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ORIGINAL ARTICLE

Toxicity assessment of agrochemical Almix in *Heteropneustes fossilis* through histopathological alterations

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ABSTRACT

The present study was designed to assess the adverse effects of the agrochemical Almix on comparative basis in gill, liver and kidney of *Heteropneustes fossilis* through histological and ultrastructural observations under field (8 g/acre) and laboratory (66.67 mg/L) conditions. Exposure duration of both experiments was 30 days. Gill showed atrophy in secondary lamellae, hypertrophied gill epithelium, damage in chloride and pillar cells, and detachment of chloride cells from gill epithelium under laboratory condition, but hypertrophy in gill epithelium and fusion in secondary lamellae were seen under field condition. In gill, scanning electron microscopy (SEM) showed fragmentation in microridges, hyper-secretion of mucus and loss of normal array in microridges, while transmission electron microscopy (TEM) displayed dilated mitochondria and rough endoplasmic reticulum (RER), abnormal sized vacuolation in chloride cells under laboratory condition. In liver, hypertrophied and pyknotic nuclei, disarrangement of hepatic cords, and cytoplasmic vacuolation were prominent under laboratory study but in field condition the liver showed little alterations. TEM study showed severe degeneration in RER and mitochondria and cytoplasmic vacuolation under laboratory condition but dilated mitochondria were prominent in field observation. Kidney showed severe nephropathic effects including degenerative changes in proximal and distal convolute tubule, damage in glomerulus under light microscopy, while deformity in nucleus, fragmentation in RER, severe vacuolation and necrosis in kidney were prominent under TEM study. The results clearly demonstrated that responses were more prominent in laboratory than field study. Thus the responses displayed by different tissues of concerned fish species exposed to Almix could be considered as indications of herbicide toxicity in aquatic ecosystem.

KEY WORDS: Almix; scanning electron microscopy; transmission electron microscopy; *Heteropneustes fossilis*

Introduction

In modern agricultural practices, the introduction of new technology for crop production and protection has several times increased the use of herbicides. Herbicides play an important role in controlling the annual grasses, broad leaved weeds and sedges from various agricultural fields. Indiscriminate uses, careless handling, accidental spillage, or discharge of untreated effluents on herbicidal uses into natural waterways, including fish farms, can cause damage in fish population and other aquatic animals or plants (Sarikaya & Yilmaz, 2003; Fonseca *et al.,* 2008). The application of environmental toxicological studies on non-mammalian vertebrates has been rapidly expanding in recent times, and for aquatic systems, fish have become indicators for the evaluation of the toxic effects of these noxious compounds. In aquatic toxicological studies, laboratory experiments are performed to estimate the potential hazards of these chemicals to establish "safe" levels of these xenobiotics (Anto´n *et al.,* 1994).

Almix® 20 WP is the new fourth generation herbicide. It is widely used to control broad-leaf weeds and sedges both in terrestrial and aquatic systems. Almix is a selective, contact as well as systematic and both pre-emergent and post-emergent herbicide of the sulfonylurea group. It is composed of 10.1% metsulfuron methyl ($C_{14}H_{15}N_5O_6S$) [methyl 2-(4-methoxy-6-methyl-1,3,5-triazin-2-yl-

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carbamoyl-sulfamoyl) benzoate], 10.1% chlorimuron ethyl ($C_{15}H_{15}ClN_4O_6S$) [ethyl 2-(4-chloro-6-methoxy-pyrimidine-2yl-carbamoyl-sulfamoyl) benzoate] and 79.8% adjuvants (DuPont Safety Data Sheet, 2012).

Documentation of Almix herbicide toxicosis to freshwater teleostean catfish, *Heteropneustes fossilis* has been started recently (Samanta *et al.,* 2014a, b; Samanta *et al.,* 2015a, b). Low concentrations of Almix, such as those used in rice fields, might cause changes in metabolic and enzymatic parameters of catfish, *H. fossilis* (Samanta *et al.,* 2014a, b) and other fish species such as *Anabas testudineus* and *Oreochromis niloticus* concerning reduction of protein level and glutathione S-transferase (GST), and enhancement of acetylcholinesterase (AChE), lipid peroxidation (LPO) and catalase (CAT) activities in different tissues of *A. testudineus* and *O. niloticus* (Senapati *et al.,* 2012; Samanta *et al.,* 2014a, b; Samanta *et al.,* 2015a, b). However, only metabolic and physiological activities alone do not satisfy the complete understanding of pathological alterations of the tissues under toxic stress. In order to know the extent of tissue damage it is thus useful to have an insight into the analysis of cellular and subcellular orientations, although the severity of damage depends on toxic potentiality of the particular toxic compound (Tilak *et al.,* 2001; Srivastava *et al.,* 2008). The great advantages of using histopathological biomarkers in environmental monitoring is that it allows an easy examination of specific target organs including gills, liver and kidney, which are responsible for vital physiological functions, such as respiration, accumulation and biotransformation, and excretion of xenobiotics (Gernhöfer *et al.,* 2001; Camargo & Martinez, 2007). A number of studies have been reported by several authors to understand the biochemical, physiological and metabolic alterations caused by exposure to different pesticides and/or herbicides on animals and fishes (Geetha *et al.,* 1999; Sambasiva Rao, 1999; Aruna *et al.,* 2000; Sornaraj *et al.,* 2005). However, studies regarding histology and ultrastructural effects of Almix herbicide on fish tissues and other aquatic invertebrates are relatively scanty (Senapati *et al.,* 2012) and still need to be evaluated when compared with mammals and was carried out only in laboratory study. Nevertheless, field studies using histopathology and ultramicroscopic observations of fish tissues as biomarkers of aquatic contamination by Almix herbicide have not so far been reported. Thus the present study was aimed to investigate the marked changes in the histological and ultrastructural architectures in gills, liver and kidney of *H. fossilis* to Almix intoxication on comparative basis under laboratory and field conditions (*i.e.,* higher *vs* lower).

Materials and methods

Chemicals

Commercial formulation of the Almix herbicide (Almix® 20 WP, DuPont India Pvt. Ltd., Gurgaon, Haryana, India) was used in both the experiments. Delafield's hematoxylin stain, eosin yellow, xylene, DPX, amyl acetate, acetone, glutaraldehyde solution, sodium hydroxide, tricaine methanesulphonate, uranyl acetate (EM grade), ethanol, disodium hydrogen phosphate, dihydrogen sodium phosphate, lead citrate (EM grade), epoxy resin (EM grade), paraformaldehyde (EM grade) and araldite CY212 (EM grade) of analytical grade were purchased from Merck Specialities Private Limited. Osmium tetraoxide was purchased from Spectrochem Pvt. Ltd., Mumbai, India.

Fish

Freshwater teleostean fish, *Heteropneustes fossilis* (Bloch) of both the sexes with an average weight of 37.91±5.43 g and total length of 18.58±0.959 cm were procured from a local fish farm and brought to the laboratory. Fish were acclimatized under congenial conditions for 15 days in aquarium (250 L). Fish were kept in continuously aerated water with a static-renewal system and experiments were conducted under natural photoperiod (12-h light/12-h dark). During the acclimatization, the average value of water parameters were as follows: temperature, 18.61±0.808°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±0.215 mg/L; total alkalinity, 260.00±16.90 mg/L as $CaCO_3$; total hardness, 177.33±5.50 mg/L as $CaCO_3$; ammoniacal-nitrogen, 2.31±0.43 mg/; and nitrate-nitrogen, 0.30±0.058 mg/L. After acclimatization, fish were divided into two groups: one group was transferred to field ponds situated at Crop Research Farm premises of the University of Burdwan and the other group was transferred to laboratory aquarium. Fish were fed once a day with commercial fish pellets (32% crude protein, Tokyu) during both acclimation and exposure periods. Therefore, the study was carried out under two different experimental conditions: field and laboratory, both for the duration of 30 days.

Field experiment

Fish were again divided into two groups as follows: control groups (triplicate cages), each cage contained 10 fish species, and Almix-exposure group with 10 fish species in three separate cages (Figure 1). The desired dose (8 g/acre) corresponds to the concentration recommended for rice culture was dissolved in water and applied once (Samanta *et al.,* 2014a; Samanta *et al.,* 2015b). Duration of the exposure period was 30 days. It was sprayed on the first day of the experiment on the surface of each Almix-treated cage. For these field experiments, special type of cage was prepared and installed separately at two different ponds of Burdwan University Crop Research Farm, University of Burdwan. Cages were prepared for the culture of experimental fish species as per Chattopadhyay *et al.* (2012) with some modifications. All the cages were square in shape having an area of 2.5×1.22 m and cage height was 1.83 m (submerged height was 0.83 m). Cages were framed by light strong bamboo. The four-sided wall, cage floor and top of the cage cover were fabricated with nylon net and embraced by two PVC nets: the inner and outer nets bearing mesh sizes of 1.0×1.0 mm^2 and 3.0×3.0 mm^2, respectively. During the experimentation

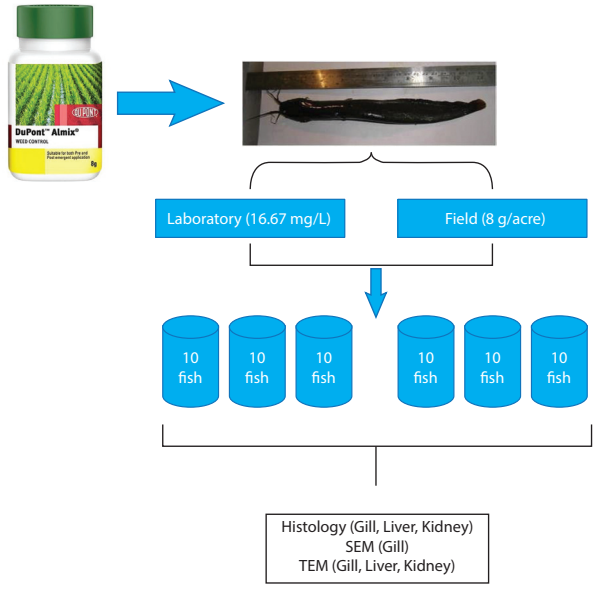

Figure 1. Experimental design of the present study.

period, pond water had the following average values: temperature 15.67±0.145 °C; pH 7.89±0.033; electrical conductivity 390.33±2.19 µS/cm; total dissolved solids 276.33±1.45 mg/L; dissolved oxygen 7.47±0.088 mg/L; total alkalinity 101.33±0.67 mg/L as $CaCO_3$; total hardness 152.00±2.31 mg/L as $CaCO_3$; ammoniacal-nitrogen 6.06±0.875 mg/L; and nitrate-nitrogen 0.58±0.016 mg/L.

Laboratory experiment

Fish were divided again into two groups (control and Almix-treated) and maintained in six aquaria (three for control and three for treatment), containing 10 fishes in each aquarium in the Ecotoxicology Lab, Department of Environmental Science, the University of Burdwan. Fish were exposed to sub-lethal dose of Almix, *i.e.,* 66.67 mg/L (40 L) for a period of 30 days (Samanta *et al.,* 2015a, b). Doses were applied every alternate day. During experimentation, Almix-treated and control were subjected to the same environmental conditions. During experimentation period, the average water parameters were as follows: temperature 19.67±0.293 °C; pH 7.48±0.052; electrical conductivity 478.33±9.70 µS/cm; total dissolved solids 341.44±6.56 mg/L; dissolved oxygen 5.82±0.394 mg/L; total alkalinity 317.30±15.60 mg/L as $CaCO_3$; total hardness 188.89±8.58 mg/L as $CaCO_3$; ammoniacal-nitrogen 6.63±1.15 mg/L, and nitrate-nitrogen 0.46±0.108 mg/L.

Sampling

During the experimentation period, water quality parameters were analyzed as per APHA (2005). After completion of the experiment, *i.e.,* 30 days, fish were collected both from aquarium and pond and were anesthetized with tricaine methanesulphonate (MS 222). After that gill, liver and kidney were taken immediately after dissection and proceeded in specific ways for histological, scanning and transmission electron microscopic study.

Histological analysis

Gill, liver and kidney from control and treatment fish were collected and fixed in aqueous Bouin's fluid solution for overnight. After fixation, tissues were dehydrated through graded series of ethanol and finally embedded in paraffin. Paraffin sections were then cut at 3–4 µ using Leica RM2125 microtome. Finally, sections were stained with hematoxylin-eosin (H&E) solution and pathological lesions were examined under Leica DM2000 light microscope. Additionally, semi-quantative analysis was also carried out by observing the frequency of pathological lesions based on Pal *et al.* (2012) with some modifications.

Ultrastructural analysis

For scanning electron microscopic study, tissues were fixed in 2.5% glutaraldehyde solution prepared in phosphate buffer (0.2 M, pH 7.4) for 24 h at 4 °C and then post-fixed with 1% osmium tetraoxide prepared in phosphate buffer (0.2 M, pH 7.4) for 2 h at 4 °C. After fixation, tissues were dehydrated through graded series of acetone, followed by amyl acetate and subjected to critical point drying with liquid carbon dioxide. Tissues were then mounted on metal stubs and sputter-coated with gold with thickness of approximately 20 nm. Finally, tissues were examined with a scanning electron microscope (Hitachi S-530) at the University Science Instrumentation Centre of the University of Burdwan.

For transmission electron microscopic study, tissues were fixed in Karnovsky fixative (mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer) for 12 h at 4 °C and then post-fixed with 1% osmium tetraoxide in phosphate buffer (0.2 M, pH 7.4) for 2 h at 4 °C. After fixation, tissues were dehydrated through graded acetone, infiltrated and embedded in epoxy resin (araldite CY212). Ultrathin sections (70 nm) were then cut using glass knife on an "Ultracut E Reichart – Jung" and collected on naked copper-meshed grids. After air-drying, grids were 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.

Ethical statement

The experiment was carried out in accordance with the guidelines of the University of Burdwan and was approved by the Ethical Committee of this University.

Results

Gill

Histologically, gill is composed of primary and secondary gill lamellae. Free edges of the lamellae are extremely thin, covered with stratified epithelium and contain a vast network of capillaries supported by pilaster cells. Primary gill lamella was supported by gill rays which were bony in nature (Figure 2.1).

Semi-quantitative evaluation of frequency of pathological lesions in gill of laboratory and field condition

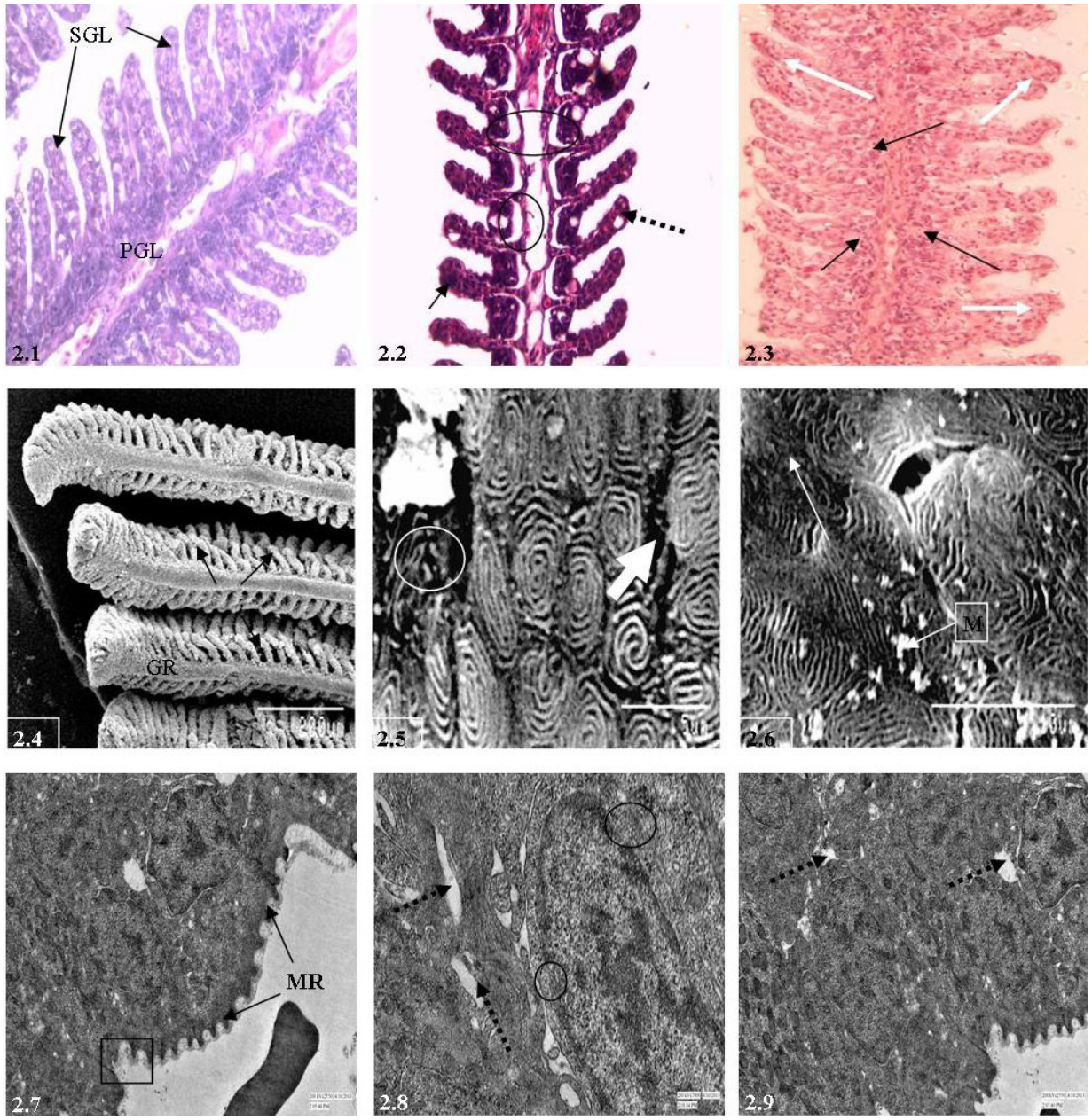

Figure 2. Histopathological photomicrographs of gill of *H. fossilis* under control condition (C), Almix treated laboratory condition (AL), Almix treated field condition (AF). **2.1** Showing normal structure of primary gill lamellae (PGL) and secondary (SGL) lamella under light microscopy (C×400). **2.2** Showing atrophy and hypertrophy of gill epithelium and SGL (arrow), damage in chloride (oval) and pillar cells (broken arrow) under light microscopy (AL×400). **2.3** Showing hyperplasia (arrow) and partial fusion of SGL (white arrow) under light microscopy (AF×1000). **2.4** Scanning electron microscopy **s**howing normal arrangement of gill rackers (GR) with primary gill lamellae (PGL) and stratified epithelial cells (SEC) on the PGL (C×150). **2.5** Gill epithelium **s**howing fragmentation of MR (oval) and loss of MR in SEC (arrow) under scanning electron microscopy (AL×6000). **2.6** Damage in MR (arrow) under SEM (AF×5000). **2.7** Gill epithelial cell under transmission electron microscopy showing normal chloride cell (CC), pavement cells (PC) with prominent mitochondria (M) (C×2550). **2.8** A double-layered nucleus (oval) and vacuolation in the chloride cells (broken arrow) under TEM (AL×7000). **2.9** Showing vacuolation (broken arrow) only under transmission electron microscopy (AF×2550).

fish compared to control fish is given in Table 1. Atrophy in secondary lamellae, hypertrophied gill epithelium and damage in chloride as well as pillar cells, detachment of chloride cells from gill epithelium and stunted growth of gill lamellae are the most common histological lesions observed under laboratory study (Figure 2.2). Contrary to laboratory findings, slight hypertrophy in gill epithelium and fusion in secondary gill lamellae are the only pathological lesions observed under field condition (Figure 2.3).

Under scanning electron microscopic study, gill epithelium showed fragmentation of microridges, swelling of microridges and loss of normal array of microridges (Figure 2.5), but under field condition hyper-secretion of

Table 1. Semi-quantitative assessment of frequency of pathological lesions in gill, liver and kidney of *H. fossilis* under laboratory and field conditions.

Pathological Lesion	Control	Laboratory condition	Field condition
Gill			
Histopathological			
Proliferated gill epithelium	–	++	+
Hypertrophy of the gill epithelium	–	+++	+
Hyperplasia of the gill epithelium	–	+++	+
Scanning electron microscopic			
Damage of microridge structures	–	+	–
Disappearance of normal array of microridges	–	+	–
Mucus secretion	–	++	–
Distortion of stratified epithelial cells	–	++	+
Swelling of stratified epithelial cells	–	+	+
Necrosis	–	++	+
Transmission electron microscopic			
Chloride cell damage	–	+++	–
Dilated mitochondria	–	+	++
Mitochondrial degeneration	–	+	–
Cytoplasmic vacuolation	–	+	–
Nuclear distortion	–	++	+
Liver			
Histopathological			
Disoriented hepatic cord	–	++	++
Hypertrophy of hepatocytes	+	+++	++
Degeneration of hepatocytes	–	++	+
Nuclear hypertrophy	+	+++	++
Cytoplasmic vacuolation	–	+++	++
Pyknotic nucleus	–	+	–
Detachment of hepatopancreatic acinar cells from hepatocytes	–	+++	++
Deformed hepatopancreas	–	++	–
Loss of zymogen granules	–	++	–
Transmission electron microscopic			
Cytoplasmic vacuolation	–	+++	+
Loss of rough endoplasmic reticulum	–	+	–
Loss of glycogen granules	–	++	+++
Dilated mitochondria	–	+	++
Kidney			
Histopathological			
Shrinkage of glomerulus	–	++	+
Lipid vacuoles in epithelial cells	–	++	+
Swelling in tubular epithelium	–	++	–
Hypertrophy in tubular epithelium	+	++	+
Fragmentation of glomerulus	–	+	+
Tubular degeneration	–	++	++
Loss of hematopoietic tissue	–	+	+
Transmission electron microscopic			
Vacuolation in epithelial cytoplasm	–	+++	+
Damage in proximal convoluted tubules	–	++	–
Dilated mitochondria	–	+	++

mucus, damage of microridges in few places were noticed after Almix exposure (Figure 2.6). Transmission electron microscopy analyses showed dilated mitochondria and endoplasmic reticulum, abnormal sized vacuolation in gill epithelium of *H. fossilis* under laboratory condition (Figure 2.8); however, in field condition gill epithelium showed almost normal appearance of pavement cells, chloride cells, mitochondria, apical pore except vacuolation in some places (Figure 2.9).

Liver

Semi-quantitative evaluation of frequency of pathological lesions in gill of laboratory and field condition fish compared to control fish is given in Table 1. The most expressive changes after Almix exposure in hepatocytes of the concerned fish species seen under light microscopy were distortion in hepatocytes with clumping of nuclei, hypertrophied and pyknotic nuclei, disarrangement of hepatic cords and cytoplasmic vacuolation under laboratory condition (Figure 3.2), while under field condition it showed only distended appearance of hepatocytes and short central vein and fat deposition in sinusoidal spaces in some places (Figure 3.3).

Ultrastructural alterations as viewed under TEM study showed severe degeneration in rough endoplasmic reticulum and mitochondria, vacuolation in cytoplasm and reduced amount of glycogen droplets in hepatocytes (Figure 3.5) under laboratory condition as compared to control (Figure 3.4), but in field condition, hepatocytes showed no significant changes in nucleus and rough endoplasmic reticulum but only dilated mitochondria in some places (Figure 3.6).

Kidney

Histologically, the kidney is made up of a large number of nephrons, each consisting of a renal corpuscle or the Malpighian body and renal tubules. Renal tubules consist of columnar epithelial cells and renal tubules which are spherical or oval in shape. Renal tubules are differentiated into proximal convoluted tubule (PCT), distal convoluted tubule (DCT) and collecting ducts (Figure 4.1).

Semi-quantitative evaluation of frequency of pathological lesions in gill of laboratory and field condition fish compared to control fish is given in Table 1. Nephropathic effects due to Almix toxicosis under laboratory condition included degenerative changes in PCT and DCT, distorted glomerulus in certain Bowman's capsules (Figure 4.2). However, under field condition no such significant alterations in PCT and DCT of *H. fossilis* were observed but aggregation and fatty deposition in hematopoietic tissues were prominent (Figure 4.3).

After 30 days of Almix exposure in laboratory condition, transmission electron microscopic study showed severe degenerative changes in mitochondria, deformity in nucleus, dilation, fragmentation and vesiculation in rough endoplasmic reticulum, severe vacuolation in cytoplasm and necrosis (Figure 4.5), while dilation in mitochondria, abundance of numerous mitochondria, lower amount of vacuolation were observed under field

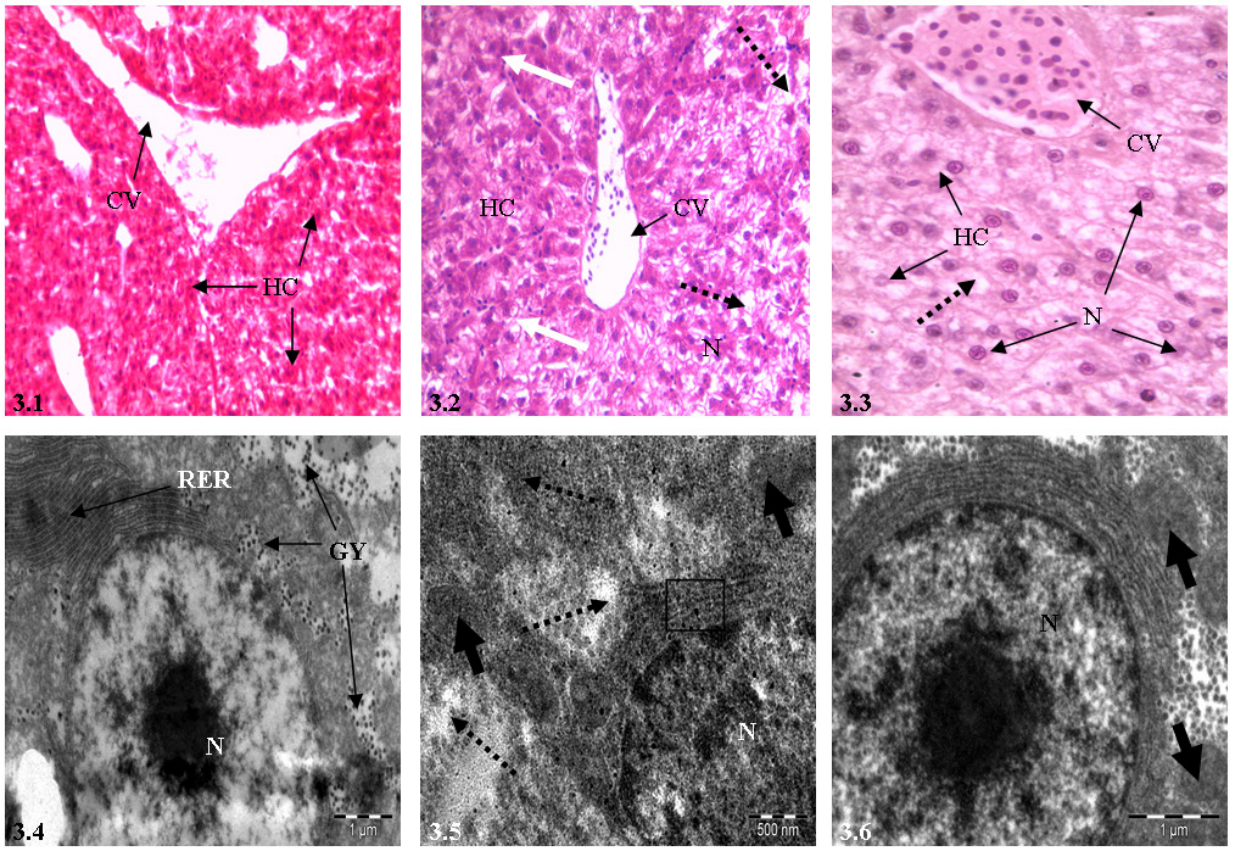

Figure 3. Histopathological photomicrographs of liver of *H. fossilis* under control condition (C), Almix treated laboratory condition (AL), Almix treated field condition (AF). **3.1** Showing normal appearance of hepatocytes (HC), compact arrangement around central vein (CV) with distinct nucleus (N) under light microscopy (C×400). **3.2** Showing hypertrophied and pyknotic nuclei (white arrow), vacuolation in hepatocytes (broken arrow) under light microscopy (AL×400). **3.3** Light microscopy showing vacuolation in HC (broken arrow) (AF×1000). **3.4** Normal appearance of hepatocytes with large number of mitochondria (M), rough endoplasmic reticulum (RER) and glycogen droplets (GY) under transmission electron microscopy (C×4000). **3.5** Hepatocytes with degenerated RER (square) and mitochondria (bold arrow) and vacuolation in cytoplasm (broken arrow) under transmission electron microscopy (AL×8000). **3.6** Under transmission electron microscopy hepatocytes showing almost normal nucleus (N) and vast amount of glycogen droplets (GY) with dilated mitochondria (bold arrow) (AF×6300).

study but damage was comparatively less compared with laboratory condition (Figure 4.6).

Discussion

The present study is a maiden attempt to report Almix toxicosis with regard to histological and ultrastructural observations in *H. fossilis*, although Senapati *et al.* (2012, 2013) reported histopathological alterations in stomach and intestine of *A. testudineus* exposed to Almix herbicide under laboratory condition.

Fish are considered a sentinel organism for ecotoxicological studies and play a significant role in evaluating the risk in aquatic ecosystem (Lakra & Nagpure, 2009). Simultaneously, cellular biomarkers including histological and ultrastructural study in tissues of pollutant-induced organism represent an intermediate level of biological organization between lower-level biochemical effects and higher-level population effects (Adams *et al.*, 2001). This will ultimately provide a better evaluation of the organism's health than a single biochemical response (Triebskorn *et al.*, 1997). They are now widely used as efficient biomarker of water quality, cellular state and mode of action of the xenobiotic contaminants under microscopic study as well as reflecting the overall health of the entire population in the ecosystem (Schwaiger *et al.*, 1997; Kammenga *et al.*, 2000).

The results of the present study showed that Almix intoxication caused serious pathological alterations in gill, liver and kidney of *H. fossilis* under laboratory study and less in field condition. Gills are considered the most vulnerable organ (Dutta *et al.*, 1996) because they are under direct contact with the surrounding contaminant medium and consequently are the first door of entrance for these contaminants (Machado & Fanta, 2003). Detailed description of each pathological lesion through light and electron microscopic observation in gills of *H. fossilis* helps to evaluate the degree of damage and potential consequences.

Hypertrophy and detachment of chloride cells from gill epithelium are the most profound alterations due

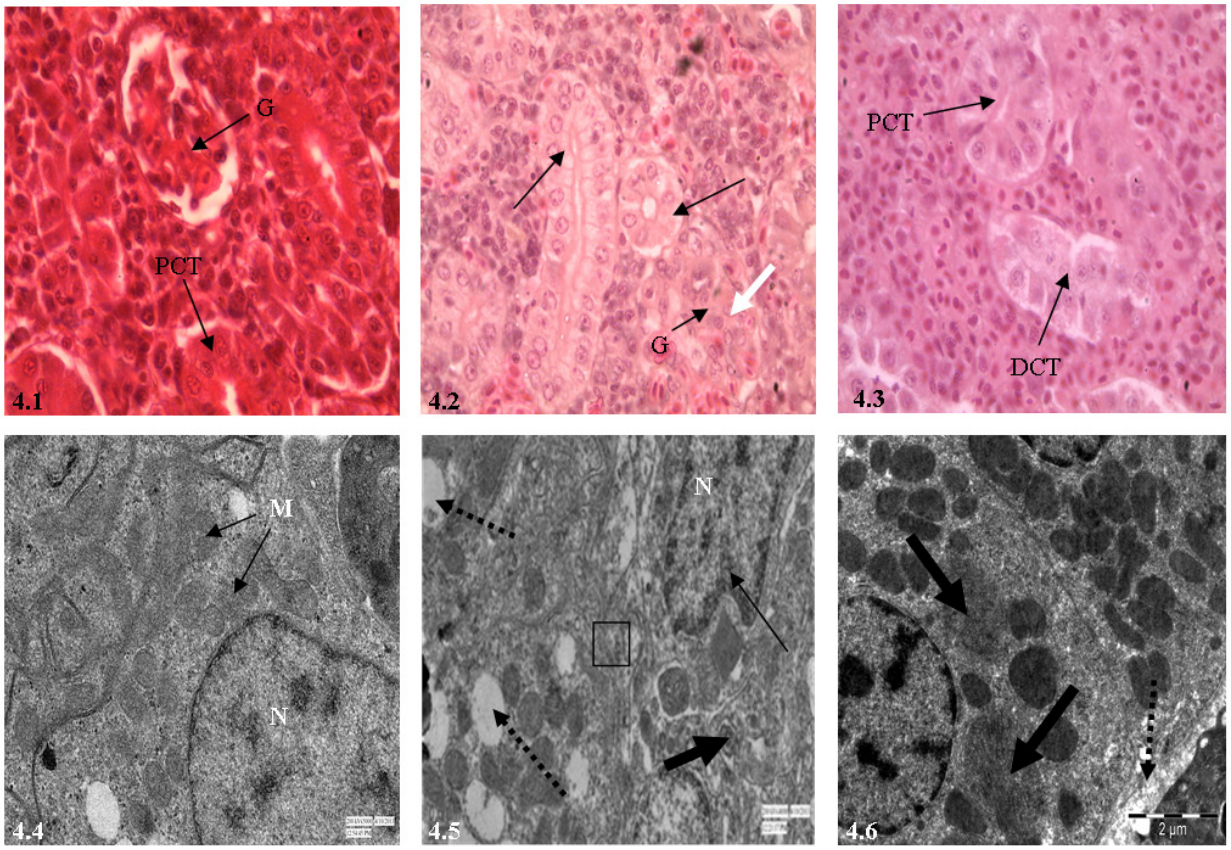

Figure 4. Histopathological photomicrographs of kidney of *H. fossilis* under control condition (C), Almix treated laboratory condition (AL), Almix treated field condition (AF). **4.1** Normal proximal convoluted tubule (PCT), distal convoluted tubule (DCT), Bowman's capsule and glomerulus (G) under light microscopy (C×1000). **4.2** Degeneration of PCT and DCT (arrow) and fragmentation of glomerulus (white arrow) under light microscopy (AL×1000). **4.3** Light microscopy showing normal structure of PCT and DCT (AF×1000). **4.4** Normal appearance of kidney with electron dense mitochondria (M), nucleus (N) and rough endoplasmic reticulum (RER) with abundant vesicular structures (V) under transmission electron microscopy (C×5000). **4.5** Degenerative mitochondria (bold arrow), deformed nucleus (arrow), dilated, fragmented and vesiculated of RER (square) and severe vacuolation (broken arrow) under transmission electron microscopy (AL×2550). **4.6** Showing dilated mitochondria (bold arrow) and lower amount of vacuolation (broken arrow) under transmission electron microscopy (AF×3200).

to Almix exposure. Similarly, Mallatt (1985) reported detachment of the gill epithelium as serious alteration. Fusion of secondary lamellae as observed in gills of *H. fossilis* indicated reduction of total respiratory area, which ultimately reduces oxygen uptake capacity (Karan *et al.,* 1998). Similar finding was also reported in *Lepomis macrochirus* after malathion exposure by Richmonds & Dutta (1989). Damage in chloride cells was also prominent under laboratory conditions. Similar results were also reported by van der Heuvel *et al.* (2000) along with loss of structural integrity of secondary lamellae and accumulation of blood cells. Stunted growth of gill lamellae is another most conspicuous change observed under the present study after Almix exposure. Scanning electron microscopic study showed fragmentation of microridges, swelling of microridges and loss of normal microridge array under laboratory condition. Light microscopic evidence as observed under the present study could be correlated with strands of secreted mucus cells over the gill surface through SEM study. Similar results of hyperplasia, loss of microridge and excess mucus secretion was also reported by Pfeiffe *et al.* (1997) in gill of juvenile goldfish, *Carassius auratus,* induced by 1-naphthyl-N-methylcarbamate (carbaryl). Excess mucus secretion as observed under field study indicated compensatory mechanism as well as defensive mechanism by the fish species against herbicidal exposure. In the present study, transmission electron micrograph showed double layered nucleus, dilated mitochondria and endoplasmic reticulum, and abnormal vacuolation in the chloride cells of gill epithelium after Almix exposure. Similar observation of chloride cell damage was also reported by Schwaiger *et al.* (2004) and indicated that these alterations might interfere with normal respiratory functions and general fish health status. Additionally, chloride cell damage might increase blood flow inside the lamellae, dilatation of marginal channel, and blood congestion or even aneurism (Rostey-Rodriguez *et al.,* 2002; Camargo & Martinez, 2007). Vacuolations in gill epithelium might impede gas exchange capacity as well as indication of swelling of mitochondria and rough endoplasmic reticulum (Ultsch *et al.,* 1980; Pawert *et al.,*

1998). Mitochondrial damage observed under the present study was also reported by Perry & Laurent (1989) and Goss *et al.* (1995) in their study after exposure to different contaminants. Although significant ultrastructural differences were observed in gill epithelium both under laboratory and field study, pathological responses were more pronounced in laboratory conditions than in field study. These alterations in gill morphology could lead to functional anomalies as well as interfere with the fundamental process such as maintenance of osmoregulation and antioxidant defence mechanism of gill epithelium (Pandey *et al.,* 2008).

Hypertrophied and pyknotic nuclei in hepatocytes of *H. fossilis* are the most pronounced lesions observed in the present study. Similar observations along with nuclear hypertrophy and cellular atrophy were reported in liver of *Cyprinus carpio* after chlorpyrifos exposure by Pal *et al.* (2012). Additionally, vacuolation in cytoplasm, infiltration of leukocytes and pyknotic nuclei were also reported by Jiraungkoorskul *et al.* (2002) in liver of *Oreochromis niloticus* after Roundup exposure. In the present study, vacuolization in hepatocytes indicated an imbalance between the rate of synthesis of substances in parenchymal cells and the rate of their release into systemic circulation. Additionally, enhanced glycolytic activity as compensatory response imposed by enhanced metabolic activity or reduction of carbohydrate absorption by intestinal part were reported (Hanke *et al.,* 1983; Gluth & Hanke, 1985; Braunbeck & Appelbaum, 1999). Disarrangement of hepatic cord is another most important hepatic lesion observed under the present study. Cytoplasmic vacuolation in hepatocytes observed under TEM study was also reported by Li *et al.* (2001). Damage in rough ER is the common response to herbicide exposure. Braunbeck & Völkl (1993) and Au *et al.* (1999) correlated damage in rough ER with higher biotransformation capacity of hepatocytes, while Ghadially (1988) demonstrated dilation of ER cisternae as enhanced storage of proteins due to reduced secretory activity. Similar findings were reported in rainbow trout after exposure to endosulfan and disulfoton (Arnold *et al.,* 1995), and in demersal fish following intraperitoneal injection of benzo(a)pyrene (Au *et al.,* 1999). Mitochondrial degeneration observed under the present study indicated impaired hepatocyte oxidative capability due to inhibition of respiratory chain enzymes function through oxidation of ATP molecule during phospholipid metabolism and fatty acid synthesis. Marked ultrastructural changes including swollen mitochondria have already been reported in liver of catfish exposed to methyl parathion by Tripathi & Shukla (1990). Reduced glycogen content is another most important cytological change associated with herbicide exposure. Cytopathological responses observed under the present investigation were more pronounced in laboratory condition compared with field study as fish are in natural condition and quickly adapt under herbicide-induced aquatic environment.

In kidney, light microscopic observation showed degenerative changes in PCT and DCT, and damage in glomerulus. The results of the present study were also in agreement with the findings of Fischer-Scherl *et al.* (1991) and Nesković *et al.* (1993). Jiraungkoorskul *et al.* (2002) in their study also reported damage in PCT, dilation of Bowman's capsule along with accumulation of hyaline droplets in epithelial cells of renal tubule of *Oreochromis niloticus* after Roundup exposure. Additionally, alterations observed under the present investigation could be correlated with disruption of several biochemical and physiological pathways including endocrine disruption (Mekkawy *et al.,* 2011; Sayed *et al.,* 2012). Degenerative changes in mitochondria and deformed nucleus observed under the present study indicated impaired metabolic activity, in particular enzyme activity. Cytoplasmic vacuolation observed under both conditions have also been reported in gold fish kidney after hexachlorobutadiene exposure by Reimschüssel *et al.* (1989). Similarly, Fischer-Scherl *et al.* (1991) reported degeneration and vacuolation in epithelial cells of kidney after lethal and sub-lethal atrazine exposure. Additionally, Fischer-Scherl *et al.* (1991) also reported dilation, fragmentation and vesiculation of RER in kidney of rainbow trout. Moreover, Bucher & Hofer (1993) reported accumulation of hyaline droplets in kidney. Abundance of large number of mitochondria, and lower amount of vacuolation observed under field condition indicated that fish are under stress. Additionally, protection against the stress-imposed conditions was observed; however, severity of damage is more pronounced under laboratory conditions than field study due to dilution capability of the natural environment.

Conclusion

In conclusion, cytopathological responses observed due to Almix intoxication indicated that laboratory study displayed higher impacts than did field study. Therefore, marked histological and ultrastructural alterations observed in gill, liver and kidney of *H. fossilis* could be considered as biomarkers of herbicidal toxicosis and might be helpful to characterize the health status of the entire aquatic ecosystem.

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