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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 inhabitant reside 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 defformis* (Linnaeus, 1836), *Frimbristylis* sp., *Eclipta alba* (Linn), *Ludwigia parviflora* (Roxb, 1820), *Cyanotis axillaris* (Don, 1826), *Monochoria vaginalis* (Presl, 1827), *Marsilea quadrifoliata* (Linn), etc., both in the terrestrial and aquatic system. It is a selective, both pre-emergent and post-emergent herbicide and destroy the unwanted plants both through contact and systematic pathway. It was applied in the field at a very low use rate i.e., 8 g per acre and did not show any volatilization property; therefore do not affect the adjacent crops [1].

Sentinel organisms play an important role in the assessment of 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 e.t.c.

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 continuously being 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 $CaCO_3$; total hardness, 177.33 ± 5.50 mg/l as $CaCO_3$; sodium, 19.20 ± 0.36 mg/l; potassium, 2.45 ± 0.22 mg/l; orthophosphate, 0.02 ± 0.002 mg/l; ammoniacal-nitrogen, 2.31 ± 0.43 mg/l and nitrate-nitrogen, 0.30 ± 0.06 mg/l. After completion of acclimatization, fish were separated into two parts: one group of fish was transferred to field ponds situated at Crop Research and Seed Multiplication Farm (CRSMF) premises of the University of Burdwan, and remaining fishes were brought to the laboratory aquarium. Fish were fed commercial fish pellets (32% crude protein, Tokyu) once a day during acclimatization and experimentation. The experiments were conducted in accordance to the guidelines of the University of Burdwan for animal experiment and were authorized by the Ethical Committee of this University.

Field experimental design

After transferring the fish to the field ponds, fish were divided into two groups: control group with 10 fish species in each cage (triplicate), and exposure group also with 10 fish species in each cage (triplicate). The recommended dose (8 g/acre) used for rice cultivation was dissolved in water and sprayed on the surface of each Almix-treated plots on the first day. Almix® was purchased from the local market (DuPont India Pvt. Ltd., Gurgaon, Haryana, India). The duration of the experiment was 30 days. Special type of cage was prepared based on the method described by Chattopadhyay with slight modifications and installed at the ponds of CRSMF premises [21]. Cages were square in shape with the dimension of 2.5 m × 1.22 m × 1.83 m (submerged height was 0.83 m) and were structured by light strong bamboo. Four-sided wall of the cage, floor and cage cover was fabricated with nylon net, which was embraced by two PVC (poly vinyl chloride) nets: the inner mesh (1.0 × 1.0 mm^2) and outer mesh (3.0 × 3.0 mm^2). The average of pond water, during the experimentation period were as follows: temperature, 15.67 ± 0.15°C; pH, 7.89 ± 0.03; electrical conductivity, 390.33 ± 2.19 µS/cm; total dissolved solids, 276.33 ± 1.45 mg/l; dissolved oxygen, 7.47 ± 0.09 mg/l; total alkalinity, 101.33 ± 0.68 mg/l as $CaCO_3$; total hardness, 152.0 ± 2.32 mg/l as $CaCO_3$; sodium, 20.56 ± 0.29 mg/l; potassium, 2.89 ± 0.11 mg/l; orthophosphate, 0.12 ± 0.01 mg/l; ammoniacal-nitrogen, 6.06 ± 0.88 mg/l and 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; dissolved oxygen, 5.82 ± 0.39 mg/l; total alkalinity, 317.30 ± 15.60 mg/l as $CaCO_3$; total hardness, 188.89 ± 8.58 mg/l as $CaCO_3$; sodium, 21.36 ± 0.76 mg/l; potassium, 2.80 ± 0.29 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 graded 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 fixed in 2.5% glutaraldehyde solution prepared in phosphate buffer (0.2 M and pH 7.4) for 24 h at 4°C and then post-fixed with 1% osmium tetra oxide solution prepared in same phosphate buffer for 2 h at 4°C. After fixation tissues were washed with phosphate buffer and dehydrated through graded series of acetone, followed by amyl acetate and finally subjected to critical point drying with liquid carbon dioxide

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 tetra oxide 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).

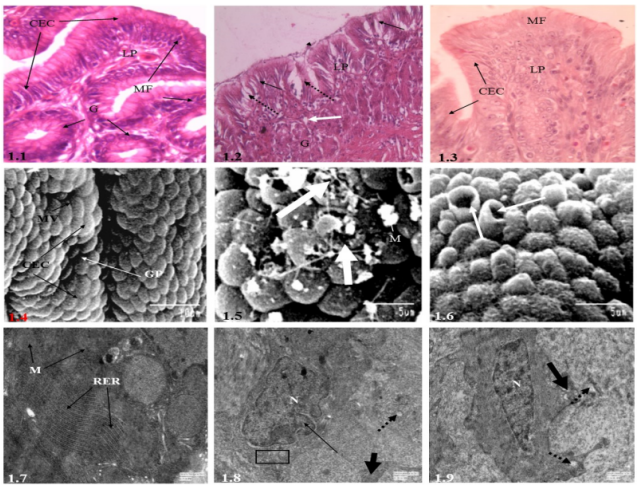

Figure 1: Photomicrographs of stomach in *A. testudineus* showing control condition (C), laboratory condition (AL) and field condition (AF).

where 1.1: Normal and compact arrangement of columnar epithelial cells (CEC) with distinct nucleus under light microscopy and presence of rounded gastric glands (G) separated by lamina propria (LP) (C × 1000), 1.2: Showing degenerated CEC (arrow), vacuolization (broken arrow), thin top plate (arrow head) and lesions in gastric gland (white arrow) under normal microscopy (AL × 400), 1.3: Compact CEC with distinct nucleus under light microscopy (AF × 1000), 1.4: SEM observation showing normal mucosal folds (MF) surrounded by oval or round shaped CEC and stubby microvilli (MV). Note presence of gastric pits (GP) (C × 3000), 1.5: Showing degeneration in columnar epithelial cells (bold arrow) and mucus secretion (M) under scanning electron microscopy (AL × 6000), 1.6: Showing damage on tip of CEC (arrow) under SEM (AF × 6000), 1.7: Showing normal gastric glands with mitochondria (M) and rough endoplasmic reticulum (RER) under TEM observation (C × 7000), 1.8: Deformed nucleus (arrow) and mitochondria (bold arrow), damage in RER (square) and vacuolization (broken arrow) under TEM study (AL × 4000), and 1.9: Presence of deformed mitochondria (bold arrow) and vacuolization (broken arrow) under TEM obsevation (AF × 5000).

Ultra structural lesions displayed severe mucus secretion over epithelial surface and necrosis under laboratory condition (Figures 2.4-2.9). While mucosal folds and CEC showed less damage in comparison to laboratory condition but in-between the primary mucosal folds debris of the fragmented secondary mucosal folds was observed under field condition under SEM study (Figure 1.6). TEM study also showed fatty deposition and vacuolations, damage in the

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).

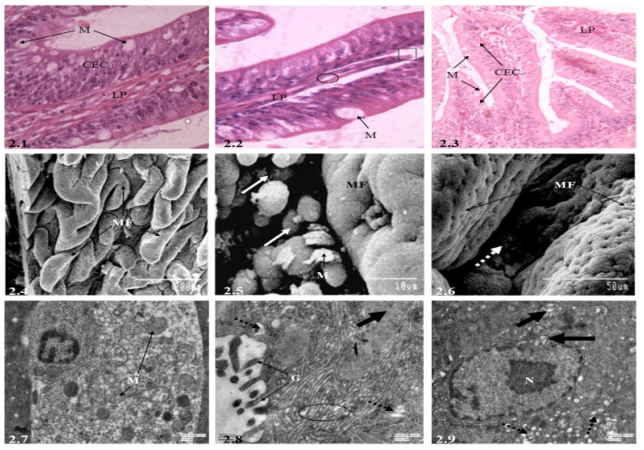

Figure 2: Photomicrographs of intestine in A. testudineus showing control condition (C), laboratory condition (AL), field condition (AF).

where 2.1: Normal lamina propria (LP), columnar epithelial cells (CEC) under light microscopy (C × 1000), 2.2: Damage in lamina propria (oval), detachment of epithelium layer from lamina propria (square) under light microscopy (AL × 1000), 2.3: Light microscopy showed almost normal CEC with distinct nucleus (AF × 400), 2.4: Distinct mucosal folds (MF) with oval or round shaped CEC and supported by microvilli (MV) under SEM observation (C × 200), 2.5: Showing deformed MV (arrow) and mucin droplets (M) under SEM observation (AL × 3000), 2.6: Showing normal mucosal folds (MF) and mucin droplets between MF (broken arrow) (AF × 1000), 2.7: TEM observation showing normal appearance of columnar epithelial cells with abundant mitochondria (M) (C × 5000), 2.8: Showing severe vacuolization (broken arrow), dilation in mitochondria (bold arrow), damaged tubular network (oval) and glycocalyx (G) under TEM (AL × 9900), and 2.9: TEM observation showed deformation in mitochondria (bold arrow) and vacuolization (broken arrow) (AF × 4000).

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 different fish species including A. testudineus but only under laboratory condition [13-20].

Stomach is one of the prime organs of fish alimentary canal and plays an important role in the digestion of ingested food stuffs for the growth and development of fish species. Histopathological study under light microscopy showed marked differences in gill epithelium between two conditions. In the present study, degenerative changes in columnar epithelial cells, vacuolated basal region, brush border disappearance, top plate thinning, distortion in gastric glands and fusion of submucosa with mucosal folds were frequently observed pathological alterations in stomach of A. testudineus. Damage in gastric glands observed under present study was also reported by Crespo and indicating lower production of mucin which ultimately reduced the protection ability of the gastric epithelium against the chemical injuries [23]. Distortion in the digestive glands also hampers the digestive enzymes production which ultimately leads to reduced absorption of food materials by intestinal part. Loss of structural integrity and vacuolation seen under this study were also reported by Establier, Sastry and Gupta [24,25]. Swelling, distortion and/or vacuolation in the mucosal epithelial cells of stomach also reported after chronic exposure of endosulfan and methyl ethyl mercurial in *Gymnocorymbus ternitzi* by Amminikutty and Rege [26]. Vacuolization in sub mucosa, shrinkage of mucosal folds observed under present study was also reported by Ghanbahadur in *Rasbora daniconius* after endosulfan exposure [27]. Prominent ultrastructural alterations under laboratory condition include severe degeneration in CEC such as fragmentation, severe mucus secretion and damage in microridge structures observed under scanning electron microscopy. Severe mucus secretion, damage in the microridge structures and CEC observed under SEM observation can also be corroborated with the findings of light microscopic observations and this might be due to herbicidal action which ultimately reduces the protection ability of gastric epithelium and triggers the activity of the gastric epithelium. The results were also agreement with the findings reported by Senapati [19]. Haque also observed damages in columnar epithelial cells which include fragmentation, profound mucus secretion and loss of microridge structure in stomach of *Channa punctatus* exposed to fluoride [28]. At the transmission electron microscopic level, gastric epithelium of stomach showed deformation in nucleus and mitochondria, damage in rough endoplasmic reticulum, and vacuolation under both conditions but the degree of changes was comparatively less in field condition. Similar result as observed under present study was also reported by Rebolledo and Vial [29]. On the other hand, Carrassón demonstrated abundance of rough endoplasmic reticulum and mitochondria, damage in tubule-vascular network, and heterochromatinic nucleus in stomach of *Dentex dentex* [30]. Comparatively less pathological alterations under field condition might be due to self-perpetuating mechanism as fish are in natural habitat and taking almost natural food.

Intestine is the next most important part of the fish alimentary canal after stomach and plays an important role in digestion and absorption of food materials 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 mucosal folds, hypertrophy, vacuolation and necrosis in *Cyprinus 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.*

mrigala after pesticidal exposure by Mandal and Sharma [37,38]. Ravanaiah and Narasimha Murthy also noticed vacuolations, lesions in villi and serosa layer, necrosis, congestion in blood capillaries and severe mucus secretion in *Tilapia mossambica* exposed to industrial pollutants [39]. Damage in brush border and blood vessels was also supported by Ghosh, which indicated reduction in the absorption of various macromolecules from the intestinal lumen [40]. Scanning electron microscopic observations depicted severe secretion of mucus and necrosis in laboratory condition. These results are also in agreement with the findings of Senapati who reported damage in mucosal folds and CEC, and degeneration in microvilli structure with profound secretion of mucus in intestine of A. testudineus afetr Almix exposure under laboratory condition [19]. Similar observations were also described by Ghosh in intestine of *Notopterus notopterus* after arsenic exposure and by Bose in A. testudineus after lead and cadmium exposure [41,42]. Severe secretion of mucus under present study indicated that fish were under stress and trying to overcome these stress as compensatory response. Debris of the fragmented secondary mucosal folds in between the primary mucosal folds was observed under SEM study in field condition. Transmission electron micrographic observation depicted severe vacuolations, damage in glycocalyx structure, dilated mitochondria and damage in the tubular network under both conditions, indicating fish were in stress and approached to protect the imposed stress. Comparatively less pathological responses in field condition might be due to dilution capability and self-regulating mechanism of the natural environment. Therefore, the alterations impaired the intestinal transportation process as well as absorption of food materials.

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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