Histopathological Study in Stomach and Intestine of *Anabas testudineus* (Bloch, 1792) under Almix Exposure

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**Abstract**

The aim of the present study was to investigate the histopathological alterations in the stomach and intestine of Indian freshwater teleost, *Anabas testudineus* (Bloch, 1792) after Almix® exposure both under laboratory and field conditions. The field (dose 8 g/acre) and laboratory (dose 66.67 mg/l) experiments was carried out for 30 days. Special type of cage was prepared and installed in the pond for the field experiment. Pathological alterations in the concerned fish organs namely stomach and intestine were assessed through light microscopy, scanning and transmission electron microscopy. Lesions observed under light microscopy also endorsed the findings of ultrastructural observations both under laboratory and field conditions. Cytopathological alterations observed under light and electron microscopy revealed that the degree of responses were different in different fish tissues as well as under conditions, here in particular effects in stomach were more prominent in laboratory condition. The overall responses registered in the fish tissues under laboratory condition were more pronounced than field condition. Therefore, these symptoms and/or alterations in the present study due to almix intoxication could be considered as biomarkers in toxicity study in aquatic ecosystem.

**Keywords:** Cytopathological; Stomach; Intestine; *Anabas testudineus*; Almix

**Introduction**

In the agricultural fields, the use of herbicides to protect the crops from the attack of pests and unwanted plants has been considered as an integral part of the modern agricultural practices worldwide. But, the indiscriminate use of it might endanger the aquatic ecosystems and fish farms close to the agricultural fields, as they ultimately reach to these aquatic bodies as runoff and caused harmful effects to the natural inhabitants residing in water especially non-target aquatic organisms such as aquatic insects, molluscs and fish. Almix is one of the most widely used herbicide in Indian agricultural fields in recent times. It is sulphonyl urea group type of herbicide, and is composed of 10.1% metsulfuron methyl, 10.1% chlorimuron ethyl and remaining 79.80% adjuvants [1]. It is used for controlling the broad leaf weeds and sedges such as such as *Cyperus iria* (Linnaeus, 1753), *Cyperus terrestial* and aquatic system. It is a selective, both pre-emergent and post-emergent herbicide and destroy the unwanted plants both from the attack of pests and unwanted plants has been considered as environmental quality and simultaneously provides a sensitive as well as reliable approach to evaluate the contamination level caused by xenobiotic substances in aquatic bodies [2]. Fish, among them considered as an excellent experimental aliquot for toxicity studies because they are the best understood organisms in the aquatic environment, held at the top of the trophic level and finally, they are directly exposed to these xenobiotic substances directly via surface run-off or indirectly through food chain [3,4]. Therefore, the use of fish for better understanding the pollution-induced environmental conditions in the aquatic environment have gained much more importance worldwide in last few decades and helps to monitor the health status of the entire aquatic environment [4,5]. In the present study, *Anabas testudineus* (Anabantidae) was selected as experimental model for toxicity study. Some of the characteristics of this fish species make them as excellent experimental model such as wide distribution in aquatic environment, non-invasive property, wide availability throughout the year, economic importance and ease acclimatization etc.

A number of studies demonstrated the histopathological alterations including ultra structural observations (scanning electron microscopy and transmission electron microscopy) which is considered as an efficient and extensively used methods to evaluate the health status of the organisms exposed to a complex mixture of environmental contaminants both in the laboratory and field conditions [6-8]. One of the most important advantage of using histopathological biomarkers in monitoring the environmental quality is that it allows only the examination in the specific target organ toxicity, in particular, stomach and intestine. In addition, histopathological biomarkers also play a pivotal role in assessing the overall health status of the entire population in the aquatic ecosystem. Furthermore, the alterations observed in these target organs are more easier and reliable to identify.
specifically than the functional ones [9], and ultimately serve as warning signal of deterioration in animal health [10,11]. These biomarkers, in last few decades, have opened up a new vista in assessing the aquatic ecosystem toxicology as the fish alimentary canal are continually exposed during the digestion of ingested food stuff contaminated with xenobiotic substances directly through primary producer organisms. In last few years, a number of studies are available on biochemical, physiological and metabolic alterations of this herbicide in different fish species [12-20]. Regarding the pathological alterations through histological and ultrastructural observations of this herbicide on various organs in different fish species are scanty [19,20] as major advancement in science has been made in recent years. Therefore, considering this scarce information of this agrochemical, the objectives of the present investigation was to characterize and compare the histological and ultrastructural alterations induced by Almix, with particular emphasis on stomach and intestine of Anabas testudineus.

Materials and Methods

Fish

Indian Freshwater teleost, Anabas testudineus (Bloch, 1792) with an average weight of 23.58 ± 2.05 g and total length of 11.15 ± 0.548 cm, respectively were purchased from the local fish farm and were acclimatized for 15 days. During acclimatization, fish were kept in continuously aerated water (250 L capacity) with a static system, and at natural photoperiod of 12 h light/12 h dark. Average value of water parameters during the acclimatization period were as follows: temperature, 18.61 ± 0.81°C; pH, 7.23 ± 0.082; electrical conductivity, 413.67 ± 0.90 µS/cm; total dissolved solids, 295.11 ± 1.16 mg/l; dissolved oxygen, 6.46 ± 0.22 mg/l; total alkalinity, 260.00 ± 16.90 mg/l as CaCO₃; total hardness, 177.33 ± 5.50 mg/l as CaCO₃; sodium, 19.20 ± 0.36 mg/l; potassium, 2.80 ± 0.02 mg/l; ammoniacal-nitrogen, 3.41 ± 0.02 mg/l; nitrate-nitrogen, 0.58 ± 0.02 mg/l.

Laboratory experimental design

After acclimatization, fish were transferred to laboratory aquarium and maintained in six aquariums, three for control and three for treatment in the Ecotoxicology Lab, Department of Environmental Science, The University of Burdwan. Each aquarium contains 10 fish (40 L capacity). Treated aquarium exposed to single sub-lethal concentration of Almix i.e., 66.67 mg/l for a period of 30 days [13-18]. On every alternate day water was replaced and after water replacement dose was applied. During experimentation almix-treated and control were subjected to same environmental conditions. Average water parameters, during the experimentation period, were as follows: temperature, 19.67 ± 0.29°C; pH, 7.48 ± 0.05; electrical conductivity, 478.33 ± 9.70 µS/cm; total dissolved solids, 341.44 ± 6.56 mg/l as CaCO₃; total hardness, 188.89 ± 8.58 mg/l as CaCO₃; sodium, 21.36 ± 0.76 mg/l; potassium, 2.80 ± 0.02 mg/l; orthophosphate, 0.02 ± 0.001 mg/l; ammoniacal-nitrogen, 6.63 ± 1.16 mg/l and nitrate-nitrogen, 0.46 ± 0.11 mg/l.

Sampling

Water quality during acclimatization and experimentation was assessed as per APHA [22]. At the end of the experiment (i.e., 30 days), fish were collected from both conditions using hand net and were anesthetized with tricaine methanesulphonate (100 mg/l). After anesthetization, fish were dissected and desired organs namely stomach and intestine were taken immediately and fixed in respective fixatives prescribed for histological, scanning and transmission electron microscopic study.

Histopathological analysis

Stomach and intestine after dissection were fixed in aqueous Bouin’s solution for overnight. Then dehydrated through gradated series of ethanol (70%, 90% and 100%) and finally embedded in paraffin for preparing the paraffin block. Tissue sections were cut at 3-4 µ using Leica RM2125 microtome and stained with haematoxylin-eosin (H&E). Finally stained sections were examined under Leica DM2000 light microscope and photographs were taken by Leica Image Organizer software to examine the pathological alterations.

Ultra structural analysis

For electron microscopic (SEM) study, stomach and intestine were dehydrated through graded series of ethanol, critical point dried with liquid carbon dioxide, then cut into small pieces and were embedded in epoxy resin. Tissue sections of interest were cut with a diamond knife in cryo-ultramicrotome (Leica). Tissue sections stained with uranyl acetate and lead citrate were examined under JEOL EM-10 electron microscope and images were captured using a digital camera (Sony). All the images were further digitally enhanced using Adobe Photoshop software.
in CPD (critical point drying) machine. After drying tissues were mounted on metal stub and sputter-coated with gold (thickness 20 nm) and examined under scanning electron microscope (Hitachi S-530) at University Science Instrumentation Centre of the University of Burdwan, Burdwan, West Bengal, India and photographs were taken for analysis by Image Organizer software.

For transmission electron microscopic (TEM) study, stomach and intestine (2 × 2 mm in size) were fixed in Karnovsky fixative prepared in 0.1 M phosphate buffer for 12 h at 4°C and then post-fixed with 1% osmium tetroxide prepared in phosphate buffer (0.2 M and pH 7.4) for 2 h at 4°C. After fixation tissues were washed with phosphate buffer and then dehydrated through graded series of acetone, infiltrated and finally embedded in epoxy resin (araldite CY212). Ultrathin sections of the respective tissues were then cut by using a glass knife on “Ultracut E Reichart-Jung” machine (thickness 70 nm). Sections were then collected on naked copper-meshed grids, and stained with uranyl acetate and lead citrate. Finally, tissues were examined under TECHNAI G2 high resolution transmission electron microscope at Electron Microscope Facility, Department of Anatomy, AIIMS, New Delhi, India and photographs were captured by Image Organizer software.

Results

Stomach

Histologically, stomach is made up of as usual of four layers viz., mucosa, submucosa, strong muscularis, and serosa. The gastric mucosa is lined with a single layer of compactly arranged columnar epithelial cells (CEC) with centrally placed nuclei. The tubular gastric glands are present at basal portion of gastric mucosa. In gastric gland, the gastric cells with centrally placed nucleus are present such as encircling the central lumen. Gastric glands are simple, tubular along with either rounded or elongated in shape. Sub mucosa is well vascularised with thick layer of loose connective tissue (Figure 1.1). Most notable changes observed under light microscopy in the laboratory condition were degenerative changes in columnar epithelial cells, fatty deposition in the basal region, brush border disappearance, top plate thinning, damage in gastric glands and mucosal folds in stomach of A. testudineus (Figure 1.2), while under field condition no such prominent changes were observed (Figure 1.3).

SEM study also confirmed the damages observed under light microscopy such as severe degeneration in CEC such as fragmented CEC, severe mucus secretion over epithelial surface and damage in the microridge structures (Figures 1.4 and 1.5), while under field condition damages were comparatively less than laboratory condition (Figure 1.6). Transmission electron microscopic observation showed deformation in nucleus and mitochondria (Figure 1.7), damage in rough endoplasmic reticulum, and vacuolations in stomach of A. testudineus (Figure 1.8), but only deformed mitochondria and vacuolations were observed under field condition and damages were less than laboratory condition (Figure 1.9).

Intestine

Intestine, histologically, also possesses prominent four histological layers like stomach. The intestinal villi are narrow and slender. The mucosa of intestine is made up of simple, long absorptive columnar epithelial cells each with basally and centrally placed nucleus. Mucous cells are present scatteredly throughout the intestinal mucosa. The loose connective tissue fibres of submucosa projected into the mucosal folds forming the lamina propria. The lamina propria is narrow, long vascular and mucous cells are dispersed. Columnar epithelial cell are prominent and nucleus are centrally placed and deep stained (Figure 2.1). The most conspicuous changes in intestine under laboratory condition were severe damage in CEC, distortion in connective tissues of lamina propria, detachment of epithelial layer from lamina propria and severe mucus secretion (Figure 2.2), while under field condition intestine showed almost normal appearance but in some places mucus secretion was prominent (Figure 2.3).

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glycocalyx structure, dilated mitochondria, and damage in the tubular network under laboratory condition (Figure 1.8), while mitochondrial deformation and vacuolations were prominent under field condition (Figure 1.9).

Figure 2: Photomicrographs of intestine in A. testudineus showing control condition (C), laboratory condition (AL), field condition (AF).

Discussion

Present study is reporting first time the toxicity of the sulfonylurea-based commercial agrochemical, Almix with regard to histological and ultrastructural observations through scanning and transmission electron microscopy in A. testudineus under field and laboratory conditions on comparative basis, although Senapati reported histopathological alterations under laboratory condition in oesophagus, buccopharynx, stomach and intestine of A. testudineus and Samanta on some biochemical parameters in fish species as well as considered as a sensitive organ for toxicity assessment of xenobiotic substances in fish species as they are directly exposed to complex mixture of toxic substances via ingestion of contaminated food stuffs or indirectly via blood and/or lymph [31]. A number of studies on histopathological effects of different pesticides on fish intestine have been reported by several authors but histological and ultrastructural studies related to intestinal epithelium due to Almix exposure, are relatively scanty [32-35]. Walsh and Velmurugan noticed degeneration in the tip of villi, loss of structural integrity in mucous cells, hypertrophy, vacuolation and necrosis in Cynicus carpio and Cirrhinus mrigala exposed to atrazine and fenvalerate, respectively [35,36]. These pathological alterations can also be resembled with our findings observed under present study such as damage in CEC, distortion of connective tissues in lamina propria, detachment of epithelial layer from lamina propria and excessive mucus secretion. Similar type of pathological alterations as observed in the present investigation was also reported in C. batrachus and C.
In summary, the present study revealed that Almix exposure caused severe pathological alterations in stomach and intestine of A. testudineus under laboratory condition. Pathological lesions displayed stronger responses under laboratory condition compared to field study. Finally, these pathological alterations to this herbicide exposure could be considered as indicators to evaluate fish health status under stressed conditions in freshwater ecosystem, and careful handling and monitoring should be taken before application of this herbicide in agricultural farms or aquatic bodies for controlling weeds.

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