Fig. 4.2). The columnar epithelial cells of stomach were fused and damaged (Fig. 4.4) due to glyphosate toxicity. The mucin masses were also found to be adhered to the epithelial surface (Fig.4.4) of mucosal epithelium.

Intestine: In *C.punctatus* the mucosal folds are irregularly arranged and the microvilli of the epithelial cells are short and compactly arranged on the apical surface of the absorptive columnar epithelial cells (Fig. 5.1 and 5.2). Due to herbicide toxicity, large areas of intestinal mucosal folds got damaged and debris of the fragmented secondary mucosal folds were noted in the concavities between the primary mucosal folds (Fig. 5.4 and 5.5). Intestinal CECs were damaged due to herbicidal contamination. The microvilli on the apical portion of the disrupted CECs (Fig. 5.4) were necrosed.

Enzymological analysis

Protease activity: Proteolytic enzymes play an important role in digestion of proteins in biological systems. In control fishes the protease activity in oesophagus showed minimum activity (0.021 µg/min/mg protein). On the other hand, stomach showed moderate quantity of protease activity (0.092 µg/min/mg protein). Intestine showed maximum protease activity (0.183 µg/min/mg protein). After 45 days of treatment by glyphosate, protease activity was moderately reduced in oesophagus (0.013 µg/min/mg protein), stomach (0.089 µg/min/mg protein) and intestine (0.166µg/min/mg protein) (Table-1).

Amylase activity: Amylase is an important carbohydrate-digesting enzyme. Maximum activity

has been shown in the oesophagus (0.248 µg/min/mg protein) in controlled condition. After treatment with glyphosate its activity was reduced up to 0.230 µg/min/mg protein. In stomach the amylase activity was moderate (0.088 µg/min/mg protein) in comparison to other parts. In intestine of controlled fishes the amylase activity was 0.102-µg/min/mg protein protease, which is slightly higher (0.086 µg/min/mg protein) than glyphosate treated fishes (Table-1).

Lipase activity: Lipase is an important lipolytic enzyme in *C. punctatus* and other carnivorous fishes. In control condition, the activity of lipase in stomach is 0.070 µg/min/mg protein but after the 45 days exposure of the experiment its activity in stomach was reduced up to 0.054 µg/min/mg protein. Intestine is another part of digestive system showing more lipolytic activities. In controlled *C. punctatus*, the lipase activity in intestine was 0.170-µg/min/mg protein; after the glyphosate treatment the activity of lipase was reduced to 0.143-µg/min/mg protein (Table-1).

Herbicide toxicosis on the aquatic organisms is a serious concern and some times poses a miserable threat to the aquatic ecosystem. The importance of its toxicity is growing rapidly due to increasing number of cumulative effect and biomagnification quality of these xenobiotics which man is constantly confronted with. Different authors like Llyod, 1960; Pickering and Henderson, 1966; Ball,1967; Sprague, 1969, 1970, 1971; McCarty *et al*.,1978; Datta and Sinha,1989; Ghosh and Chakraborty, 1990, in different occasions studied the effects of different xenobiotics on fish alimentary canal. But the study of herbicidal toxicity on fish health is very scanty. Present study shows severe pathological lesions in the various regions of the alimentary canal because of glyphosate toxicity. In carnivorous fishes, the mucosa of buccopharynx and oesophagus are intimately connected with stratified epithelial cells provided with various pattern of microridges leaving narrow depressions. Similar types of microridges in the aforesaid regions have also been reported in other carnivorous teleost (Sinha and Chakrabarti, 1986, Chakrabarti and Sinha, 1987). Such microridges located on the epithelial cells play a major role on the anchorage of thin mucous film over the soft mucous membrane and this film is associated with lubrication of ingested food from these regions to stomach and also withstand the trauma resulting from ingested material.

In the present study, in *Channa punctatus* cytolysis in microridges of the stratified epithelial cells of the buccopharynx and oesophagus has been found to be affected due to this herbicide toxicity. It is known that microridge structure of the epithelial cells of the aforesaid regions anchors mucous film and plays an important role for the lubrication of the food and protects the mucosa layer from mechanical rubbing (Sperry and Wassersug, 1976; Sinha, 1983; Sinha and Chakrabarti, 1986; Chakrabarti and Sinha, 1987). Therefore, obliteration of microridges of the epithelial cells of the aforesaid regions can reduce the retention ability of mucus film. This may affect the ingestion of foodstuffs and transmission of the same to the next regions in the fishes concerned ultimately resulting in to deterioration of fish health with consequent production.

The present study shows that herbicide adversely affect the microridge structure located on the apical portions of the columnar epithelial cells of stomach in *C.punctatus.* Therefore, it is obvious that the action of this herbicide on gastric epithelium concomitantly reduces the protection ability of gastric epithelium from chemical injuries and cell lysis. In some places excessive secretion of mucus has been found from the epithelial cells, which indicates that herbicide (Glyphosate) exposure triggers the activity of the aforesaid cells.

The mucosal fold in the intestine of *C. punctatus* in the present study also modify accordingly, resulting in the increase of mucosal surface for effective digestion and absorption, which is necessitated due to possession of a comparatively short gut. The luminal surface of the entire intestine is lined with well-developed columnar epithelial cells. However, major feature of the intestine is the scalping of the luminal plasma membrane into microvilli. In the intestine, microvilli of the plasma membrane of epithelial cells are normally arranged in regular fashion. It is probable that disruption of mucosal folds and disarray of microvilli structure in the intestinal portion were caused by herbicide (Glyphosate) toxicity. This probably impair the storage and digestive capacity of the fish concerned.

The exaggerated secretion of mucin by exocytosis from the different parts of the alimentary canal was the common effect of herbicide (Glyphosate) toxicity in *C. punctatus*. It is possible that the aforesaid herbicide entering the alimentary tract of fish may cause the changes of the luminal environment which influences the exaggerated mucus secretion throughout the length of the alimentary tract. Teleosts ingest food materials which comprise asset of complex molecular components. For break down of these components into simpler forms, specific enzymes are essential. The physiological processes of digestion take place in the different portions of the alimentary canal in teleost fishes. Digestion of foodstuff followed by absorption and ultimately utilization of the metabolic products are the fundamental function of the alimentary tract of fishes.

Removal of unwanted xenobiotics including pesticides, heavy metals into the exterior are also the important function of GI tract. Therefore activity of different digestive enzyme like amylase, protease, and lipase were studied in this work. Oesophagus, the anterior part of the alimentary canal passes the undigested but lubricated food materials with mucus into the stomach. The carnivorous fishes macerates the animal foodstuff of larger forms into smaller forms in stomach. The anterior part of the stomach, called cardiac stomach has many gastric gland cells which secrets digestive enzymes. The gastric glands also secrete hydrochloric acid, and pepsinogen which are most important to split large portion of molecules particularly for carnivorous and predacious fishes. (Lagler *et al*., 1977). Intestine has highly absorptive capacity due to its cell linings *i.e*., presence of absorptive columnar epithelial and compact infoldings of brush border which increases absorptive capacity area. In *C. punctatus* the gastric acidity ranges between 2.5 to 3.7. In GI tract, the proteolytic digestion of endogenous proteins within cells involves proteases and peptidases. Intracellular proteases hydrolyse the internal peptide bonds of protein, forming peptides. These peptides are then degraded to free amino acids by peptidases. Endopeptidases cleave internal bonds in peptides, forming shorter peptides. Aminopeptidases and carboxypeptidases then remove amino acids from the N-and C-terminals of peptides, respectively. Protease activity in many carnivorous fishes has been recorded by various authors and this has been correlated with the diet of the fish concerned (Kitamikado and Tachino 1960, Croston 1966, Kapoor *et al*., 1975; Fal'ge and Spannkhof 1976; Yoshinaka *et al*., 1981; Tue 1983). In the present study protease activity was found to be higher in intestine than in stomach, which was slightly or moderately reduced in treated condition.

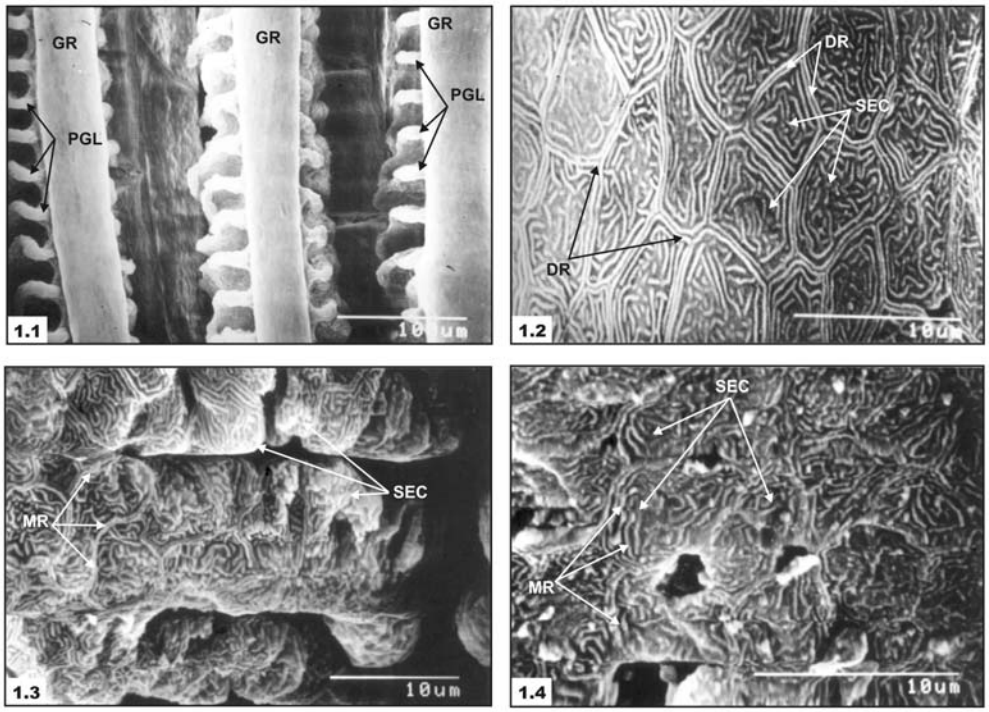
The carnivorous fish normally ingest higher proportion of fatty food with their normal food and this is ultimately assimilated in gastro-intestinal tract (Kapoor *et al*., 1975). The intestinal region offers a good source of lipase which breaks down fat into fatty acids and glycerin. Fatty acids are absorbed in the anterior part of the intestine. In the present study it was observed that lipase activity was higher in intestinal portion than in stomach. The activity of lipase enzyme was also reduced in glyphosate treated fish. Intestine also helps in digestion of carbohydrate due to presence of amylase as in *Tilapia* (Lagler *et al*., 1977), in case of carnivorus fish zymogen granules in the hepatopancreas is the source of amylase. In the present study maximum amylase activity was observed in oesophagus. Stomach showed minimum amylase activity. In intestine moderate amylase activity was observed. Due to toxicity of glyphosate herbicide amylase activity was found to decrease.

Table 1: Enzymological analysis

Enzymes / Tissues	Protease activity (µg/min/mg protein)		Amylase activity (µg/min/mg protein)		Lipase activity (µg/min/mg protein)	
	Control	**Treated**	**Control**	**Treated**	**Control**	**Treated**
Oesophagus	0.021	0.013	0.248	0.230	ND	ND
Stomach	0.092	0.089	0.088	0.079	0.070	0.054
Intestine	0.183	0.166	0.102	0.086	0.170	0.143

ND Not detected

PLATE 1: GILL



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Figs. 1.1-1.4: Scanning Electron micrographs of gill of control (C), glyphosate treated *C.punctatus*

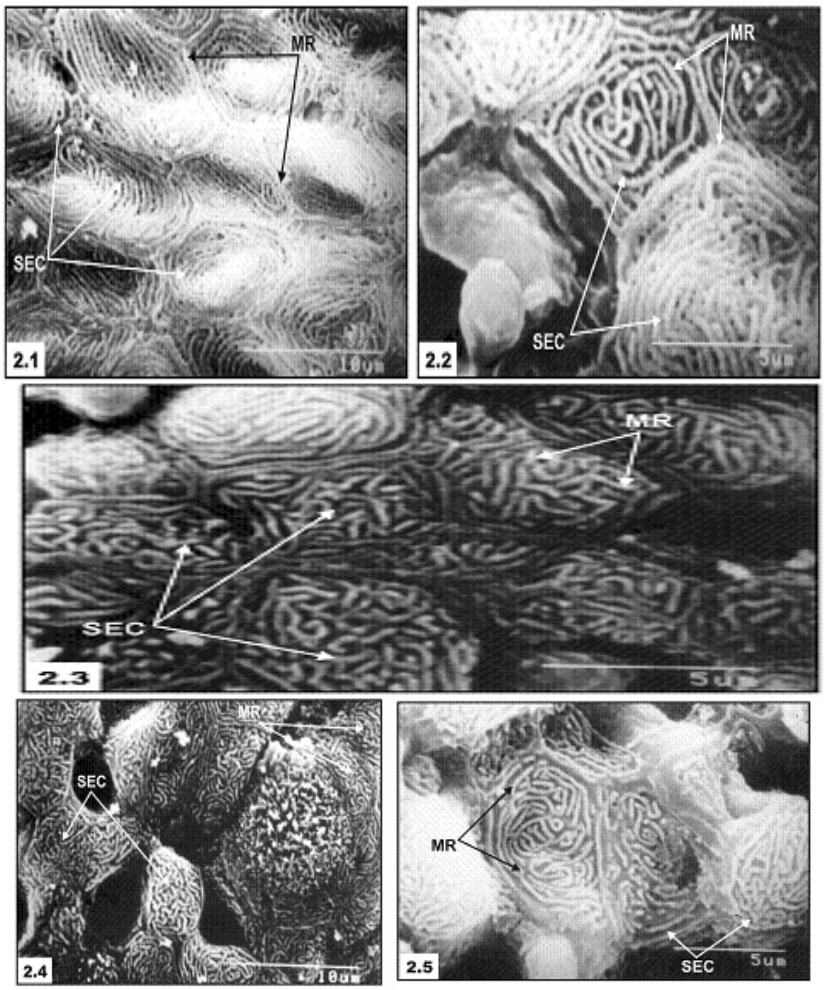
Fig.1.1. Showing bony gill arches (GR) and the number of double rows gill filaments, primary gill lamellae (PGL). (C) X 4000

Fig. 1.2. Showing oval and pentagonal stratified epithelial cells (SEC) provided with prominent double ridge structure make cell to cell connection (DR) and prominent micro ridges (MR). (C) X 4000

Fig.1.3. Showing concentrically arranged MR in SEC of each PGL. Note – deep channels in between two lamellae and obliteration of DR structure of adjacent SEC. (GP) x 5000

Fig. 1.4. Showing severe damage of the MR of SEC, Shrinkage and degeneration of pentagonal cellular contour of SEC. Note appreance of vacuoles in the SEC. (GP) X 4000

PLATE 2: BUCCOPHARYNX



Figs. 2.1-2.5 Scanning Electron micrographs of buccopharynx of control (C), glyphosate treated *C.punctatus*

Fig. 2.1. Showing oval pentagonal stratified epithelial cells (SEC) provided with prominent microridges (MR) and demarketed by double ridges. Note the retention of mucin by MR. (C) x 4000

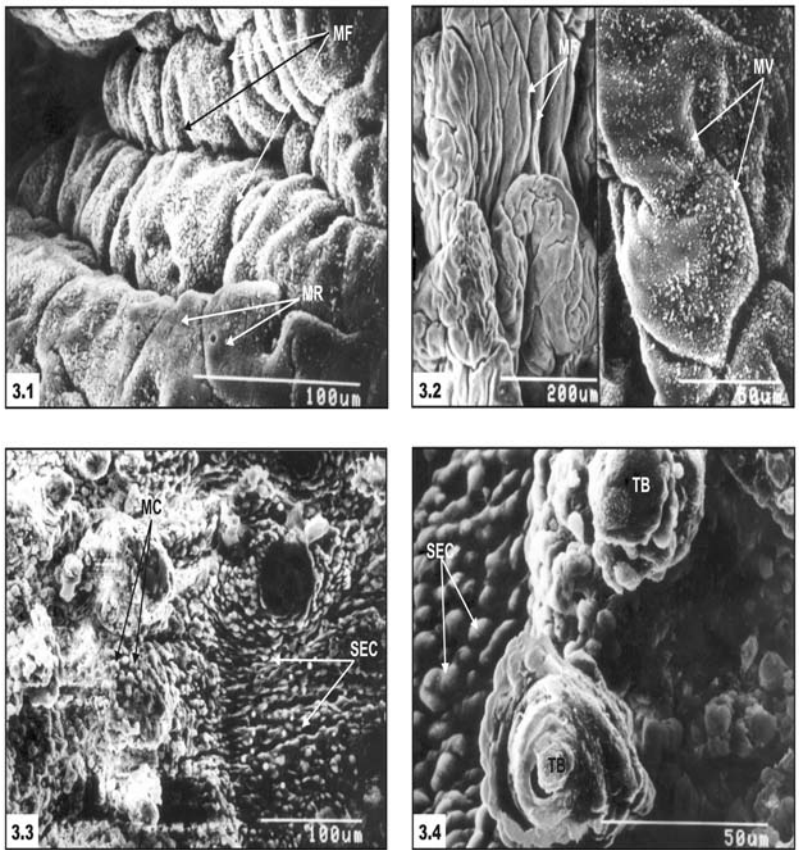
Fig. 2.2. Concentrically arranged MR in SEC Note presence of mucous cell. (C) x 8000

Fig. 2.3. Showing concentrically arranged MR in SEC. Note deep channels in between MR. (C) x 6000

Fig. 2.4. Shrinkage of SEC resulting severe damage of MR in SEC. Note deterioration of double ridge structure of adjacent SEC. (GP) x 4000

Fig. 2.5. Shrinkage of SEC resulting degeneration of MR of the SEC. Showing fragmentation and disarray in the neuro epithelial cells of TB. (GB) x 6000

PLATE 3: OESOPHAGUS



Figs. 3.1-3.4. Scanning Electron micrographs of oesophagus of control (C), glyphosate treated *C.punctatus*.

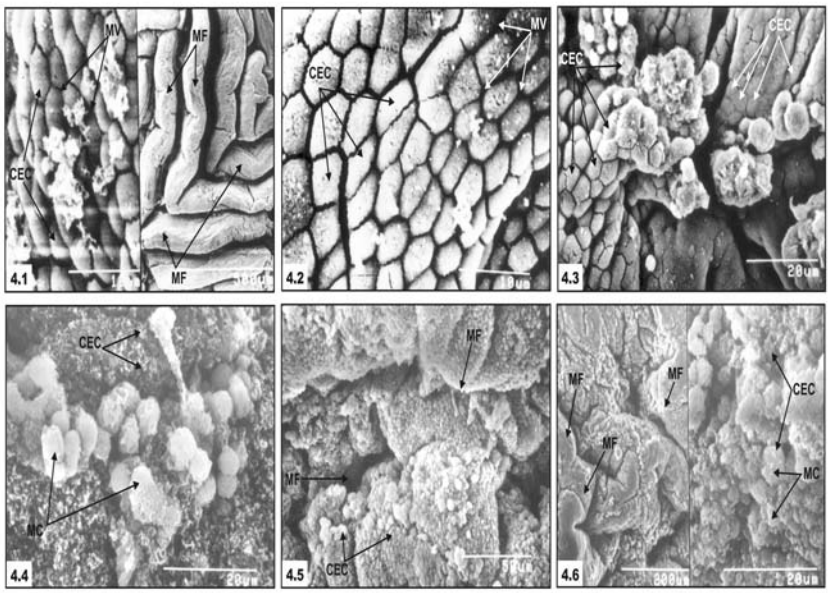
Fig. 3.1. Showing thick mucosal folds provided with microridges structure (C) x 1000. Note retention of mucus.

Fig. 3.2. Showing MR (C) and MV of treated fish.

Fig. 3.3. Showing the absence of MR on the apical surface of mucous cells (MC) surrounded SEC. Note secreted mucus over the SEC. (treated)

Fig. 3.4. Showing SEC (treated) Note appearance of mucus.

PLATE 4: STOMACH



Figs. 4.1-4.6 Scanning Electron micrographs of stomach of control (C), glyphosate treated *C.punctatus*

Fig 4.1. Showing the regularly arranged mucosal folds (MF). Each fold is supported with oval or rounded columnar epithelial cells (CEC) provided with stubbymicrovilli (MV). Note the retention of mucus on the surface of the SEC. (C) x 1000

Fig.4.2. Presence of gastric pit encircled by group of CEC. Note stubby MV on the epical surface of the CEC. (C) X 1000

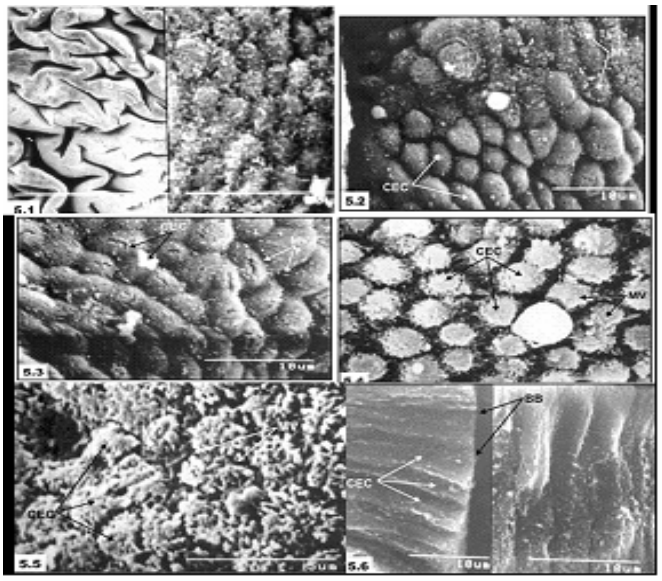
Fig 4.3. Showing disarrangement of mucosal surface. (GP) x 2000

Fig.4.4. Showing errrosion and damage of CEC. Note complete degeneration of CEC in some places. Note deposition of mucus on epical portion of CEC. (GP) x 2000

Fig. 4.5. Fragmentation and congregation of MF on CEC. Severe necrosis of CEC on MF. (GP) x 1000

Fig. 4.6. Showing erosion on the apical surface of MF and CEC. Note deposition of necrosed CEC. (GP) x 150

PLATE 5: INTESTINE



Figs. 5.1-5.6 **Scanning Electron micrographs of Intestine of control (C), glyphosate treated *C.punctatus*.**

Fig. 5.1. Showing prominent Zigzag pattern of mucosal folds and concavities in between the wavy MF.Each fold is regularly packed with oval rounded CEC provided with prominent microvilli.

Fig.5.2. Regularly arranged CEC is provided with MV. Note the presence of mucus. (C) x 4000

Fig .5.3. Elongated CEC showing microvilli zones continued as microridges on the luminal surface. (C) x 4000

Fig. 5.4. Necrosed MV on apical portion of disrupted CEC. Note the deep furrows and channels in the disrupted arrangement of CEC. (GP) x 5000

Fig .5.6. Showing obliteration of CEC along its entire length from basement membrane. (GP) x 4000

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