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Histopathological Alterations in Muscle, Liver and Gill Tissues of Zebra Fish Danio Rerio due to Environmentally Relevant Concentrations of Organochlo Rine Pesticides (OCPs) and Heavy Metals

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ABSTRACT: The study monitored the toxicity of environmentally relevant concentration of pesticides and metals in zebra fish Danio rerio. Zebra fish were exposed to environmentally relevant concentration of pesticides and metals for a period of 14 days. The individual and the combined toxicity of pesticides and metals were evaluated. The pesticides and metals treated groups showed histopathological alterations in the muscle, liver and gill tissues. Significant alterations were seen in liver and gill compared to muscle tissue. Among groups, the changes in group III (pesticides treated) were severe followed by group II (metals treated) and then the group IV (metals and pesticides treated, combined toxicity). Splitting of muscle fibers was observed in muscle tissue of fish treated with pesticides (group III). In the liver tissues severe damages such as dilation and congestion of blood sinusoids (group IV), cytoplasmic vaculoation in both group II & III was observed. Pronounced hyperplasia and necrosis in gill of fish treated with pesticides (group III). In conclusion the evidence of pathological alterations in gills and livers of zebra fish Danio rerio appeared to be a useful bio-marker to assess the impact of combined toxicity of pesticides and metals.

Key words: Zebra fish, Pesticides, Metals, Muscle, Liver, Gill

INTRODUCTION

Water pollution has now become an international issue. As water is scarce and its demand is likely to intensify, it mandates more attention. Pollution of water is mainly due to contamination with hazardous chemicals from agricultural runoff and wastewater from house hold and industries. One of the major chemicals from agricultural runoff is pesticides which play important role in increasing agricultural productivity through controlling pest. But on the other hand, they cause severe damage to the non target organisms both in terrestrial and aquatic environment (Magar & Shaikh, 2013). Pesticides pose potential health hazard not only to livestock and wild life but also to fish, birds, mammals and even human beings. Subsequent to pesticides are the heavy metals from both natural and anthropogenic sources are continually released into aquatic ecosystems, which could be a serious threat because of the toxicity, long persistence, bioaccumulation, and biomagnification of metals in the food chain (Langston *et al.,* 1999; Pandey *et al.,* 2003). Aquatic organisms, including fish, accumulate pollutants directly from contaminated water and indirectly via food chain (Sasaki *et al.,* 1997). So, it is necessary to study the histopathology of fish and other aquatic organisms in detail. Histological investigation appears to be a very sensitive parameter and is crucial in determining cellular changes that may occur in target organs, such as the gills, liver and gonads (Dutta, 1996). The organ most associated with the detoxification and biotransformation process is the liver, it is also one of the organs most affected by contaminants in the water (Camargo & Martinez, 2007). This in turn provides toxicologists with a definitive site for the investigation of the hepatotoxic potential of a chemical. Gills play an important role in the capture, accumulation and transfer of metal toward internal compartments via blood transport. The gills are the site of respiration and transport system involved in osmoregulation, and it has been confirmed that accumulation of metal ions within them may have an effect on their functions (Fernandes & Mazon, 2003).

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They are vulnerable to pollutants in water because of their large surface area and remain in close contact with the external environment and particularly sensitive to changes in the quality of the water. For this reason, they are considered to be appropriate indicators of water pollution (Alazemi *et al.,* 1996). Muscle is the tissue composed of elongated muscle fibers and muscle cells are held together by connective tissues. Histopathological study provides factual data concerning tissue changes prior to external manifestation.

It is generally assumed that histopathological biomarkers are valuable indicators of the general health of fish and mirror the effects of exposure to a variety of anthropogenic pollutants (Hinton *et al.,* 1992). These histopathological biomarkers are closely related to other biomarkers of stress since many pollutants have to undergo metabolic activation in order to be able to provoke cellular change in the affected organism. Pesticides exposure can ultimately affect structural and functional levels of the exposed animals due to a combination of direct and indirect effects. For example, the mechanism of action of several xenobiotics could initiate the formation of a specific enzyme that causes intoxication and death, at a cellular level, whereas this manifests as necrosis (Velkova *et al.,* 2009). Numbers of studies have reported several histopathological alterations in different tissues of fish exposed to individual chemicals. Hence, the present investigation aims at studying the histopathology of combined and individual toxicity of organochlorine pesticides and heavy metals in the muscle, liver and gill of Zebra fish.

MATERIALS & METHODS

Zebrafish (D. rerio) were purchased from a commercial fish supplier and acclimatized for one week in the laboratory conditions in 50 L glass tanks before the start of the experiments. The animals were treated with 16 hours of light and 8 hours of dark photoperiod under controlled conditions of temperature (25 - 27 °C). The acclimatized fish were divided into four different experiment groups. During the acclimatization period, the fish were fed twice a day with commercial fish food. Static renewal method was employed and the chemical concentrations supplied daily to maintain a constant toxic media. The entire water was replenished daily for maintaining the oxygen level in the water. The experimental groups were exposed for a period of 14 days. All groups were matched for body weight, food intake and food efficiency as determined during one week baseline period before group formation.

Four different groups (each 20 animals of mixed sex) were used for the experiment: control with acetone (Group I), Group II represents the mixture of metals such as Ni in the form of Nickel chloride (0.05 mg/L), Cd in the form of Cadmium chloride (0.02 mg/L) and Pb as Lead nitrate (0.32 mg/L). Group III comprises the mixture of six pesticides Viz., α-HCH (47 ng/L), o,p'-DDE (46.4 ng/L), aldrin (36 ng/L), dieldrin (16 ng/L), heptachlor (259 ng/L) and mirex (17.5 µg/L). Group IV includes mixture of both metals and pesticides. The concentration of pesticides and metals used for the toxicity study reflect those detected in the surface water of River Cauvery and Veeranam Lake (Bhuvaneshwari, 2011).

HPLC grade acetone was used for preparation of pesticide mixture (Qualigens Pvt. Ltd, India) and for metals deionized ultrapure water (Elga water purification system) was used. Nickel Chloride, Lead Nitrate and Cadmium Chloride were purchased from Rankem Pvt. Ltd, India. The pesticides α-Hexachlorocyclohexane (α-HCH), o,p α-Dichlorodiphenyldichloroethylene (o,p'-DDE), aldrin (36 ng/L), dieldrin (16 ng/L), heptachlor (259 ng/L) and mirex (17.5 µg/L) were procured from AccuStandard Inc, USA.

At the end of the exposure period of 14 days five fishes from each group were randomly collected and blotted dry with soft absorbent paper, body length (3.15± 0.05cm) and weight (1.94 ± 0.04g) was measured. Each fish was dissected to collect muscles, gills and livers. These organs were weighed individually, washed with ultrapure water and drained on a good quality filter paper. For histological analysis gills and liver tissues were fixed in 10% formalin. Then tissue was washed and dehydrated in descending grades of isopropanol and cleared in xylene. The tissue was then embedded in molten paraffin wax. Sections were cut at 5 µm thickness using semi automatic rotary microtome (Microtome India) and stained with hematoxylin and eosin. The sections were then viewed under light microscope (Olympus Medical Systems India Pvt. Ltd.) at 400x for histopathological changes. The remaining tissues of gills, liver and muscles were transferred into marked sterilized polythene bags for their storage in a freezer at -20°C until analysed.

RESULTS & DISCUSSION

Fish are extensively used to assess the health of aquatic ecosystems and their physiological changes serve as biomarkers to monitor the environmental pollution (Kock *et al.,* 1996). In this study, the liver and gill was the prime target for the evaluation of metal accumulation in zebra fish Danio rerio as compared to the muscles. The present study revealed that zebra fish treated with pesticides and metals manifest histopathological changes in muscles, liver and gills.

The muscle of the control group (I) showed normal structure with nucleus (N) at the periphery of fibers (Fig. 1A). Degeneration (D) in muscle bundles was spot-

ted in the muscle tissue of zebra fish treated with metals (group II: Fig. 1B). Splitting (S) of muscle fibers was observed in muscle tissue of fish treated with pesticides (group III: Fig. 1C). Fig.1D showed vacuolar degeneration (VD) in muscle fibers in pesticide and metal treated group (IV). The histopathological alterations in the muscles of zebra fish were in agreement with those observed by many investigators who have studied the effects of different pollutant on fish muscles (Nour & Amer, 1995; Das & Mukherjee, 2000; Mohamed & Gad, 2008; Mohamed, 2009). Splitting of muscle fibers and vacuolar degeneration in muscle bundles were considered to be significant histopathological changes. Initial stimulus of pesticides and metals can induce hyperactivity and excitability in animals. All these changes were clearly evident as clinical signs at the initial stage of the experiment and were subsequently reflected through histopathological changes (Das,1998). Mohamed (2009) reported several histological alterations in the muscles of T. Zillii and S. vulgaris .

from Lake Qarun during summer and winter and the pathological findings included degeneration in muscle bundles with aggregations of inflammatory cells between them and focal areas of necrosis. Also, vacuolar degeneration in muscle bundles and atrophy that are observed in the muscle bundles might be due to the various concentrations of mixture of heavy metals. Miranda et al. (2008) reported the physiological disturbances and morphological damages in the muscle tissue of freshwater fish Hoplias alabaricus collected from Ponta Lake in southern Brazil caused due to bioaccumulation of chlorinated pesticides and PCBs. Mohamed (2009) observed the degeneration of muscle

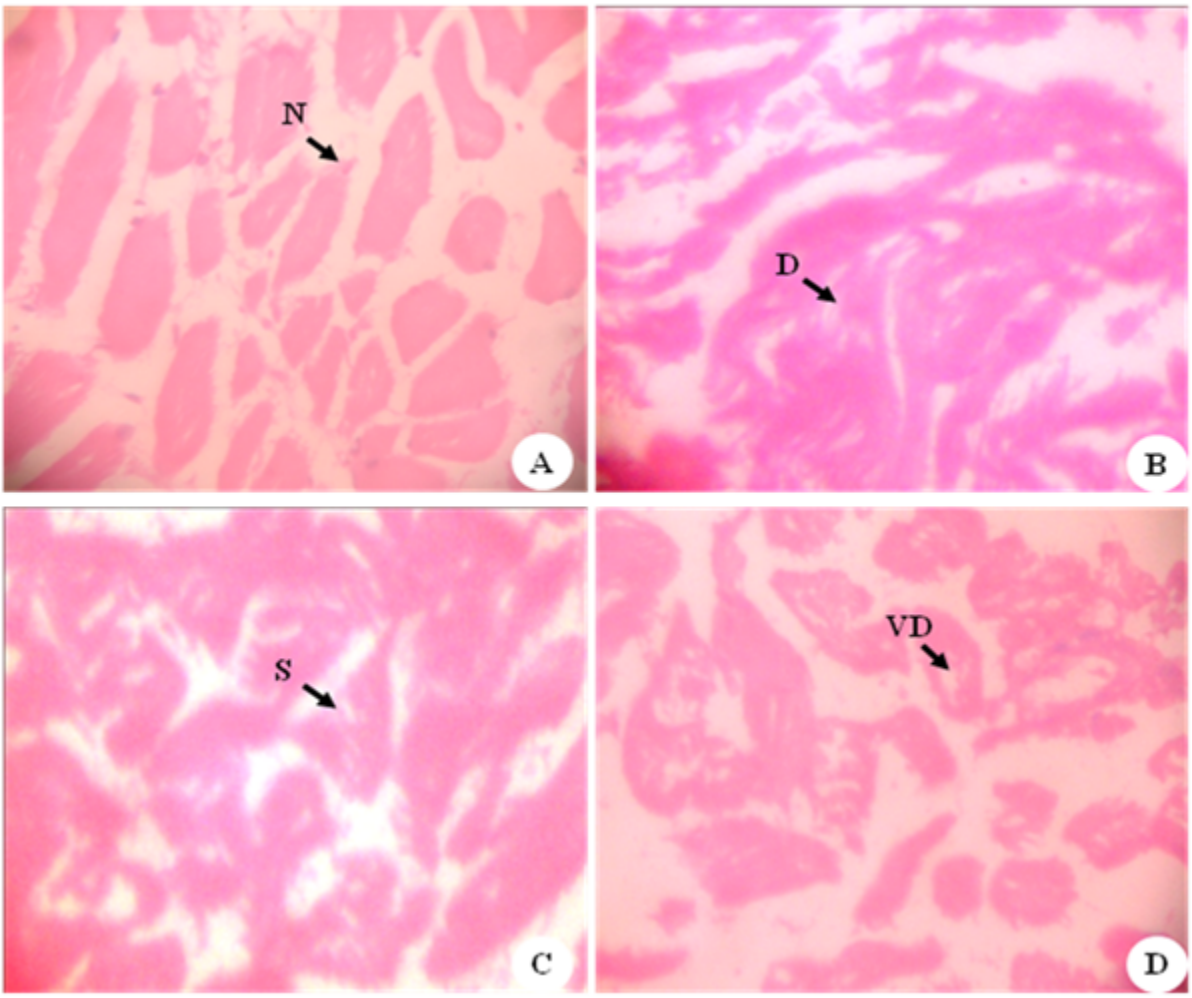

Fig.1. Photomicrograph of muscle tissue section of zebra fish Danio rerio (Stain: H&E; Magnification: ×400); control group (A): normal structure with nucleus (N) at the periphery of fibers; treated with metals (B): Degeneration (D) in muscle bundles; treated with pesticides (C): Splitting (S) of muscle fibers; treated with both pesticides and metals (D): vacuolar degeneration (VD)

bundles with aggregation of inflammatory cells between them and focal areas of necrosis.

In the present investigation, liver of a control fish (group I) exhibited a normal architecture with hepatocytes presenting a homogenous cytoplasm and a large spherical nucleus (N) (Fig. 2A). Hepatocytes were located among blood capillaries called sinusoids (SS) forming cord-like structures. The liver of metal treated group (II) exhibited focal area of necrosis (NC), degenerative nuclei (DN) and cytoplasmic vacuolation (CV) (Fig.2B). Anomalies such as irregular shaped hepatocytes and nucleus in a lateral position, close to the cell membrane were also observed.

The liver of zebra fish treated with pesticides (group III) also showed necrosis (NC), cytoplasmic vacuolation (CV) and vascular dilation (D) (Fig. 2C). The vascular dilation (D) may be also responsible for the cellular degeneration and necrosis in the liver (Mohamed, 2001). The combined pesticide and metal treated group (IV) showed less damage compared to metal and pesticide treated groups individually. Only congestion and dilation of sinusoids (CSS and DSS; Fig. 2D) were observed in group (IV). According to Olurin et al. (2006) when the liver is damaged excessive amounts of blood flows into the liver blocking the sinusoids. Thus the blood flow from the hepatic artery

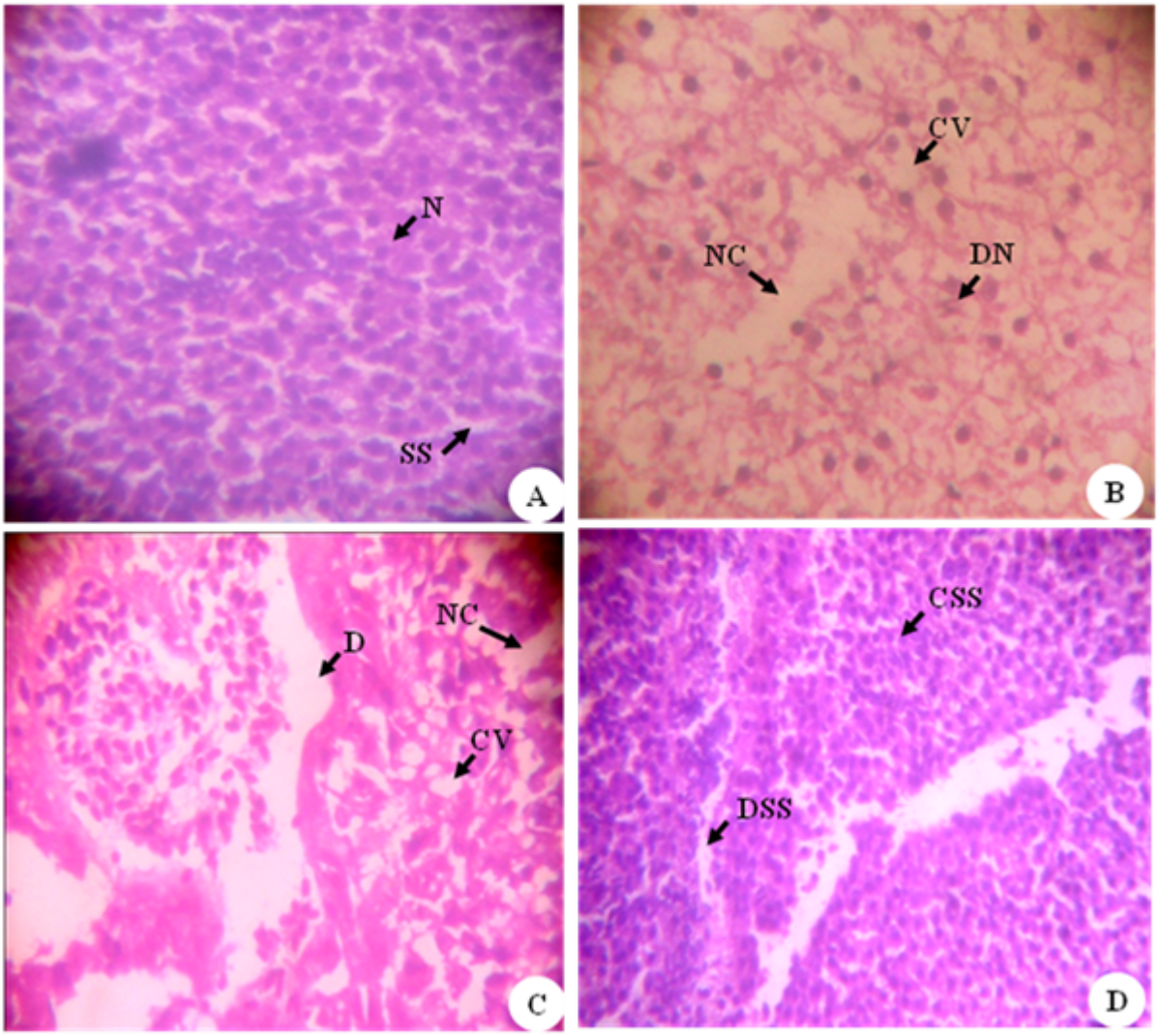

Fig.2. Photomicrograph of liver section of zebra fish Danio rerio (Stain: H&E; Magnification: ×400); control group (A): homogenous cytoplasm and a large spherical nucleus (N) and blood capillaries called sinusoids (SS); treated with metals (B): focal area of necrosis (NC), degenerative nuclei (DN) and cytoplasmic vacuolation (CV); treated with pesticides (C): necrosis (NC), cytoplasmic vacuolation (CV) and vascular dilation (D); exposed to both pesticides and metals group (D): congestion and dilation of sinusoids (CSS and DSS)

and veins into the central vein, and the sinusoids are dialated in order to facilitate this blood flow. The vacuolization of hepatocytes might indicate an imbalance between the rate of synthesis of substances in the parenchymal cells and the rate of their release into the circulation system. Also, oxygen deficiency as a result of gill degeneration is the most common cause of the cellular degeneration in the liver (Gingerich, 1982). The present results are in agreement with those observed by many authors who have studied the effects of different pollutants on fish liver (Mohamed, 2001; Ptashynshki *et al.*, 2002; Fanta *et al.*, 2003; Moneim *et al.*, 2008; Mohamed, 2009). Morphological and histopathological alterations related to pesticide presence in the liver of fish have been studied, showing that these toxic compounds cause severe damage to the liver cells (Ahmad & Srivastava, 1985; Dutta *et al.*, 1993; Ortiz *et al.*, 2002). Hepatocytes are the most abundant cell types within the liver and perform most of the liver's essential functions such as the conversion of glucose to glycogen, regulation of lipids and deamination of amino acids (Wright *et al.*, 2004). Structural damage of hepatocytes in response to xenobiotics such as pesticides can result in the impairment of liver function (Hopwood *et al.*, 2004).

The gill is made up of filaments of primary lamellae arranged in double rows. Secondary lamellae arise from these filaments. The secondary lamellae are lined by a squamous epithelium. Control gill tissues (group I) con-

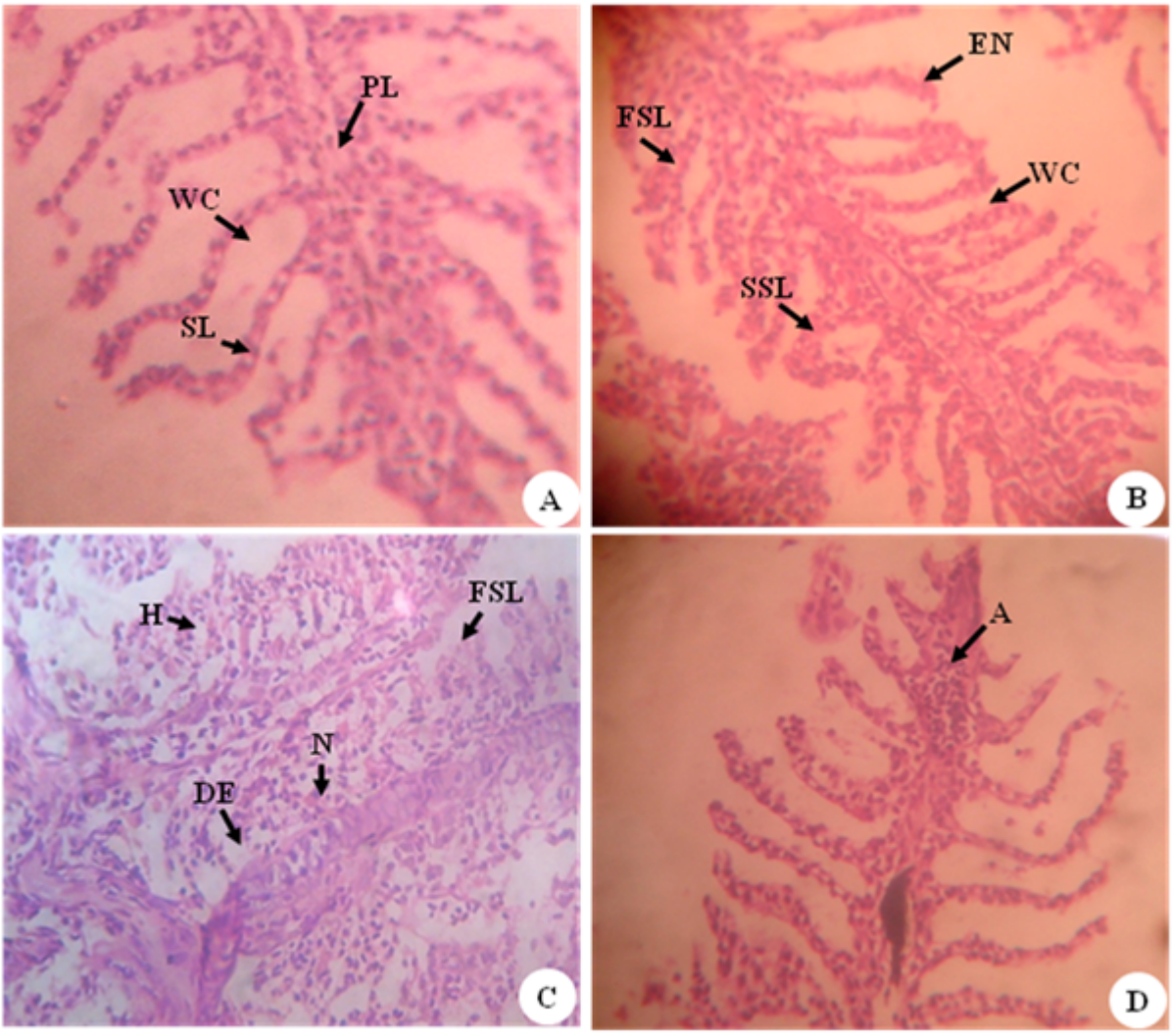

Fig.3. Photomicrograph of gill section of zebra fish Danio rerio (Stain: H&E; Magnification: ×400); control group (A): primary lamellae (PL) and secondary lamellae (SL) and wide water channel (WC); treated with metals (B): fusion of secondary lamellae (FSL), shortening of secondary lamellae (SSL), epithelial necrosis (EN) and narrowed water channels (WC); treated with pesticides (C): hyperplasia (H), degenerative epithelium (DE), focal area of necrosis (N), fusion of secondary lamellae (FSL); exposed to both pesticides and metals (D): showing aneurysm (A) in the primary lamellae

sisted of a primary lamellae (PL) and secondary lamellae (SL) and wide water channel (WC). The secondary lamellae composed of a single layer of epithelial cells (Fig. 3A). In the metal treated groups (II) (Fig. 3B) fusion of secondary lamellae (FSL), shortening of secondary lamellae (SSL), epithelial necrosis (EN) and narrowed water channels (WC) were observed. Similar histopathological changes were observed in the Oreochromis niloticus and Lates niloticus from Lake Nasser, Egypt contaminated with metals (Younis et al., 2013). The histological changes such as fusion of secondary lamellae and narrowed water channels in the gills of Danio rerio in the meal treated groups of this study may be an indication of their either reaction to toxicant such as metal intake or adaptation to prevent the pollutant entry through the gill surface. Arellano et al. (2000) reported the fusion of adjacent lamellae after exposure to heavy metals, such as cadmium and copper which is in line with the present findings.

The alterations in the pesticide treated group (III) (Fig. 3C) were hyperplasia (H), degenerative epithelium (DE) and focal area of necrosis (N) in the epithelium of gill filaments and secondary lamellae, and oedema in secondary lamellae accompanied with separation of their epithelium from the lamellar supporting cells. In addition to that thickening of primary lamellae and fusion of secondary lamallae were also observed. Jayachandran & Pugazhendy (2009) reported similar alteration in the gill of Labeo rohita (Hamilton) fingerlings exposed to Atrazine. Similar effects in the gills of insecticide exposed fish were reported by Elezaby et al. (2001). Banee et al. (2013) observed dilatation of blood capillaries, hyperplasia of the epithelial lining of the secondary lamellae, necrosis and shortening of the secondary lamella, abnormal raising or swelling of the epithelium, as well as fusion of the secondary lamellae and excessive mucus secretion in fish exposed to diazinon. Group IV (pesticides and metals treated) showed aneurysms (A) in the primary lamellae (Fig. 3D). Filament cell proliferation and lamellar cell hypertrophy reduce the interlamellar space and may cause a complete lamellar fusion reducing the total surface area for gas exchange (Nowak, 1992). The injury to the gill epithelium is a common response observed in fish exposed to a variety of contaminants (Jabeen & Chaudhry, 2013). The changes in appearance of the secondary lamellae result from the collapse of the pillar cell system and breakdown of vascular integrity with release of large quantities of blood that push the lamellar epithelium outward (Alazemi *et al.*, 1996). Epithelial oedema increases distance between the contaminant and the bloodstream, while secondary lamellae fusion significantly reduces the gill surface and thus decreases the contact between the pollutant and gill epithelium.

CONCLUSIONS

It must be emphasized that histopathology studies are able to evaluate the early effects and the responses to acute exposure to chemical stressors. The muscle tissues of treated groups exhibited changes such as splitting of muscle fibers and necrosis. In the liver tissues severe damages were observed such as dilation and congestion of blood sinusoids, cytoplasmic vacuolation, necrosis etc. The most severe was vascular dilation caused by pesticides (group III) that may result in cellular degeneration, necrosis and ultimately leads cell death. In gill, the higher damage was found in group III (pesticide treated group) with pronounced hyperplasia and necrosis. From the present investigation it can be concluded that individual pollutant has higher competence to cause severe effect on organism and their ecosystem and in actual fact in the environment merely combination of pollutants exists. Plausibly, fish histopathology imparts valuable contribution in the surveillance of aquatic ecosystem and act as an important part of environmental management process.

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