International Journal of Food, Agriculture and Veterinary Sciences ISSN: 2277-209X (Online)
An Online International Journal Available at http://www.cibtech.org/jfav.htm
2013 Vol. 3 (2) May-August, pp. 92-103/Senapati et al.
Research Article

STUDY ON HISTOPATHOLOGICAL, HISTOCHEMICAL AND ENZYMOLOGICAL ALTERATIONS IN STOMACH AND INTESTINE OF *ANABAS TESTUDINEUS* (CUVIER) EXPOSED TO ALMIX 20WP HERBICIDE

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ABSTRACT
Almix 20WP, a sulfonylurea herbicide is frequently used to control weeds in the agricultural fields. Almix 20WP herbicide is the combination of 10% metsulfuronmethyl and 10% chlorimuronethyl. Indiscriminate use of herbicide may cause harmful effects on non-target aquatic organisms *e.g.*, fish in the adjacent aquatic bodies. Histopatological, histochemical and enzymological alterations in the stomach and intestine of non-target teleost *Anabas testudineus* (Cuvier) were studied after chronic exposure of Almix 20WP herbicide in the laboratory condition. Fishes were treated with Almix 20WP herbicide at a sublethal dose 66 mg/l for 45 days. Histopathological alterations included distortion of columnar epithelial cells (CEC), damage of gastric glands in the stomach. In intestine, histopathological changes included distortion of columnar epithelial cells (CEC) and secretion of mucus. Acid and neutral mucin content slightly reduced in stomach as confirmed by PAS-AB test in the histochemical study. Acid mucin content was reduced but neutral mucin content was present in a good amount. Digestive enzymes (amylase, protease, and lipase) activities were significantly reduced ($p<0.05$) after under the laboratory condition after exposure of Almix 20 WP herbicide.

Key Words: *Almix 20WP, Histopathology, Histochemistry, Digestive Enzyme*

INTRODUCTION
Herbicides are frequently used to control weeds in terrestrial as well as aquatic systems. Sulfonylurea group of herbicides are now becoming popular due to their effectiveness and low toxicity. These are selective herbicide, used to kill broad leaf weeds in terrestrial and aquatic systems. Almix 20WP is a very common sulfonylurea group herbicide. It is combination of 10% metsulfuronmethyl and 10% chlorimuronethyl (DuPont, 2001). Due to the high leaching potential metsulfuronmethyl is easily transferred from the application site to the nearby surface water *via* surface runoff (Belfroida *et al.,* 1998; Fogg and Boxall, 2004; Sondiha, 2009). So there is a risk of contamination in the aquatic system. Chlorimuronethyl persists for a longer time in soil. So it can causes significant damage to nontarget plants/crops and affects the function of soil microbes and soil enzymes (Wagner *et al.,* 1995; Boldt and Jacobsen, 1998; EL-Ghamry *et al.,* 2001; Gigliotti and Allievi, 2001; Soltani *et al.,* 2005; Wang and Zhou, 2005; Teng and Tao, 2006, 2008; Yang *et al.,* 2007; Nemat Alla *et al.,* 2008). It is highly water soluble and K_{ow} value is high, that is why, it could be leached to groundwater through soil and may cause groundwater pollution (Briggs *et al.,* 1981; Afyuni *et al.,* 1997) as well as surface water pollution. Jabeen *et al.,* (2008) observed biochemical and ezymological alterations in *Cyprinus carpio* after exposure of Almix 20WP herbicide. Samanta *et al.,* (2010) studied the digestive enzyme activity of *Anabas testudineus* and *Channa punctatus* in field condion after application of Almix 20WP herbicide. Senapati *et al.,* (2012) studied the ultrastructural changes in the alimentary canal of *Anabas testudineus* due to Almix 20WP exposure in laboratory condition. The objective of this research work is to study the histopathological, histochemical changes and alterations in the digestive enzyme activity in the stomach and intestine of non-target organism, *Anabas testudineus*.

MATERIALS AND METHODS

The adult healthy fishes *viz.*, *Anabas testudineus* were collected from local pond they were treated with 0.1% potassium permanganate solution and kept in aquarium for seven days for necessary acclimatization in the laboratory environment. Foods (*Tubifex* sp.) were supplied regularly. Two sets of experiments were designed separately in the laboratory, *i.e.*, one for treatment and another for control. After the acclimatization treatment was given by using Almix 20WP herbicide at a sub lethal dose of 66.66 mg/l for 45 days in every alternate day (Senapati *et al.*, 2012). Control set of the experiment was maintained side by side where herbicide was not applied. Water was changed in every alternate day in laboratory aquarium. Physicochemical quality of aquarium water was monitored regularly. Fishes were sacrificed for chronic toxicity test of herbicides *i.e.*, Almix 20WP before herbicide application (0-day) and on 45th day of application of herbicide. Stomach and intestine were removed from the sacrificed fish and prepared for the histopathological, histochemical and enzymological observations. Dissected portion of the tissues were fixed in Buin's fluid for 24 hrs then washed with 70% ethyl alcohol. Then tissues were dehydrated through graded ethyl alcohol and embedded in the paraffin. Tissues were sectioned by microtome 3-5µm thick. Then tissues were stained with haematoxylin and eosin (H&E). For histochemical observation 8-10 µm thick tissue sections were stained with Periodic-Acid Schiff-Alcian Blue (PAS-AB). Digestive enzyme activities were measured by using following methods *viz.*, for amylase activity (Bernfeld, 1955), protease activity (Snell and Snell, 1971), lipase activity (Cherry and Crandall, 1932) and for protein content by Lowry (1951).

RESULTS AND DISCUSSION

Histopathological Study of Stomach

Control Condition

The stomach of *A. testudineus* was composed of four histological layers *viz.*, mucosa, submucosa, muscularis and serosa. Mucosa layer was folded into variable depths. It was composed of superficial and glandular epithelium. The superficial epithelium contained a single layer of columnar epithelial cells with large oval and basal nuclei (Figures 1.1 & 1.2). The glandular epithelium was provided with gastric glands (Figures 1.1, 1.2 & 1.3). The gastric glands were simple and tubular and were rounded or elongated in shape. The cells of the gastric glands were closely arranged within the lumen and were provided with centrally placed nuclei (Figures 1.1, 1.2 & 1.3). The submucosa layer was highly vascularized with a thick layer of connective tissue (Figure 1.1). The submucosa layer was projected into the lamina propria (Figures 1.1, 1.2 & 1.3). Muscularis layer was thin and penetrated by blood capillaries (Figure 20.1).

Treated Condition

Degeneration and vacuolation in the basal region of the gastric epithelium were pronounced after chronic exposure of Almix 20WP herbicide (Figures 1.4 & 1.5). Columnar epithelial cells were degenerated in some areas (Figure 1.6). Damage of gastric glands was also noticed after Almix 20WP exposure (Figures 1.4, 1.5 & 1.6).

Histopathological Study of Intestine

Control condition: In *A. testudineus* intestine was composed of four histological layers *e.g.*, mucosa, submucosa, muscularis and serosa. The intestinal mucosal layer was formed of the intestinal villi. The intestinal mucosa was composed of columnar epithelial cells with centrally and basally placed nuclei, mucous cells and leucocytes. Mucous cells were present all over the intestinal mucosa (Figures 2.1 & 2.2). Intestinal villi were covered by thin layer of tissue matrix. Lamina propria was formed by the loose connective tissue fibers of submucosa layer. Blood cells were present in the lamina propria and submucosa layer. Muscularis layer was formed by the inner circular muscle fibres and outer longitudinal muscle fibres. The serosa layer was composed of a single layer of flat cells with blood capillaries and connective tissue fibers (Figures 2.1 & 2.2).

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Treated condition: Histopathological alterations in the intestine of *A. testudineus* after Almix toxicosis included degeneration of columnar epithelial cells, degeneration of lamina propria (Figures 2.3 & 2.4) and prominent luminal mucus secretion (Figure 2.4).

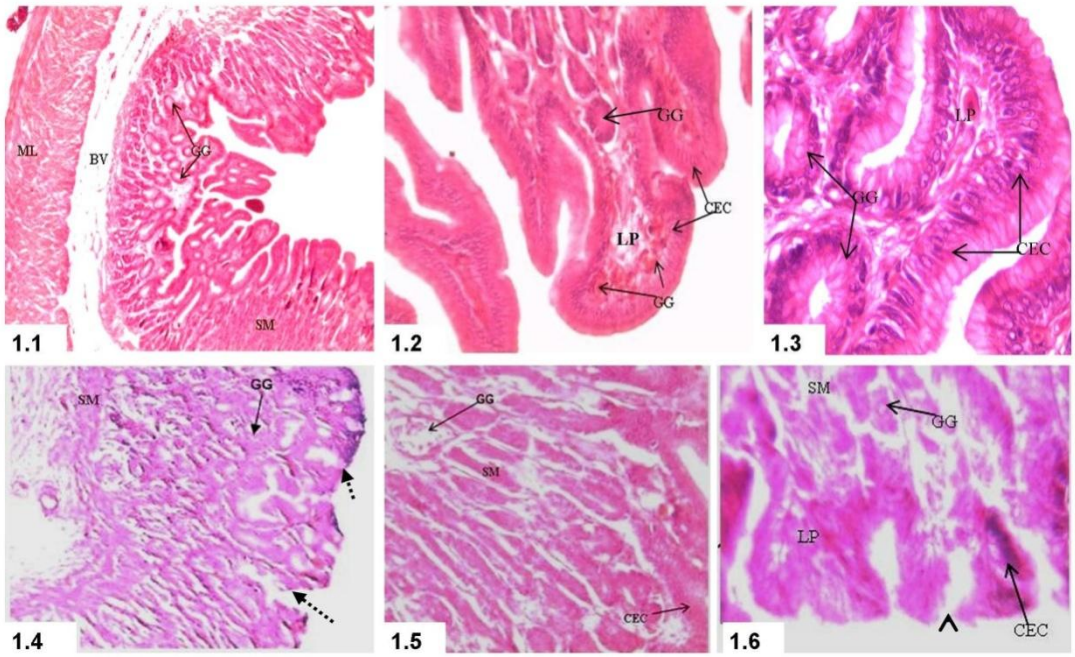

Figure 1: Histopathology of Stomach

Figures 1.1 -1.6: Photomicrographs of transverse section of stomach of *A. testudineus* under condition (C), Almix treated condition (AL) (H- E staining)
Figure 1.1: Showing superficial epithelium provided with columnar epithelial cells (CEC) and randular epithelium provided with tubular gastric glands (GG). Note regular connective tissue network in submucosa (SM). (C) X 100
Figure 1.2: Showing compactly arranged single layer of columnar epithelial cells (CEC). Note blood vessels in lamina propria and presence of oval or rounded gastric glands (GG). (C) X 400
Figure 1.3: Showing compactly arranged single layer columnar epithelial cells (CEC) with prominent nucleus at higher magnification. Note presence of compactly arranged gastric cells surrounding the central lumen in the gastric gland (CG). (C) X 1000
Figure 1.4: Showing degeneration and vacuolation in the basal region of gastric epithelium. Note distortion of CEC (broken arrow). (AL) X 100
Figure 1.5: Showing degeneration of GG. (AL) X 400
Figure 1.6: Showing distortion of CEC (arrow head). Note distortion of GG. (AL) X 400

In the present study, pathological lesions were also detected in the various regions of alimentary canal *e.g.*, stomach, intestine due to chronic exposure of Almix 20WP herbicide at a sublethal dose. In the present study, distortion of columnar epithelial cells and secretion of mucus were the frequently occurred histopathological changes in the stomach of *A. testudineus* due to herbicide toxicity. In the stomach, the secretion of mucus may protect the surface layer from gastric acidity and other chemical reaction (Ghosh, 1990). In *A. testudineus*, gastric glands were present in the submucosa layer and these were supported by lamina propria. The secretion of gastric glands played a significant role in the digestion of protein food. In the present study, it was revealed Almix 20WP herbicide severely affected the digestive gland thus mucin

secretion might be affected. Therefore, gastric epithelium gradually reduced the protection ability of underlying epithelial cells from chemical injuries and cell lysis, which resulted into the destruction of epithelial cells. The distortion of digestive glands could hamper the production of digestive enzymes.

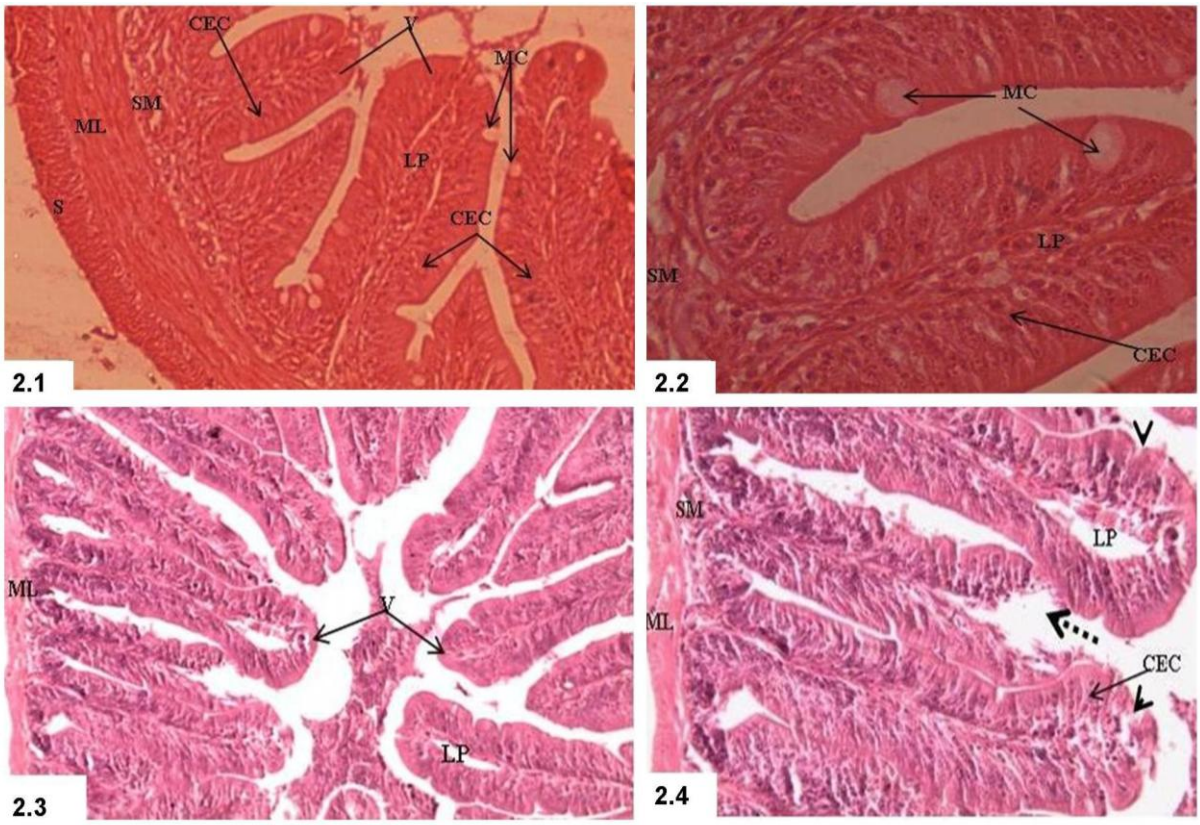

Figure 2: Histopathology of Intestine

Figures 2.1 – 2.4: Photomicrographs of transverse section of intestine of *A. testudineus* under control condition (C), Almix treated condition (AL). (H-E staining)
Figure 2.1: Showing four histological layers namely, mucosa (M), submucosa (SM), muscularis (ML), serosa (S) and arrangement of columnar epithelial cells (CEC) in the mucosa layer. Note presence of prominent mucous cells (MC). (C) X 400
Figure 2.2: Showing arrangement of CEC with prominent nucleus in the mucosa (M) of intestinal villi ai higher magnification. Note presence of lamina propria (LP) and prominent mucous cells (MC). (C)X 1000
Figure 2.3: Histological structure of intestinal villi (V). Note narrow SM and ML. (AL) X 200
Figure 2.4: Histological structure of intestinal villi at higher magnification. Note degeneration of CEC (broken arrow) and exocytosis of MC (arrow head). (AL) X 400

And this was also evident in the present study by assaying digestive enzyme activity in the fishes after herbicide exposure in the laboratory. In a similar type of chronic toxicity study, Amminikutty and Rage (1977) reported swelling, distortion and/or vacuolation with a tendency to necrotization in the mucosal epithelial cells of stomach of *Gymnocorymbus ternitzi* after chronic exposure of endosulfan and methyl ethyl mercurial. In a recent study, Ghanbahadur and Ghanbahadur (2012) reported vacuolization in the submucosa, shrinkage of mucosal folds in the stomach of larvivorous fish *Rasbora daniconius* due the toxic effect of endosulfan. Intestine is the important part of fish alimentary canal for absorption. It is

exposed to different types of toxicants directly *via* drinking and feeding or indirectly *via* blood and lymph (Karuppasamy, 1999). Digested food stuffs and different toxic materials are absorbed in the intestine. So intestine can be used as a sensitive organ in the toxicity study (Muniyan, 1999). In the present study, the concomitant changes due to herbicide toxicity in the intestine of *A. testudineus* were damage of submucosa layer, destruction of lamina propria, distortion of columnar epithelial cells which led to the damage of mucosa layer and severe mucus secretion.

Mandal and Kulshrestha (1980) reported similar type of histopathological changes in the intestine of *Clarias batrachus* due to exposure of sublethal concentration of sumithion. Sharma *et al*., (2001) also showed similar histological alteration in the intestine of *Cirrhinus mrigala* due to toxicological effects of different pesticides. Ravanaiah and Narasimha Murthy (2010) reported vacuolization, damage of villi and serosa layer, necrosed mucous epithelium, congested blood capillaries and hyperactivity of mucous cells in fish *Tilapia mossambica* exposed to industrial pollutants.

The destruction of mucosa and particularly the columnar epithelial cells in the intestine of *Rasbora daniconius* due to endosulfan toxicity was reported by Ghanbahadur and Ghanbahadur (2012). Degeneration of mucosal epithelium, damages of lamina propria and submucosa of the intestine might deteriorate the secretion of digestive enzymes into the lumen of the alimentary canal. So impairment of food digestion might occur in the fishes. Damage of brush border on the luminal surface of the intestinal villi could reduce the ability of absorption of various macromolecules from the intestinal lumen to tissue interior. Furthermore, the disruption of blood vessels in the submucosa might impair its ability of absorption (Ghosh, 1990).

Histochemical Study of Stomach

Control Condition

In the control condition, intense PAS-AB reaction occurred in the densely packed columnar epithelial cells of gastric mucosa, whereas submucosa, lamina propria and gastric glands showed moderate to mild reaction (Figures 3.1 & 3.2). PAS positive reaction produced bright purple colour and AB positive reaction produced blue colour. PAS positive reaction indicated presence of neutral mucin and AB positive reactions indicated presence of acid mucin. Presence of combination of acid and neutral mucin in the gastric epithelium of *A. testudineus* was confirmed by PAS-AB test. Secreted luminal mucin was PAS-AB positive and produced purple and blue colour thus indicated presence of acid and neutral mucin (Figures 3.1 & 3.2).

Treated Condition

In the stomach of the Almix treated *A. testudineus* in the laboratory condition, positive PAS-AB reaction was observed in the columnar epithelial cells along with secreted luminal mucin. PAS-AB test confirmed the presence of acid mucin and neutral mucin in the columnar epithelial cells and luminal secretion. Intensity of PAS-AB reaction was slightly reduced in the treated condition (Figures 3.3 & 3.2).

Histochemical Study of Intestine

Control Condition

The intestine of *A. testudineus* in the control condition was found to be associated with secretory and non-secretory mucous cells in varying intensities. PAS-AB test confirmed the presence of profuse quantity of neutral and acid mucin in the mucous cells (Figures 4.1 & 4.2). A weak PAS-AB reaction was noticed in the submucosa layer whether columnar epithelial were negative to this test (Figures 4.1 & 4.2).

Treated Condition

In the laboratory condition, intestine of the Almix treated *A. testudineus* showed slight reduction in acid mucin content (Figures 4.3 & 4.4). Profuse quantity of neutral mucin was observed through PAS positive test. There was significant amount of acid and neutral mucin present in secreted luminal mucin (Figures 4.3 & 4.4). Intensity of the PAS reaction was almost same as compared to the control condition (Figures 4.3 & 4.4).

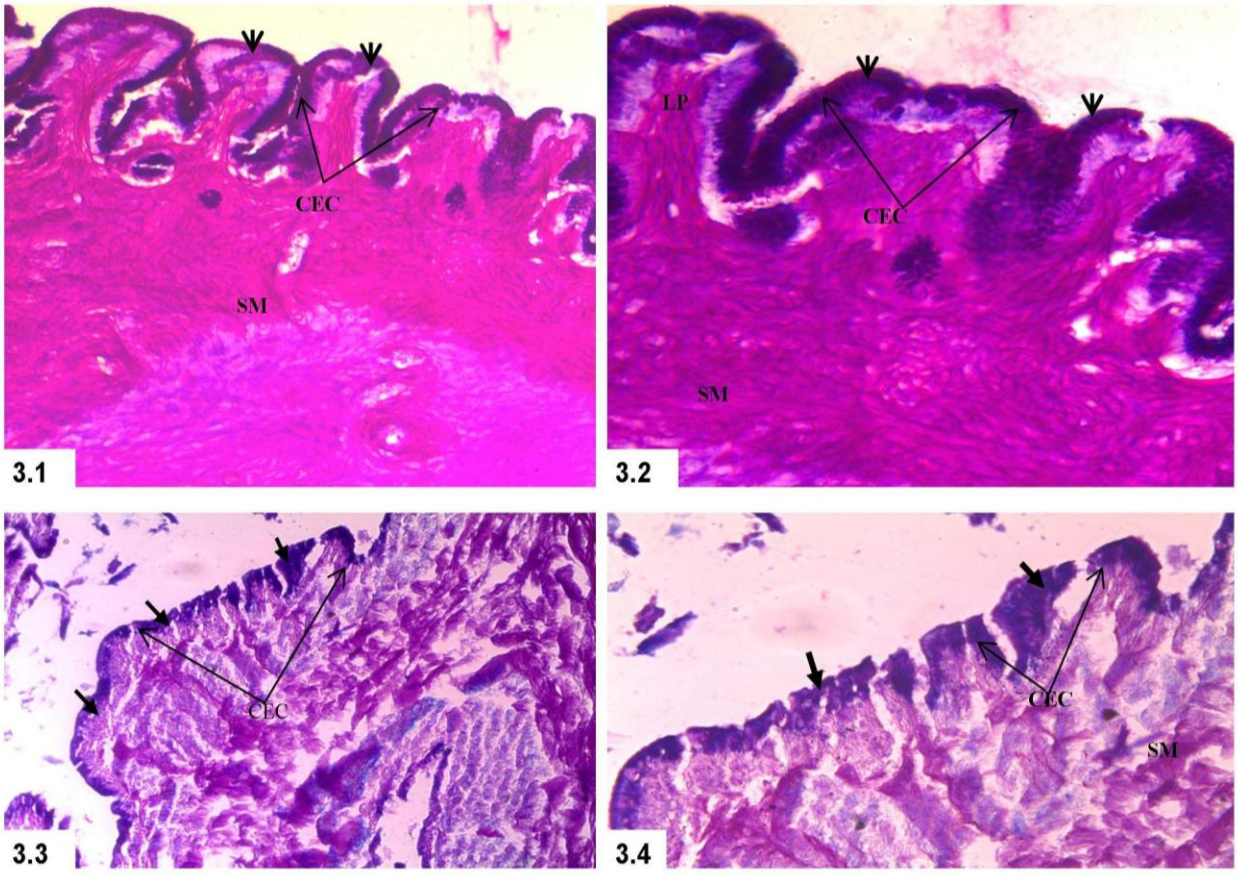

Figure 3: Histochemistry of Stomach

Figures 3.1 – 3.4: Photomicrographs of transverse section of stomach of *A. testudineus* in control condition (C), Almix treated condition (AL). (PAS-AB)
Figures 3.1 & 3.2: Showing intense PAS-AB reaction in the mucosal surface of the stomach. Note presence of combination of acid and neutral mucin in the secreted luminal mucin. (C) X 200 & (C) X 400
Figures 3.3 & 3.4: Showing positive reaction in the epithelial surface of gastric mucosa. Note slight reduction in the intensity of reaction. (AL) X 200 & (AL)X 400

In the present study, Almix 20WP herbicide induced excessive secretion of mucus throughout the length of alimentary canal in *A. testudineus*. In response to herbicide exposure, the mucous cells in the alimentary canal of *A. testudineus* became hypersecretory in nature rather than increasing in number. This finding was similar to the report of Ghosh (1990), on cadmium and arsenic triggered mucous cell activities in *H. fossilis* and *N. notopterus*. In the present study**,** Almix 20WP herbicide induced the activity of mucous cells by changing the luminal environment. The affected mucous cells secreted profuse quantity of mucin from the cell interior and thus the mucin masses was reduced in volume within the mucous cells. The reduction of neutral and acid mucin *viz.*, carboxylated and sulphated mucin was of variable intensity as evident due to toxicity of Almix 20WP herbicide in the treated condition. Consequently, intense reaction in external luminal mucin has been detected by PAS-AB test. In the present study, the occurrence of combination of neutral and acid mucin in the columnar epithelial cells of the gastric mucosa of *A. testudineus* was confirmed by employing the PAS-AB test.

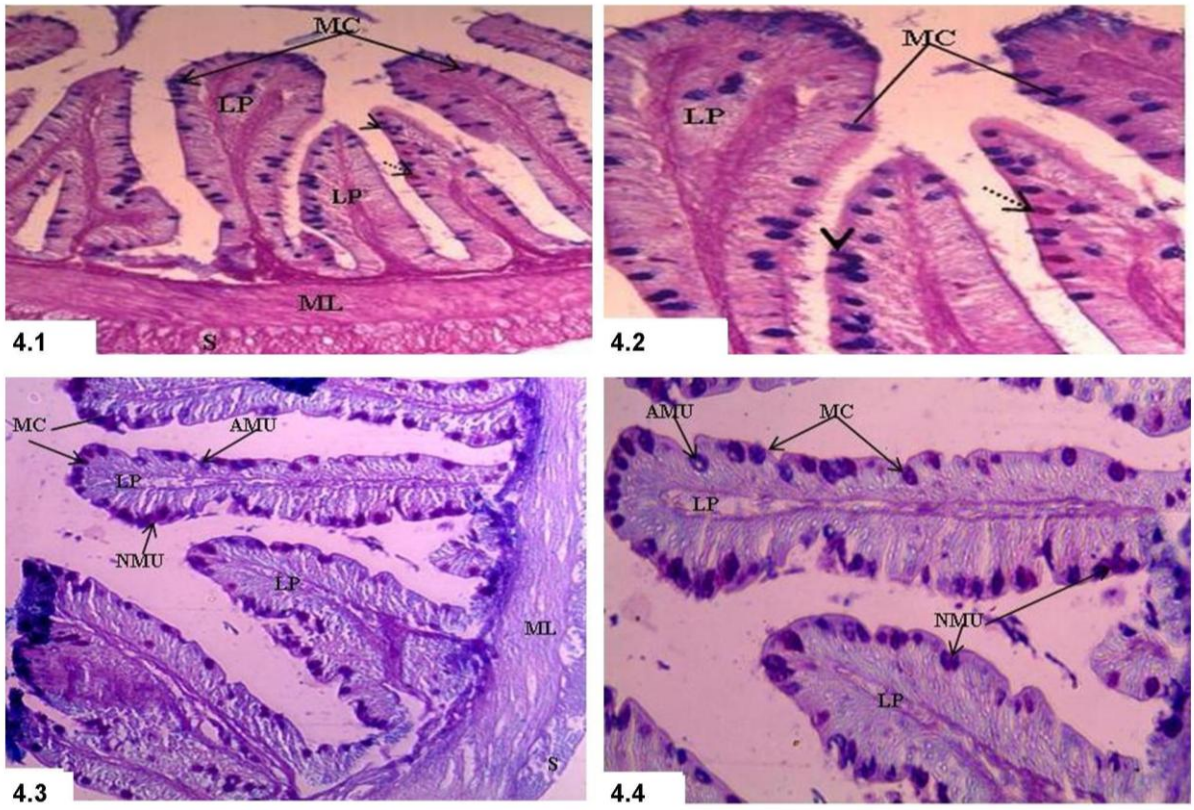

Figure 4: Histochemistry of intestine

Figures 4.1- 4.4: Photomicrographs of transverse section of intestine of *a. testudineus* in control condition (C), Almix treated condition (AL). (PAS-AB)
Figures 4.1 & 4.2: Showing intense PAS-AB reaction in the MC of intestinal villi. Note presence of profuse quantity of acid mucin (arrow head). Note also presence of neutral mucin (broken arrow). (C) X 200 & (C) X 400
Figures 4.3 & 4.4: Denoting reduction of acid mucin (AMU) in the MC. Note presence of profuse quantity of neutral mucin (NMU). (AL) X 200 & (AL) X 400

Reifel and Travill (1978) found mixture of acid and neutral mucin in the stomach of eight teleostean species of different families, *e.g.*, Centrarchidae, Cyprinidae, Esocidae, Ictaluridae and Percidae. Sinha *et al*., (1988) described that the neutral mucopolysaccharides of gastric mucosa of the fishes protected the epithelial cells from chemical injuries. The neutral mucin also has a buffering effect on the acid and enzymes secreted by the gastric mucosa, thus, protecting the epithelial lining from the chemical injuries including autodigestion (Ghosh, 1990). The mucin mass helps in the movement of large food particles and protects the gastric mucosa from mechanical injury (Domeneghini *et al*., 1999). Therefore, it can be assumed that profuse secretion of neutral mucin due to the toxic effect of Almix 20WP herbicide might provide a defensive mechanism against aforesaid herbicide and also protected the gastric epithelium from cell lysis. The mucin mass, secreted from different regions of intestine performs some important physiological functions like transportation of food materials, absorption of the ingested material and finally defaecation of undigested food materials through rectum. The presence of luminal mucin in the intestinal region keeps it moist and enabling early transport of ingested material apart from protecting the epithelial cells from mechanical injury (Sinha and Chakrabarti, 1982). In the intestinal region of *A. testudineus* acid and neutral mucins played the important role as lubricant and in addition to this intestinal

surface epithelium were coated with mucin mass. Thus, it can be suggested that this coating ensures a favourable environment for ionic and molecular diffusion and so it is essential to study this region. Acid mucin plays a significant role in ion exchange (Van Oosten, 1957; Hughes and Wright, 1970; Olson and Fromm, 1973). It also acted as buffering agent for controlling pH of the ingested food medium in the intestinal region thus creating a favourable environment for subsequent action of various digestive enzymes. In the present study, intestinal region of *A. testudineus* was affected due to Almix exposure. The excessive secretion of mucin mass was notable observation of toxic effect of Almix 20WP herbicide. Excessive secretion of mucin over the intestinal villi might provide protection ability from irritation due to ingestion of herbicide. According to Ghosh (1990), the excessive secretion of mucin in the lumen of intestine may alter the luminal pH and thus affects the enzymatic break down polymer substances in the intestinal region and subsequently impair the physiology of digestion. Reports on such effects on fish due to toxicity of herbicides or pesticides are very scanty but similar reports on heavy metal toxicity are available, *viz.*, copper sulphate and zinc sulphate (Lewis and Lewis, 1971), lead nitrate (Chakrabarti *et al.*, 1986), cadmium chloride (Datta and sinha, 1988, 1989), arsenic and cadmium (Ghosh, 1990), lead and cadmium (Bose, 2005).

Enzymological Study

Stomach

The amylase activity in stomach was 1.315±0.087 unit/mg protein/min in the control condition whereas; in treated condition it was 1.255±0.051 unit/mg protein/min after 45 days (Figure 5). Protease activity was 1.140±0.005 unit/mg protein/min in the control condition. After 45 days it was reduced to 1.121±0.008 unit/mg protein/min. Lipase activity in the stomach was 0.164±0.016 unit/mg protein/min in control condition whereas; after treatment it was lowered to 0.140±0.018 unit/mg protein/min (Figure 5).

Intestine

In intestine the amylase activity in control condition was 10.431±0.060 unit/mg protein/min; after 45 days of herbicide exposure, amylase activity was 9.918±0.461 unit/mg protein/min. Protease activity was 5.393±0.025 unit/mg protein/min in control condition. After treatment of Almix 20WP herbicide, it was reduced to 0.496±0.016 unit/mg protein/min. The activity of lipase was 0.328±0.027 unit/mg protein/min and after 45 days of herbicide exposure it was reduced to 0.313±0.026 unit/mg protein/min (Figure 6).

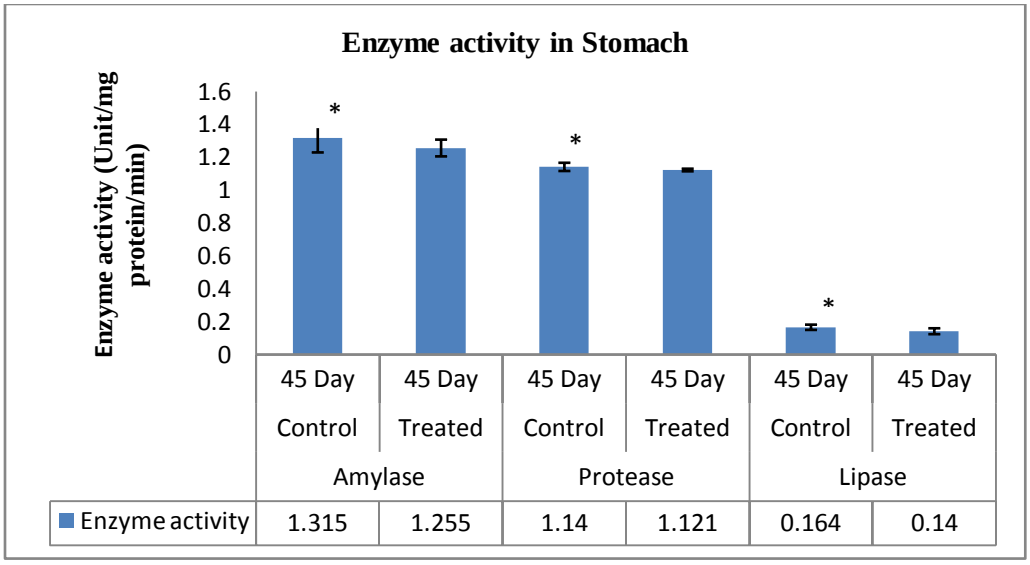

Figure 5: Enzyme activity in Stomach

The food items of teleosts comprise of complex molecular components. Digestive enzymes play an essential role to break down these complex food materials into simpler form through a process, is known

as digestion. Digestion of food stuff followed by absorption and ultimately utilization of the metabolic products are the fundamental function of the alimentary tract of fishes. Removal of xenobiotics including pesticides, heavy metals, into the exterior is also the important function of GI tract. Carnivorous fishes *e.g*., *A. testudineus* consume complex food items. In *A. testudineus* the activity of digestive enzymes *i.e*., amylase, lipase, protease are playing a vital role in their growth and development. So the study of digestive enzyme activity in different regions of alimentary tract *i.e*., stomach, intestine *A. testudineus* is very important in the toxicological study.

In the present study, the digestive enzyme activity was significantly ($p<0.05$) reduced in comparison with control fishes, in the Almix treated fish. In the present study, amylase activity was found to be higher in the intestine but was low in stomach of *A. testudineus*. Amylase activity in the stomach was low probably due to low pH of the gastric juice. Ghosh (1990) recorded higher amylase activity in intestine and hepatopancreas of *N. notopterus* and *H. fossilis*. In the present study, significant reduction of amylase activity was found in the alimentary canal of *A. testudineus* after chronic exposure of Almix 20WP herbicide to a sublethal dose.

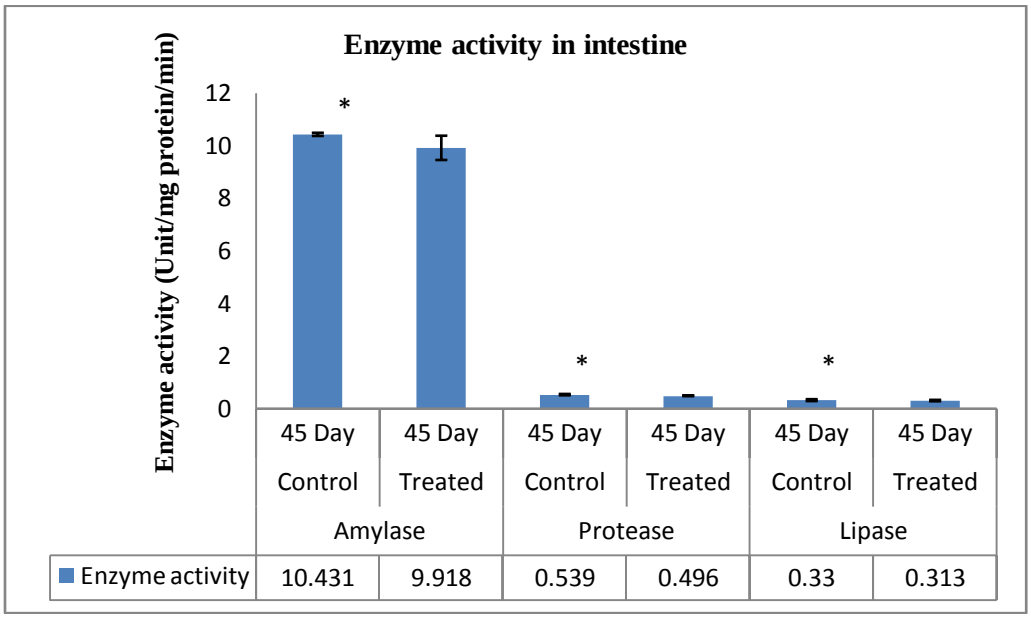

Figure 6: Enzyme activity in intestine

- Significant differences from controls are indicated by an asterisk ($P < 0.05$,'t' test). Values are (means ± SD).

In another study Bhattacharya *et al.*, (1975) reported reduction of amylase activity in *C. batracus* when exposed to sublethal dose of endrin. Senapati *et al.*, (2009) observed alteration of amylase activity in *C. punctatus* after exposure to glyphosate in the laboratory condition. In the present study, the highest protease activity was found in stomach of *A. testudineus*. Highest protease activity in the stomach may be correlated with carnivorous food habit of the experimental fishes. In the stomach of experimental fish's proteolytic enzyme, mainly pepsin partly hydrolyses protein food in the acidic pH environment. Samanta *et al.*, (2010) reported that there was no significant alteration in protease activity of *C. puntatus* and *A. testudineus* after Almix exposure in field condition. Nemesok and Boross (1981) reported that paraquat and $ZnCl_2$ induced reduction of proteolytic enzyme activity in the intestine of common carp and silver carp. In another study Senapati *et al.*, (2009) also reported alteration of protease activity in the alimentary canal of *C. punctatus* due to chronic exposure of glyphosate herbicide in the laboratory condition. The reduction of protease activity in the stomach might be due to alteration of gastric pH due to excessive

secretion of neutral mucin and disruption of zymogen granules in the gastric gland which ultimately culminate the production of proteolytic enzymes (Ghosh, 1990). In the intestinal region, distortion of mucosal epithelial cells and deterioration of intestinal brush border might cause the reduction of protease activity. The food items of carnivorous fishes are normally composed of higher proportion of fatty food and this is ultimately assimilated in the gastrointestinal tract (Kapoor *et al.*, 1975). In the present study, intestine and stomach of both the fishes showed moderate lipase activity. In this study, the lipase activity was significantly lowered in the different parts of alimentary canal *i.e.*, stomach and intestine of *A. testudineus* in laboratory condition after Almix exposure. Senapati *et al.*, (2009) reported that lipase activity was reduced in alimentary canal of *C. punctatus* treated with glyphosate herbicide in the laboratory. From the histopathological study it was evident that Almix herbicide can cause damage of epithelial cells along with brush border in the intestine. The damage of brush border on the epithelial cells of the intestine might be responsible for reduction of lipase activity. From this study it can be assumed that Almix 20WP herbicide definitely have some toxic effects in non-target organisms *e.g.*, *A. testudineus* when it was constantly exposed to the herbicide.

ACKNOWLEDGEMENT
Authors are grateful to Dr. NK Mandal, HOD, Dept. of Environmental Science, The University of Burdwan. Principal author is also grateful to Mr. Sashikant Singhi, Director general, Poornima University, Dr. Rakesh Duggal, Academic director, Poornima University, Dr. Manoj Gupta, Dean, Poornima University for their support and cooperation.

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