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The Significant of Poisson Load on Tissue Alteration with Reference to Histopathological Changes in Induced *Oreochromis niloticus*

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Abstract: The correlation between endosulfan accumulation and tissue damages was examined on induced *Oreochromis niloticus*. The organization for economic cooperation and development No. 407 recommended static renewal bioassay was employed during the test. The test fish were exposed to sub-lethal concentrations separately for a period of 7, 14, 21 and 28 days. At the end, of each exposure period the liver tissues were isolated and analyzed using high performance liquid chromatography for bioaccumulation study while alterations observed in exposed fish were determined in the laboratory by light microscopy using standard technique of haematoxylin and eosin staining. No mortalities occurred in any group during the test. The severity of the histological alterations observed in the liver of the treated fish, reflects the concentrations of the toxicant, level of depuration and exposure durations. An indication that that the regulating mechanisms of the liver has been overwhelmed resulting in the structural damages. The findings showed that the body could be liberated of contaminants after longer exposure period, however the effects on the body's tissues remain lethal.

Key words: *Oreochromis niloticus*, liver, histopathology, endosulfan, Nigeria

INTRODUCTION

The mode of action for a pesticide is determined by its chemical structure. Chlorinated pesticides have low water solubility but a high affinity for hydrocarbons and fats and a strong tendency to attach to soil particles as their use expanded (Park *et al*., 2004). Endosulfan is an organochlorine and highly toxic pesticide in EPA toxicity. The solubility of endosulfan is 0.3 mg L^{-1} with a half-life of 50 days in soil and 5 weeks in water but β isomer has longer half-life, i.e., 150 days under neutral conditions. Though endosulfan is banned in many countries but it is extensively used in Africa because of its economical value (Ezemonye *et al*., 2010). As a result of its incessant usage and potential transport, endosulfan contamination is frequently found in the environment at considerable distances from the point of its original applications (Palaniappan and Karthikeyan, 2009).

Most of bioaccumulation studies in fish, both experimental and theoretical, generally follow a whole body and tissues distribution (Arnot and Gobas, 2004) without considering the effects of elimination on tissues such as liver, kidney, gills and muscles. Information on concentrations in the edible part of the fish and effects can be used for the estimation of bioaccumulation and histopathological effects of chemicals in the human food chain. To date, it is unclear to what extent only organ specifics bioaccumulation investigation estimate the overall welfare of the fish.

The goal of the present study is to quantify the concentration of endosulfan in the liver and assess the effects of depuration on liver's tissues of *O. niloticus*. The was chosen as it is one of the main species produced in aquaculture, high growth rates, efficiency in adapting to diverse diets, great resistance to diseases and to handling practices and good tolerance to a wide variety of environmental conditions (Fontainhas-Fernandes, 1998).

During fish growth, adaptation to a new environment and seasonal variation may induce internal redistribution of fish lipids and organochlorine contaminants (Debruyn *et al*., 2004). When concerning organochlorines toxicity, specific organs (liver and thyroid) are more sensitive (Safe, 2003). If concentrations have a higher increase in liver than the predicted this would result in a higher toxic effect of the compound. Besides, the liver was chosen for this investigation in view of the fact of its roles in detoxification, storage processes and redistribution and as an active site of pathological effects induced by contaminants hence, a better indicator of water pollution than other organs in fish (Feeley and Jordan, 1998).

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MATERIALS AND METHODS

Collection and acclimatization of the test organisms: Total 120 juveniles of *O. niloticus* of mean weight (320.40±1.2 g) and length (50.00±2.13 cm) from fresh water environment were collected from the Department of Fisheries, Faculty of Agriculture, University of Benin, Benin City, Nigeria. They were acclimatized to laboratory conditions in holding glass tanks containing unchlorinated tap water for 2 weeks before they were used for the experiments. The holding tanks were aerated with the help of air pump, cleaned and water renewed daily. Fish were fed on 30% protein pellets.

Experimental design: The LC_{50} values for endosulfan was determined by using OECD Guideline No. 203 for static-renewal test conditions (OECD, 1992) and was found to be 0.21 μg L^{-1}. The sub-lethal test was conducted under OECD test guideline 407 (OECD, 1995) with some modification. The physico-chemical parameters were measured according to American Public Health Association (APHA, 2005) and maintained at optimum level. Water temperature remained between 27.00±0.58 and 26.67±1.15°C; pH between 7.27±0.25 and 7.16±0.25 and the rate of dissolved oxygen was kept between 8.73±0.12 and 8.12±0.10 with the help of an air pump. Dechlorinated tap, tap water was used for preparing test solutions.

From the LC_{50} determination, lower concentrations of the pesticide were prepared for sub-lethal test. Total 5 fish per test concentration in three replicates were exposed to varying concentrations of endosulfan 0.00, 0.0025, 0.005, 0.0075 and 0.01 μg L^{-1} in water. The test fish were exposed to the above-mentioned sub-lethal concentrations separately for a period of 7, 14, 21 and 28 days. During these periods, the water was changed along with waste feed and fecal materials daily at 8 a.m. by a siphoning system which caused minimal disturbance to the fish. Daily the containers were refilled and redosed with the toxicant. Endosulfan analysis of water was carried out prior to replacement and was found to be with 98% of the required concentration. At the end of each exposure period the liver tissues were isolated and kept in a freezer (-20°C) prior to analysis. Pesticide residues were analyzed using high performance liquid chromatography (model CECIL 1010) while alterations observed in the liver of the exposed fish were determined in the laboratory by light microscopy using standard technique of haematoxylin and eosin staining. No mortalities occurred in any group during the test.

Pesticide residue analysis

Equipment: The glassware; cecil HPLC system comprised of CE 1200 high performance variable wavelength monitor and CEII00 liquid chromatography pump, UV detector with variable wavelength and stainless steel column (C18 reverse phase) packed with octasilica, vacuum pump and ultrasonic check.

Chemicals: Special chemicals, such as technical grade endosulfan (93.5% purity) and methanol (analytical grade) for High-Performance Liquid Chromatography (HPLC) were obtained from chemical service (West Chester, PA, USA), petroleum ether (analytical grade) was supplied by Sigma-Aldrich (USA). All other reagents were of chemical grade.

Extraction and analysis: Endosulfan was extracted using Solid Phase Extraction (SPE) technology (IST, 1995). About 50 g of the tissue was thoroughly macerated and homogenized in 150 mL of petroleum ether in a metallic blender at high speed until thoroughly mixed. About 100 g anhydrous sodium sulphate (Na_2SO_4) was added to combine with water and to disintegrate the sample. Petroleum ether supernatant was decanted in a glass fitted funnel with filter paper into a 500 mL flask fitted with a suction apparatus. The residue in the blender cup was re-extracted with two 100 mL portions of petroleum ether blended for 2 min each time, filtered and combined with the first extract. The combined extract equivalent to 20 g of fish tissue was passed through column (25×50 mm long) of anhydrous Na_2SO_4 and collected as petroleum ether extract. The above extract was concentrated on a rotary vacuum evaporation at steam bath temperature to obtain fat taken up for clean-up procedure.

Clean-up: About 15 mL of petroleum ether and 30 mL of acetonitrile was added to the extracted sample and transferred to a 125 mL separatory funnels (Bancroft and Cook, 1994). The mixture was shaken vigorously for 1 min, the layers were allowed to separate and the acetonitrile layer was drained into a beaker. This procedure was repeated thrice. The solution obtained was transferred to 1 L separatory funnel containing 650 mL water, 40 mL saturated NaCl solution and 100 mL petroleum ether. The extract in the separating funnel was mixed thoroughly for 45 sec. The layers were allowed to separate and the aqueous layer was drained into another 1 L separating funnel. About 100 mL petroleum ether was added to the second 1 L separating funnel and the mixture was shaken vigorously for 20 sec and layers allowed to separate. Aqueous layer was discarded and the petroleum ether layer was combined with the previous one in the first separatory funnel and washed with two 100 mL portions of water. The washing was discarded. The petroleum

ether layer was passed through column (25×50 mm long) of anhydrous sodium sulphate (Na_2SO_4). The dried petroleum extract was evaporated to 10 mL in a rotary vacuum evaporator.

Florisil column clean-up: A swab of washed cotton was placed at the base of the chromatographic column and carefully rinsed with petroleum benzene. The column (pre-wetted with 50 mL petroleum ether) was filled with 4 g activated florisil topped with anhydrous sodium sulphate to about 3 cm. The concentrated petroleum ether solution of sample extract rinsed with two 5 mL, portions of petroleum ether was transferred to the column.

The column was eluted with 200 mL HPLC methanol. The elute in the round bottomed flask was collected and concentrated under rotary vapour to complete dryness. About 2 mL HPLC methanol was added to round bottom flask and shifted in labeled glass stoppered vials for analysis in high performance liquid chromatography.

Preparation of standard stock solution: About 1 mg of the standard per gram of stock solution was prepared by adding 0.1 g of endosulfan standard into a 100 mL volumetric flask. About 5 mL of methanol were then added to the volumetric flask to dissolve the standard. Distilled water was then used to fill the flask to the 100 mL mark.

Activation of the HPLC system: The target wavelength for the analysis was determined using UV-visible equipment. A small quantity of the stock solution was diluted with methanol and its wavelength of 202 nm was determined by scanning. The instrument wavelength was then set at 202 nm with a sensitivity of 0.05 nm and a flow rate of 1 mL min^{-1}. The instrument was purged to remove air and charge the column. Purging was conducted using a washing solution of 30% methanol and 70% distilled water.

Degassing the mobile phase solution: Helium gas was bubbled into the solution to degas the mobile phase. The mobile phase was then injected into the instrument and allowed to run through the system for 20 min. The system was then separated following the procedures outlined in the instrument operating manual.

Determination of retention time for standard: The endosulfan standard was injected into the chromatograph to determine the retention time. A series of concentrations ranging from 0.025-100 mg L^{-1} where then injected. The resulting peak areas were plotted against concentrations to determine the linearity of detector response to the standard. Using this approach, the retention time for the endosulfan standard was 4.26 min.

Histopathological study: The tissues were fixed in 10% phosphate-buffered formalin for 48 h immediately after dissection. Thereafter, the tissues were dehydrated in Periodic Acid Schiff's reagent (PAS) following the method of Hughes and Perry in graded levels of 50, 70, 90 and 100% isopropanol for 3 days to allow paraffin wax to penetrate the tissue during embedding. The tissues were then embedded in malted wax. The tissues were sectioned into thin sections (5 μm) by a rotatory microtome, dehydrated and stained with harris haematoxylin-eosin (H&E) stain (15), using a microtone and each section was cleared by placing in warm water (38°C) where it was picked with clean slide and oven-dried at 58°C for 30 min to melt the wax. The slide containing sectioned materials/tissue was cleared using xylene and graded levels of 50, 70, 90, 95 and 100% alcohol for 2 min each. The section was stained in haematoxylin eosin for 10 min. The stained slide was observed under a light microscope at x100 magnification. Sections were examined and photographed using an Olympus BH2 microscope fitted with photographic attachment (Olympus C35 AD4), a camera (Olympus C40 AB-4) and an automatic light exposure unit (Olympus PM CS5P).

Statistical analysis: One-way analysis of variance SPSS (14.0 version), SPSS Inc, Chicago, USA, was employed to calculate the significance of the differences between control and experimental means. p-values of 0.05 or less were considered statistically significant (Fisher, 1950). Multiple line graphs were also used in this study for the pictorial representation of assessment endpoints.

RESULTS AND DISCUSSION

Concentrations and depuration: Bioaccumulation and depuration of endosulfan in the liver of *O. niloticus* after days of exposure to sub-lethal concentrations of endosulfan is shown in Fig. 1. At the lower concentrations of 0.0025 and 0.005 μg gdw^{-1}, the pesticide residues in the liver decreases with increase in

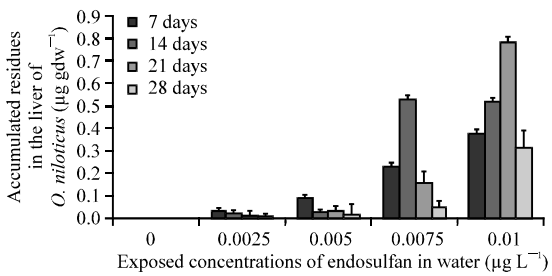

Fig. 1: Bioaccumulation and depuration of endosulfan in the liver of *O. niloticus* after days of exposure to sub-lethal concentrations of endosulfan

exposure duration. The maximum amount of bioaccumulation was observed on day 7 than other intervals. It may be due to rapid induction of the detoxifying enzymes at such concentrations in order to neutralize and depurate the pesticide. However when the concentration was increased to 0.0075 μg gdw^{-1}, accumulated residues was higher at day 14 than day 7 and drop abruptly with day 28 having the least residue. At the highest concentrations of 0.01 μg gdw^{-1}, there was spontaneous increased in the pesticide residue in the fish tissues with the exposure duration till day 21 and dropped on day 28. Bioaccumulation levels of endosulfan by the liver were significantly higher than the control ($p<0.05$) in all the concentrations and exposure durations. This indicated that the higher the concentrations, the difficult the depuration of the pesticide. The consequence is that the liver would be over stressed to secret more detoxifying enzyme to neutralize the effects of the toxicant in the process destroying the tissues. The organs in the visceral region (liver, intestine, etc.) carry out the primary activities related to absorption, distribution and elimination.

The rate of depuration is more in visceral part due to the enzymes induced or enhanced by the toxicant stress which decrease the lipid solubility of organic contaminant facilitating assimilation and excretion of the contaminant. The enzymes involved mainly are cytochromes P-450s, glutathione-s-transferases, rhodanese, sulfotransferase, etc. and other enzymes mainly belonging to mono-oxygenase system (Begum *et al*., 1994; Gill *et al*., 1988, 1991). Similar observation on concentration dependent depurations in visceral organs were observed when fish, *Labeo rohita* and *Saccobrachus fossilis* exposed to metasysntox, *Mugil cephalus* and *Mystus gulio* to endosulfan and *Clarias batrachus* to sub-lethal concentrations of dimethoate, respectively (Glenn and Gandolfi, 1986; Guzelian *et al*., 1980).

Histopathological abberation: Liver is the organ most associated with the detoxification and biotransformation of any foreign compounds entering the body and is highly exposed to a wide variety of exogenous and endogenous products (IST, 1995). This investigation revealed that the accumulation and elimination of endosulfan varies with the concentration of the toxicant and it correlated with the severity of pathological alteration of the liver. The histopathological investigation revealed that the higher the concentrations, the slower the depuration, the more the severity of the pathological changes of the liver's tissues. The pathological severities in the liver reflect its multifunctional role in detoxification process (Jimenez and Stegeman, 1990). The severity reported at higher concentrations showed that the regulating mechanisms of the liver has been overwhelmed with increased concentration of the toxicant that resulted in the structural damage. Similar pathological changes induced by pesticides and metals contaminants has been reported (OECD, 1992).

Histopathological studies of the liver revealed morphologic changes in the liver of exposed fish and were not observed in the control fish. Figure 2-5 showed the normal histological structures of the liver.

In the fish exposed to 0.0025 μg L^{-1} endosulfan, the cells of liver showed necrosis, dilation of sinusoids and light hypertrophy after 7 days exposure (Fig. 2b). Necrosis and dilation of sinusoids were still apparents togther with focal lymphotic infiltration after 14 days exposure (Fig. 2c). Pale stained in the portal area, dilation of sinusoids, focal necrosis and mild hypertrophy were observed after 21 days (Fig. 2d). Severe fatty degenerative changes of hepatocyte, severe dilation of sinusoids and a clear congestion position were observed after 28 days (Fig. 2e).

At the higher concentration (0.005 μg L^{-1}), degenerative changes of hepatocytes, mild hypertrophy and focal necrosis were observed after 7 days exposure (Fig. 3b). Examination of livers in the fish exposed for 14 days, revealed, pale stained and degenerative changes of hepatocytes (Fig. 3c). Severe desquamation and degeneration of hepatocyte were observed after 21 days exposure (Fig. 3d). About 28 days exposure revealed, cellular rupture, presence of cell debris and severe desquamation of hepatocyte (Fig. 3e).

With higher concentration (0.0075 μg L^{-1}) endosulfan, blood congestion and cellular rupture were observed after 7 days exposure (Fig. 4b). After 14 days exposure, the changes observed are focal necrosis, desquamation and severe cellular degeneration (Fig. 4c). Severe cellular degeneration was still apparent after 21 days together with cellular rupture (Fig. 4d). Examination of liver in the fish exposed for 28 days showed focal necrosis, pale stained hepatocytes and severe cellular rupture (Fig. 4e).

When the concentration was raised to 0.01 μg L^{-1}, severe dilation of sinusoids and cellular degeneration were observed after 7 days exposure (Fig. 5b). After 14 days, the pathological changes in the fish's liver revealed, cellular degeneration, severe dilation of sinusoids and pale stained hepatocyte (Fig. 5c). Assessment of liver in the fish exposed for 21 days, reaveled severe intravascular haemolysis (Fig. 5d). Necrosis, severe dilation of sinusoids, pale stained

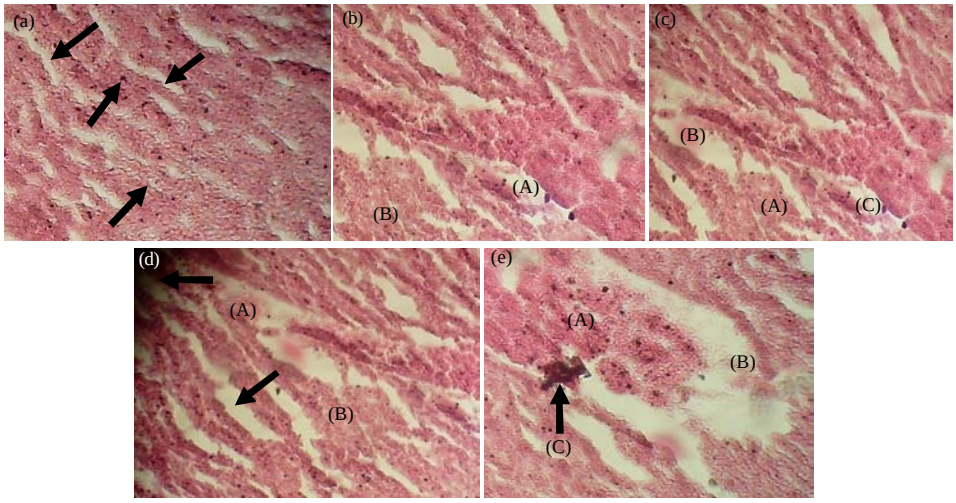

Fig. 2: a) Liver of control fish showing the architecture of a hepatic lobule. The hepatocytes (arrow head) and between the hepatocytes, the hepatic sinusoid. No visible lesion, lesion was observed after 7 days exposure (H&E stain x300); b) Liver of fish exposed to 0.0025 μg L^{-1} endosulfan. Dilation of sinusoids (A) and light hypertrophy (B) were observed after 7 days (H&E stain x300); c) Liver of fish exposed to 0.0025 μg L^{-1} endosulfan. Focal lymphotic infiltration (A), necrosis (B), dilation of sinusoids (C) were observed after 14 days (H&E stain x300); d) Liver of fish exposed to 0.0025 μg L^{-1} endosulfan. Pale stained in the portal area (arrow head), dilation of sinusoids (arrows), focal necrosis (A) and mild hypertrophy (B) observed after 21 days (H&E stain x300); e) Photomicrograph of liver exposed to 0.0025 μg L^{-1} endosulfan. Severe fatty degenerative changes of hepatocyte (A). Severe necrosis and dilation of sinusoids (B) and a clear congestion position (C) were observed after 28 days (H&E stain x300)

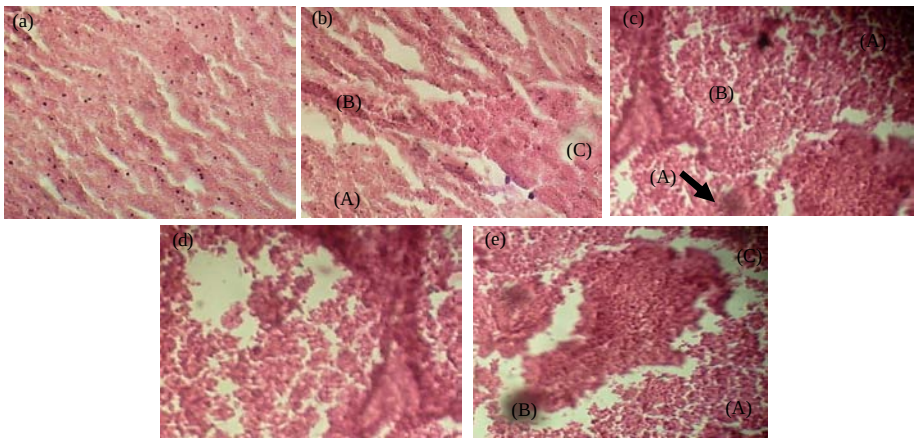

Fig. 3: a) Photomicrograph of liver of control fish. No significant lesion observed after 7 days exposure (H&E stain x300): b) Photomicrograph of liver of fish exposed to 0.005 μg L^{-1} endosulfan. Degenerative changes of hepatocytes (A), mild hypertrophy (B) and focal necrosis (C) were observed after 7 days (H&E stain x300); c) Photomicrograph of liver of fish exposedto 0.005 μg L^{-1} endosulfan. Pale stained (A) and degenerative changes of hepatocytes (B) were observed after 14 days H&E stain x300); d) Photomicrograph of liver of fish exposed to 0.005 μg L^{-1} endosulfan. Severe degeneration and desquamation of hepatocyte were observed after 21 days (H&E stain x300); e) Photomicrograph of liver of fish exposed to 0.005 μg L^{-1} endosulfan. Cellular rupture (A), presence of cell debris (B) and severe degeneration of hepatocytes (C) were observed after 28 days (H&E stain x300)

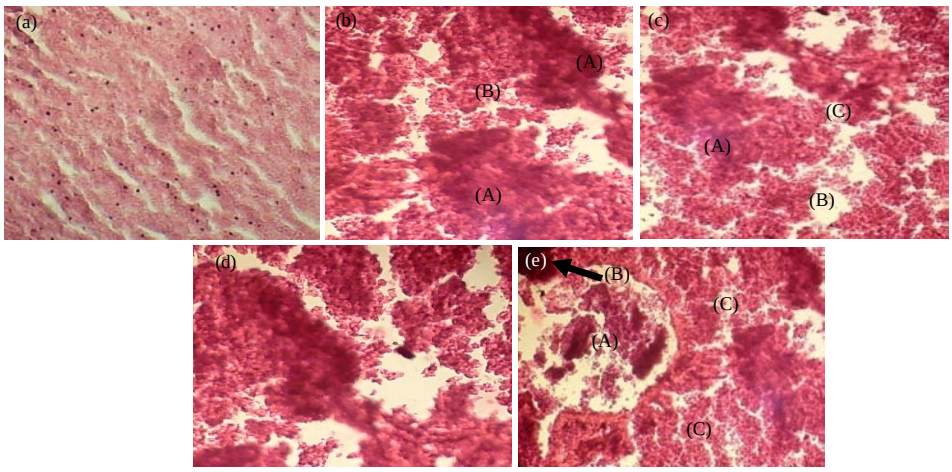

Fig. 4: a) Photomicrograph of liver of control fish. No significant lesion observed after 7 days (H&E stain x300); b) Photomicrograph of liver of fish exposed to 0.0075 μg L^{-1} endosulfan. Blood congestion (A) and cellular rupture (B) were observed after 7days (H&E stain x300); c) Photomicrograph of liver of fish exposed to 0.0075 mg L^{-1} endosulfan. Necrosis (A), desquamation of hepatocyte (B) and severe cellular degeneraion (C) were observed after 14 days; d) Photomicrograph of liver of fish exposed to 0.0075 μg L^{-1} endosulfan. Severe degenerative changes and rupture of blood cells were observed after 21 days (H&E stain x300); e) Photomicrograph of liver of fish exposed to 0.0075 μg L^{-1} of endosulfan. Focal necrosis (A), pale-stained hepatocytes (B) and cellular rupture (C) were observed after 28 days (H&E stain x300)

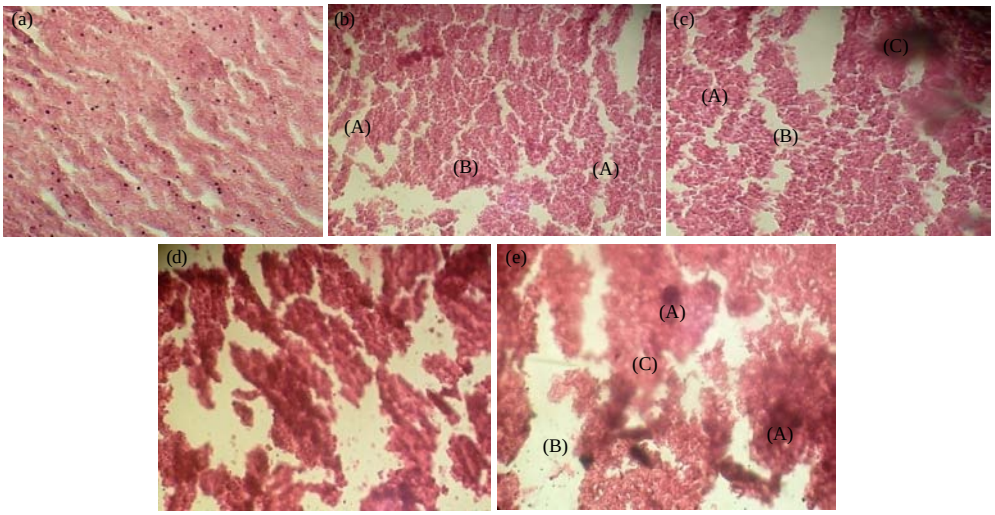

Fig. 5: a) Photomicrograph of liver of control fish. No significant lesion observed after 7 days exposure (H&E stain x300); b) Photomicrograph of liver of fish exposed to 0.01 mg L^{-1} endosulfan. Severe dilation of sinusoids (A) and cellular degeneration (B) were observed after 14 days (H&E stain); c) Photomicrograph of fish's liver exposed to 0.01 μg L^{-1} endosulfan. Cellular degeneration (A), dilation of sinusoids (B) and pale-stained hepatocytes (C) were observed after 14 days (H&E stain x300); d) Photomicrograph of liver of fish exposed to 0.01 μg L^{-1} endosulfan. Severe intravascular haemolysis were observed after 21days (H&E stain x300); e) Photomicrograph of liver of fish exposed to 0.01 μg L^{-1} endosulfan. Necrosis (A), severe dilation of sinusoids (B) and rupture of blood vessels (C) were observed after 28 days (H&E stain x300)

hepatocytes and rupture of blood vessels were observed in the liver of the fish at a longer exposure period of 28 days (Fig. 5e).

The vacuolar degeneration in the hepatocytes, dilation and congestion in blood sinusoids, hypertrophy, focal lymphatic infiltration, fibrosis, pale stain, fatty

degeneration and changes of hepatocyte. Fibrosis, dilation and congestion in blood sinusoids reported in this study were similar to the findings of OECD (1995) who observed histological alterations when an increasing concentration of carbaryl applied to *Puntius conchonius*. In another study, Palaniappan and Karthikeyan (2009) found that there were hepatic lesions including hypertrophy, vacuolization, nuclear pycnosis, karyolysis and fatty degeneration of hepatocytes in a the same fish, *P. conchonius* which was chronically exposed to sublethal concentrations of three pesticides. Induction of prehepatomatous lesions was reported Popp and Cattley (1991) in *Salmo clarki* exposed to endrin in water. The pathological findings in this species included liver cord disarray and intrazonal and periportal inflammatory foci, Rajendran and Venugopalan (1991) also reported degeneration of hepatocytes, sinusoidal dilation and congestion in the blood vessels of the liver of *Oncorhynchus mykiss* treated with copper sulphate. The dilated blood sinusoids of the liver were, also recorded in the liver of *Gambusia affinis* exposed to commercial deltamethrin (Safe, 2003). Similar observation was also observed in rats under the influence of dursban intoxication (Strucinski *et al*., 2000) and by Van der Oost *et al*. (2003) in experimental animals poisoned with chlorodecone.

CONCLUSION

The present study showed that the liver tissue is a very good biomarkers for toxicological study and it responses to low levels of contamination is exceptional and has shown the potential relationships between the structural changes and environmental contamination. The substantiation of pathological alterations in organs sequentially in contact with toxicants appear to be more sensitive biomarker of pollutant exposure and effect. The ealriar mentioned findings, clearly indicated that endosulfan is very toxic, the incessant use can endanger aquatic flora and fauna and can be magnified along the food chain and affect human which is at the top of the trophic level. The finding revealed that the higher the concentration of endosulfan, the slower the depuration of the toxicant and the more the severity of the effects on the liver. Further study is required to quantity the enzyme (s) induced that resulted in the structural damage of the liver.

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