Original Article

Bioaccumulation and Histopathological Alterations in Liver Caused by Endosulfan Toxicity in *Oreochromis Niloticus*

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A R T I C L E   I N F O

A B S T R A C T

Objective: The toxic effects of sublethal concentrations of endosulfan on *Oreochromis niloticus* was examined using bioaccumulation and histopathology studies of the liver as toxicological endpoint. **Methods:** The Organization for Economic Cooperation and Development #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. **Results:** 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 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.

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 (Daesik 2004) .Endosulfan is an organochlorine and highly toxic pesticide in EPA toxicity. The solubility of endosulfan is 0.3 mg/l 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

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is to quantify the concentration of endosulfan in the liver and assess the effects of depuration on liver's tissues of *O.niloticus* . This fish 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).

2. MATERIALS AND METHODS

2.1. Collection and Acclimatization of the Test Organisms

One hundred and twenty juveniles of *O. niloticus* of mean weight (320.40 $\pm$ 1.2g) and length (50.00 $\pm$ 2.13cm) 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 two 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.

2.2. Experimental Design

The LC_{50} value for endosulfan was determined by using OECD Guideline No. 203 for static-renewal test conditions (OECD 1992) and was found to be. 0.21µg / L .The sub-lethal test was conducted under OECD test guideline 407 (OECD 1997a) 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 $\pm$ 0.58 ∘C and 26.67 $\pm$ 1.15∘C; pH between 7.27 $\pm$ 0.25 and 7.16 $\pm$ 0.25 and the rate of dissolved oxygen was kept between 8.73 $\pm$ 0.12mg/l and 8.12 $\pm$ 0.10mg/l with the help of an air pump. Dechlorinated tap water was used for preparing test solutions. From the LC_{50} determination, lower concentrations of the pesticide were prepared for sub-lethal test.. Five 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 in

water: The test fish were exposed to the above-mentioned sub-lethal concentrations separately for a period of 7, 14, 21, and 28 day. During these periods, the water was changed along with waste feed and fecal materials daily at 8a.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, a fish was removed from each aquarium. A total of three- fishes per test concentration were dissected and the liver tissues 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.

2.3. Pesticide Residue Analysis

2.3.1. 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.

2.3.2. 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**.**

2.3.3. Extraction and Analysis

Endosulfan was extracted using solid phase extraction (SPE) technology (IST 1995). Fifty (50) gram of the tissue was thoroughly macerated and homogenized in 150ml of petroleum ether in a metallic blender at high speed until thoroughly mixed. 100g 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 500ml flask fitted with a suction apparatus .The residue in the blender cup was re-extracted with two 100ml portions of petroleum ether blended for 2 minutes each time, filtered and combined with the first extract. The combined extract, equivalent to 20 g of fish tissue, was passed through column (25mm x 50mm 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.

2.3.4. Clean-up

Fifteen ml (Bancroft and Cook 1994) of petroleum ether and 30ml of acetonitrile was added to the extracted sample and transferred to a 125ml separatory funnels .The mixture was shaken vigorously for 1 minute, 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 litre separatory funnel containing 650ml water; 40ml saturated NaCl solution and 100ml petroleum ether. The extract in the separating funnel was mixed thoroughly for 45 seconds .The layers were allowed to separate and the aqueous layer was drained into another 1litre separating funnel. 100ml petroleum ether was added to the second 1litre separating funnel and the mixture was shaken vigorously for 20 seconds 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 100ml portions of water. The washing was discarded. The petroleum ether layer was passed through column (25mm x 50mm long) of anhydrous sodium sulphate (Na_2SO_4).The dried petroleum extract was evaporated to 10ml in a rotary vacuum evaporator.

2.3.5. 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 50ml petroleum ether) was filled with 4g activated Florisil topped with anhydrous sodium sulphate to about 3cm. The concentrated petroleum ether solution of sample extract rinsed with two 5ml, portions of petroleum ether was transferred to the column. The column was eluted with 200ml HPLC methanol. The elute in the round bottomed flask was collected and concentrated under rotary vapour to complete dryness. 2ml HPLC methanol was added to round bottom flask and shifted in labeled glass stoppered vials for analysis in high performance liquid chromatography

2.3.6. Preparation of Standard Stock Solution

One milligram of the standard per gram of stock solution was prepared by adding 0.1 g of endosulfan standard into a 100 ml volumetric flask. Five milli litres 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.

2.3.7. 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. 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.

2.3.8 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 minutes. The system was then separated following the procedures outlined in the instrument operating manual.

2.3.9. 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 mg/l to 100 mg/L were 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 minutes.

2.4. Histopathological Study

The tissues were fixed in 10% phosphate-buffered formalin for 48 hours immediately after dissection .Thereafter, the tissues were dehydrated in periodic acid Schiff's reagent (PAS) following the method of Hughes and Perry (1976) 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 melted wax and sectioned into thin sections (5 μm) using a wax microtome and mounted on glass slides. Sections were deparaffinized in xylene, hydrated in decreasing concentrations of isopropanol (100% - 90% - 80% - 70%- 50% - water) and stained with hematoxylin. Sections were differentiated in 1% isopropanol rinsed in running tap for 5 minutes, counter stained with eosin, dehydrated, cleared in xylene and dried in ovum. A drop of Canada balsam was added .Changes induced by treatment in these tissues (10 sections of each tissue from each fish) were photographed and analyzed by light microscopy (Nikon® Labophot) (Bancroft and Cook 1994, Bernet et al.1999)

2.5. Statistical Analysis

The difference between the control and experiment were analyzed with the help of student t test. One-way analysis of variance SPSS (14.0 version), SPSS Inc, Chicago, USA, was employed to evaluate the differences among various experimental tests. 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 bioaccumulation and depuration assessment endpoints

3. Results

3.1 Bioaccumulation 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 figure 1 .At the lower concentrations of 0.0025 µg/gdw and 0.005 µg/gdw, the pesticide residues in the liver decreases with increase in exposure duration. The maximum amount of bioaccumulation was observed on day-7 than other intervals .However, when the concentration was increased to 0.0075 µg/gdw, accumulated residues was higher on day -14 than day 7, and drop abruptly, with day 28 having the least residue. At the highest concentration of 0.01 µg/gdw, there was spontaneous increased in the pesticide residue in the fish tissues with the exposure duration till day 21 and dropped on day -28.
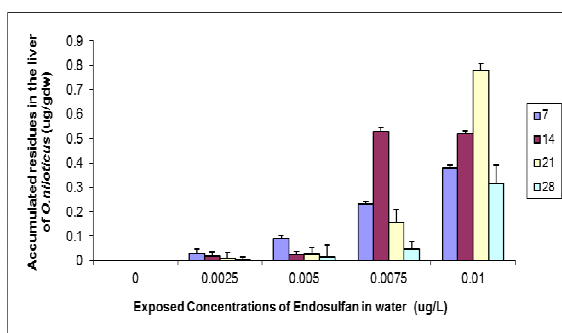
Figure 1. Endosulfan concentration (µg/gdw) in liver of control fish and fish exposed to different concentrations of endosulfan for 28 days. The graph presents the average of endosulfan concentration in the liver of O.niloticus exposed to four concentrations (0.0025, 0.005, 0.0075 and 0.01 µg/gdw) including control for different exposure time (7, 14, 21 and 28 days). Each sample was replicated.

3.2 Histopathology Changes

Histopathological studies of the liver revealed morphologic changes in the liver of exposed fish and were not observed in the control fish. Figures 2a, 3a, 4a and 5a showed the normal histological structures of the liver. In the fish exposed to 0.0025 µg/L 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 apparent together 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); 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).Twenty eight (28) days exposure revealed- cellular rupture, presence of cell debris and severe desquamation of hepatocyte (Fig.3e) With higher concentration (0.0075 µg/L) 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 after 28 days, showed focal necrosis, pale stained hepatocytes and severe cellular rupture (Fig.4e)

When the concentration was raised to 0.01µg/L, 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, revealed severe intravascular haemolysis (Fig.5d). Necrosis, severe dilation of sinusoids, pale stained hepatocytes, and rupture of blood vessels were observed in the liver of the fish at a longer exposure period of 28 days (Fig.5e).
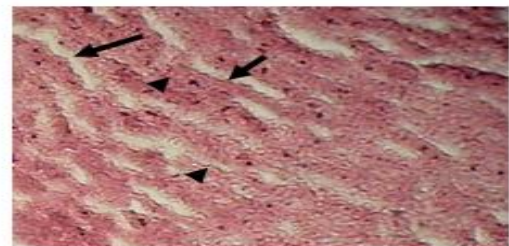

Fig. 2a: Liver of control fish showing the architecture of a hepatic lobule .The hepatocytes (arrow head) and between the hepatocytes, the hepatic sinusoid. No visible lesion lesion was observed after 7 days exposure (H & E stain x300)

Fig. 2b: Liver of fish exposed to 0.0025µg/L endosulfan. Dilation of sinusoids (A) and light hypertrophy (B) were observed after 7 days (H & E stain x300)
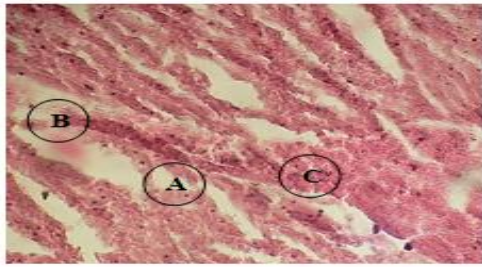
Fig. 2c: Liver of fish exposed to 0.0025 μg/L endosulfan . Focal lymphotic infiltration (A), necrosis (B),dilation of sinusoids (C) were observed after 14 days (H &E stain x300)
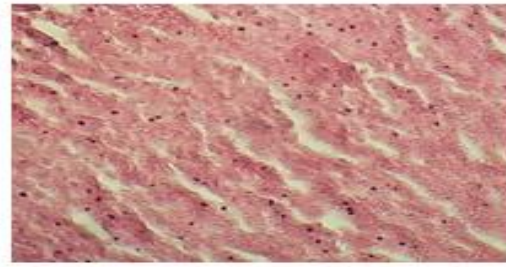

Fig. 3a: Photomicrograph of liver of control fish. No significant lesion observed after 7 days exposure (H &E stain x300)
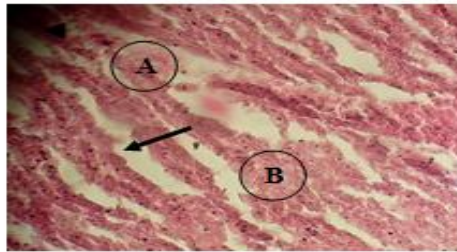

Fig. 2d. Liver of fish exposed to 0.0025μg/L endosulfan . Pale stained in the portal area (arrow head), dilation of sinusoids (arrows),focal necrosis (A) and mild hypertrophy (B) observed after 21 days (H &E stain x300)
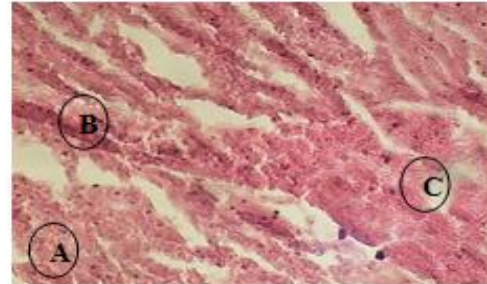
Fig. 3b: Photomicrograph of liver of fish exposed to 0.005μg/L endosulfan. Degenerative changes of hepatocytes (A), mild hypertrophy (B) and focal necrosis (C) were observed after 7 days (H &E stain x300)
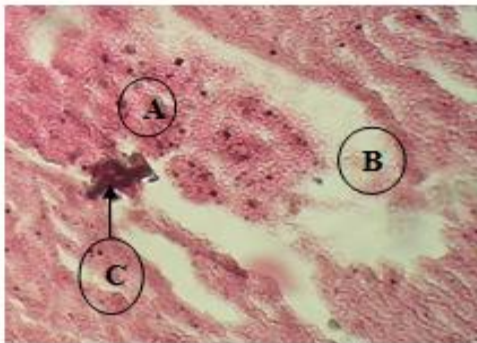

Fig. 2e: Photomicrograph of liver exposed to 0. 0025μg/L endosulfan.Severe fatty degenerative changes of hepatocyte (A).severe necrosis and dilation of sinusoids (B), and a clear congestion position (C) were observed after 28 days (H &E stain x300)
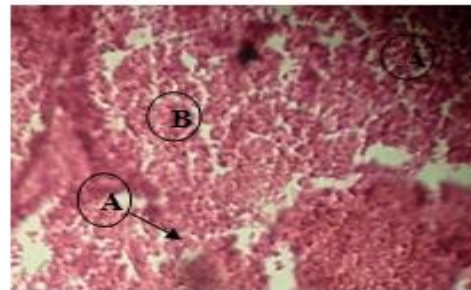

Fig. 3c: Photomicrograph of liver of fish exposed to 0.005μg/L endosulfan. Pale stained (A), and degenerative changes of hepatocytes (B) were observed after 14 days H &E stain x300)
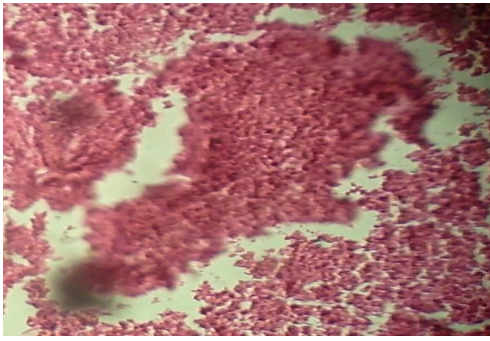
Fig.3e : Photomicrograph of liver of fish exposed to 0.005µg/L endosulfan. Cellular rupture (A), presence of cell debris (B) and severe degeneration of hepatocytes (C) were observed after 28 days (H &E stain x300)
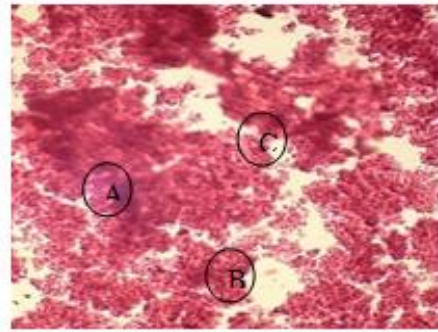

Fig.4a: Photomicrograph of liver of control Fish. No significant lesion observed after 7 days (H &E stain x300)

Fig.4b: Photomicrograph of liver of fish exposed to 0.0075µg/L endosulfan. Blood congestion (A) and cellular rupture (B), were observed after 7days (H &E stain x300)

Fig. 4c: Photomicrograph of liver of fish exposed to 0.0075µg/L endosulfan. Necrosis (A), desquamation of hepatocyte (B) and severe cellular degeneraion (C) were observed after 14days
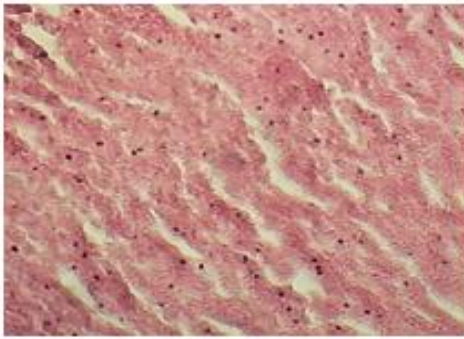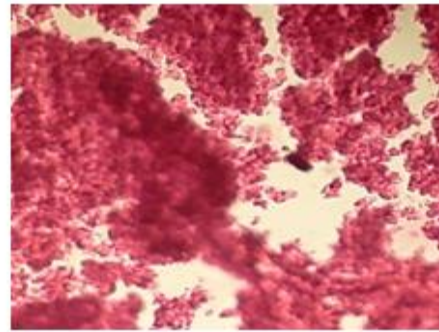

Fig. 4d :Photomicrograph of liver of fish exposed to 0.0075µg/L endosulfan . Severe degenerative changes and rupture of blood cells were observed after 21 days (H &E stain x300)
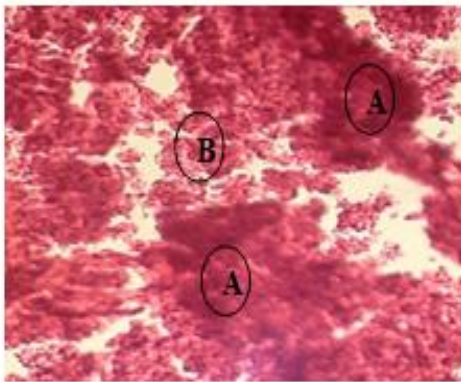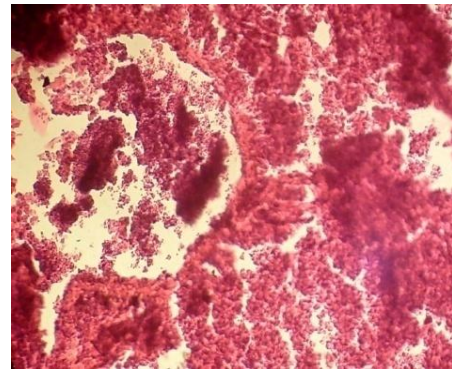

Fig. 4e: Photomicrograph of liver of fish exposed to 0.0075µg/L of endosulfan. Focal necrosis (A), pale-stained hepatocytes (B) and cellular rupture (C),were observed after 28 days (H &E stain x300
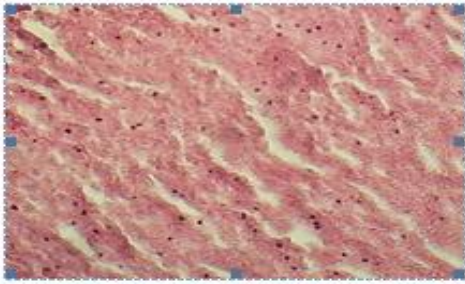
Fig.5a: Photomicrograph of liver of control fish .No significant lesion observed after 7 days exposure (H &E stain x300)
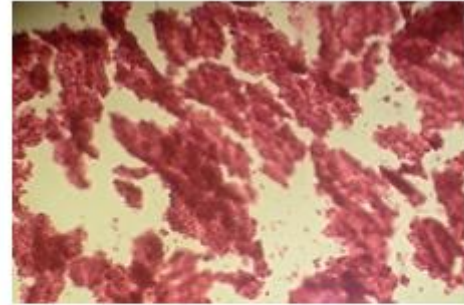

Fig. 5d: Photomicrograph of liver of fish exposed to 0.01µg/L endosulfan. Severe intravascular haemolysis were observed after 21days (H &E stain x300)
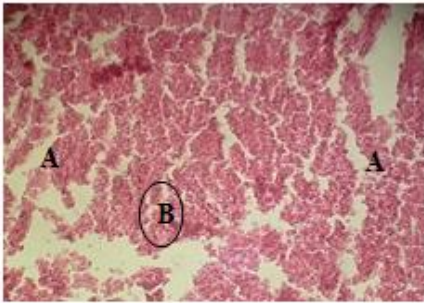

Fig. 5b:Photomicrograph of liver of fish exposed to 0.01µg/L endosulfan. Severe dilation of sinusoids (A) and cellular degeneration (B) were observed after 14 days (H & E stain)
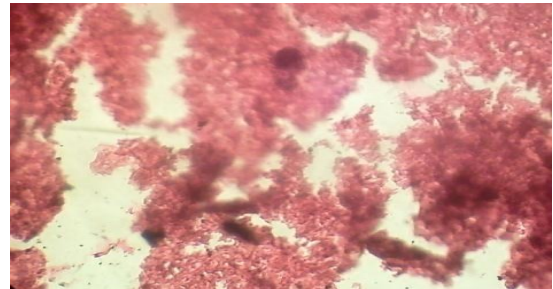

Fig. 5e:Photomicrograph of liver of fish exposed to 0.01µg/L endosulfan. Necrosis (A), severe dilation of sinusoids (B) and rupture of blood vessels (C) were observed after 28 days (H &E stain x300)
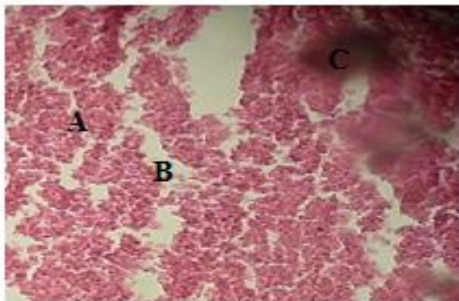

Fig. 5c:Photomicrograph of fish's liver exposed to 0.01µg/L endosulfan. Cellular degeneration (A), dilation of sinusoids (B), and pale-stained hepatocytes (C) were observed after 14 days (H &E stain x300)

4. DISCUSSION

4.1 Bioaccumulation and Depuration

Bioaccumulation occurs when an organism take in a toxic substance at a rate greater than that at which the substance is lost (Liao 2006). Chlorinated compounds have been detected in various organs and fluids of the human body as a result of consuming contaminated food and their bioaccumulation along the trophic chain. In toxicological study, each step results in increased bioaccumulation, an animal living at the top of the food chain, through its regular diet and longer lifespan, may accumulate a much greater concentration of a chemical than the organisms found lower in the food pyramid (Góralczyk and Struciński 1996). Humans situated at the top of the trophic pyramid, are particularly exposed to the harmful effects of these pesticides .Pesticides have been detected in such body fluids as blood, milk, urine, in tissues (e.g. fat tissue), and in the liver, kidneys, lungs, brain and skin. Most of them cause functional disorders of many organs, leading to various types of pathological changes Schinas et al. 2002).

Tissues and organs analysis to determine chemical uptake, accumulation, biotransformation, and elimination are basic toxicological indices to understand the effects of organic chemicals in an organism. In this investigation, bioaccumulations of endosulfan in the liver

of the treated fishes were significantly higher than the control (P < 0.05) in all the concentrations. The higher the concentrations the more difficult the depuration of the pesticide and the consequence is that the liver would be over stressed to secret more detoxifying enzyme to neutralize the effects of the toxicant and 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 and other enzymes mainly belonging to mono-oxygenase system (Glenn and Gandolfi 1986; Jimener and Stegman 1990; Van der Oost .2003) .Similar observation on concentration dependent depurations in visceral organs were observed, when fish, Labeo rohita and Saccobrachus fossilis exposed to metasystox (Rajendran and Venugopalan 1991), Mugil cephalus and Mystus gulio to endosulfan and Clarias batrachus to sub-lethal concentrations of dimethoate respectively (Ghousia et al.1994).

4.2 Histopathological Alteration

Pathological changes in the tissue of test organisms induced experimentally to toxicants provide information on the mode of action of the toxicant on them. The liver performs vital functions in animal's life. Besides being the major metabolic centre for detoxifying toxic substances, it secrets bile that emulsify fat, plays important role in carbohydrate, protein and lipid metabolism. In this study, the architecture of the liver of the exposed fish was altered remarkably. The histopathological investigation revealed that, the higher the concentrations, the more the severity of the pathological changes in the liver's tissues. The pathological severities in the liver reflect its multifunctional role in detoxification process (Al- Yousuf et al .2000) .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 observations in the liver of rats exposed to chlorpyrifos and cypermethrin was reported by Latuszynska et al. (1999) 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 (Gill and Pant 1988) who observed histological alterations when an increasing concentration of carbaryl applied to Puntius conchonius. In another study, (Gill et al.1991) 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 (Eller 1971) in Salmo clarki exposed to endrin in water. The pathological findings in this species included liver cord disarray, and intrazonal and periportal inflammatory foci. Atamanalp et al. (2008), 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 (Cengiz and Unlu, 2006). Similar observation was also observed in rats under the influence of dursban intoxication (Popp and Cattley 1991) and by (Guzelian et al.1980) 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's response 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 above 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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