JST

Journal homepage: http://penerbit.uthm.edu.my/ojs/index.php/jst
ISSN : 2229-8460 e-ISSN : 2600-7924

Journal of Science and Technology

Histopathological Biomarkers of exposure to Monocyclic Aromatic Hydrocarbons in *Clarias gariepinus* (African Catfish)

Funmilayo V. Doherty[1], Abdullahi Adeola[1*], Idowu A. Aneyo[1], Adebayo A. Otitoloju[2]

[1]Environmental Biology Unit, Department of Biological Science,
P.M.B 2011, Yaba College of Technology, Lagos, NIGERIA

[2]Ecotoxicology unit, Department of Zoology,
P.O. BOX 156, University of Lagos, NIGERIA

*Corresponding Author

DOI: https://doi.org/10.30880/jst.2019.11.01.004
Received 16 April 2019; Accepted 1 June 2019; Available online 19 June 2019

Abstract: The pollution of the environment with petroleum products through spills in oil producing countries has resulted in the widespread distribution of benzene, toluene, ethylbenzene and xylene. The aim of this study was to identify histopathological alterations in *Clarias gariepinus* (catfish) that can be used as biological markers for detection of petroleum hydrocarbon contamination which can be included in monitoring programmes. The toxicological evaluations of benzene, toluene, ethylbenzene and xylene were carried out against *Clarias gariepinus.* The histopathological effects of benzene, toluene, ethylbenzene and xylene (BTEX) on different organs were investigated in *C. gariepinus*, *Tilapia zillii*, and *Chrysichthys nigrodigitatus* taken from the impacted areas of the Lagos Lagoon. Toxicological evaluations of the monocyclic aromatic components, benzene, toluene, ethylbenzene and xylene on *Clarias gariepinus* showed that ethylbenzene (0.479 ml/l) was the most toxic compound tested followed by xylene (0.519 ml/l), benzene (0.666 ml/l) and toluene (1.190 ml/l). The results from the histological study identified necrosis and deformation of the gills, inflammations in the liver, and wrinkling of the oocyte membrane in the gonads of fish, as good histopathological biomarkers of hydrocarbon related stressors. The combination of chemical analysis with these identified biomarkers can be used during environmental monitoring programmes for the protection of aquatic ecosystem.

Keywords: Biomarkers; BTEX; fish; histopathological alterations, African catfish

1. Introduction

The hydrocarbon pollution of Nigeria's water bodies especially the Lagos lagoon is a major problem, as many people depend on these water bodies as a source of generating income through fishing and other recreational activities. Some researchers have observed high concentrations of hydrocarbons in the Lagos lagoon **[1, 2]**. Doherty and Otitoloju **[3]** reported total hydrocarbon content varying from 2.03 mg/l - 31.38 mg/l and total Benzene, Toluene, Ethylbenzene and Xylene (BTEX) values as high as 596.98 µg/l in the Lagos lagoon. Monocyclic aromatic hydrocarbons such as BTEX can cause harm to fauna and flora in the aquatic ecosystem **[4]**. There has been a decline in fish organisms in the

*Corresponding author: adesalam84@gmail.com

Lagos lagoon which is attributed to hydrocarbon pollution as reported by **[5]**. Reactive oxygen species (ROS) are produced when fishes are susceptible to hydrocarbon pollution, and these ROS can lead to cellular damage. Histological indicators can be generated by ROS, and these indicators can be used as biological markers in fishes exposed to toxicants **[6]**. It is significant to research on effect of ROS on histology of fishes in order to understand if fish species are affected by exposure to contaminants. Oxidative stress resulting from pollution has been reported in different organisms **[7].**

Various histological changes in aquatic organisms especially fish have been identified and recommended as biomarkers in environmental monitoring programs to determine the effects of pollution in water bodies. For example, studies such as the National Oceanic and Atmospheric Administration NOAA's National Status and Trends Program in the United States have confirmed that there is an association between fish diseases and levels of contaminants in water bodies **[8]**. Histological alterations in fish were employed as the measure used to determine exposure to pollutants in these programs and certain disorders have been confirmed to be reliable biomarkers of effects evolving from the exposures. For adequate pollution diagnosis and control, there is also the need to identify biological responses or biomarkers which can serve as early indicators of petroleum hydrocarbon related stress in oil impacted ecosystems in developing countries. These biomarkers will complement chemical analysis of pollutant levels in environmental media during monitoring programmes. Though fishes are suitable indicators of contaminants accumulation, in any pollutant research on aquatic toxicity due to their high sensitivity to environment **[9]**, few researches have been conducted to identify indicators of toxic responses for fish species exposed to monocyclic aromatic hydrocarbons particularly the BTEX compounds. Biomarkers are not used in pollution monitoring by regulatory agencies in developing countries **[10]**. The implementation of biomarkers of effects in national monitoring programmes in developing countries is unquestionably an important near-future step. To this end, biomarkers of effects identified in this study will be an important contribution. These biomarkers can be useful tools for decision makers to improve the impact assessment of accidental pollution and they can be used to detect impacts when chemicals cannot be measured or are no longer detectable. The Biomarkers identified in this study have a huge potential for use in monitoring programmes. The aim of this study was to identify histopathological changes in the gills, gonads and livers of the fishes that can be used as biological markers of BTEX exposure and to establish the lethal concentration (LC_{50}) of BTEX.

2. Materials and Method

2.1 Collection of Samples

Tilapia zillii (Gervais, 1848) and *Chrysichthys nigrodigitatus* (Lacapede, 1803) were caught from the Lagos Lagoon. The fish species were collected with the assistance of fishermen using a fish cast net (31 mm – mesh size) which was used to form a complete fence. African catfish *Clarias gariepinus* (Burchell, 1822) were used for the ecotoxicological bioassay. The catfish were purchased from the Nigerian Institute for Oceanography and Marine Research (NIOMR). The fish in the farm were of known stock history. The animal stocks are not affected by any form of pollutants.

2.2 Test Chemicals

Laboratory Reagent of the following chemicals: benzene, toluene, xylene and ethylbenzene were obtained from Sigma Aldrich. The focus on BTEX in this study is because they form major constituents of petroleum products mostly released into the Lagos lagoon.

2.3 Acute Toxicity Studies

The fishes collected were of similar sizes. The juveniles (200 to 300 g- body weight) and fingerlings (1.5 to 2.0 g) were kept in separate square holding tanks in which they were allowed to acclimatize for two weeks. Rectangular glass tanks (Vol. 4.5 liters; 15 cm ×15 cm ×15 cm) were used as bioassay containers. Twelve active fish of similar sizes were randomly distributed into bioassay tanks already holding aromatic hydrocarbon (BTEX) and untreated control medium. Each treatment had 3 replicates and *Clarias gariepinus* were exposed to the following concentrations:

(a) Benzene: 0.65, 1.0, 1.8, 3.24, 5.83, 10.49 ml/l.
(b) Toluene: 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 ml/l.
(c) Ethylbenzene: 0.2, 0.3, 0.5, 0.65, 0.85, 1.0 ml.
(d) Xylene: 0.1, 0.3, 0.75, 1.0, 1.5, 1.8 ml/l.

The 96 hr LC_{50} obtained for benzene, toluene, ethylbenzene and xylene were 0.666 ml, 1.190 ml, 0.479 ml, 0.519 ml respectively.

2.4 Chronic Toxicity Studies

Rectangular glass tanks (58 cm by 39 cm by 37 cm) were used as bioassay containers. The chronic studies were carried out for a period of 60 days, to determine the sub-lethal (histopathological) effect of BTEX. Sub lethal concentrations of BTEX compounds were obtained using this formula -$LC_{50} \times 1/10^{th}$, according to Otitoloju **[11]**.

(a) Benzene against juvenile *Clarias gariepinus* at: 0.666 ml × 1/10 = 0.066 ml/l

(b) Toluene against juvenile *Clarias gariepinus* at: 1.190 × 1/10 = 0.119 ml/l

(c) Ethylbenzene against juvenile *Clarias gariepinus* at: 0.479 ml/l × 1/10 = 0.0479 ml/l

(d) Xylene against juvenile *Clarias gariepinus* at: 0.519 ml/l × 1/10 = 0.0519 ml/l

The liver, gonad and gills were quickly removed, and preserved for histopathological analysis according to standard methods.

2.5 Statistical Analysis

The Probit analysis was carried out using SPSS 14.0. Data was analysed with one-way analysis of variance (ANOVA). Differences at $P < 0.05$ were considered significant. This was used to compare several treatment means in appropriately designed experiments. When significant variations were detected, a post hoc test was performed. Significant differences at $P < 0.05$ were used to compare treatment means. SPSS 14.0 computer software package and Excel 2007 was used to analyse data.

3. Results and Discussion

3.1 Acute toxicity of BTEX compounds against *Clarias gariepinus*

The acute toxicity studies conducted within 96 hours reveal the LC_{50} values following this order with the most toxic compound being ethylbenzene: Ethylbenzene (0.479 ml/l) > Xylene (0.519 ml/l) > Benzene (0.666 ml/l) > Toluene (1.190 ml/l). The least toxic compound tested against *C. gariepinus* was toluene.

3.2 Chronic toxicity studies

3.2.1 Histopathological studies in gills of *Clarias gariepinus*

The structure of the control gills shows normal gill arch, gill filament and nucleus. No pathological effects such as lesion, necrosis, or inflammation were observed in the control animals. The histological alterations after 60 days exposure to sub-lethal concentrations of $1/10^{th}$ 96 hr LC_{50} of benzene, toluene, ethylbenzene and xylene is shown in Fig. 1. Benzene: distortion and loss of shape, loss of primary and secondary lamellae, fusion of secondary lamellae, severe pigmentation, severe necrosis, and inclusion bodies were observed. Toluene: fusion, distortion and loss of shape of secondary lamellae, atrophy of lamellae, severe necrosis, lesion and pigment were noted. Ethylbenzene: Severe areas of necrosis and pigment were noted with nuclear abnormality, lost shape and fusion of secondary lamellae. Xylene: Nuclear abnormality, area of inflammation, decreased lamellae length, distortion and loss of shape, fusion of secondary and primary lamellae, and loss of secondary lamellae were observed. The primary lamellae were also noted to be curved.

3.2.2 Histopathological studies in livers of *Clarias gariepinus*

Control: The structure of the liver of C*larias gariepinus* consisting of normal cellular pattern, normal central vein, bile duct, hepatic vein, hepatic artery and hepatocytes (Fig. 2). No changes were observed: No pathological changes such as lesion, necrosis, pigments, malignancy, inflammation and inclusion bodies were observed in the control animals, as the hepatic organization was intact.

Histopathological changes in the livers of C*larias gariepinus* after exposure to sub-lethal concentration of BTEX compounds for 60 days is shown in Fig. 2. Benzene: Moderate area of lesions, nuclear abnormality and area of inflammation, irregular shaped hepatocytes, nuclear vacuolisation, and bile stagnation as brownish- yellow were observed. Toluene: Severe area of lesion, nuclear abnormality, nuclear degeneration, vacuolization, bile stagnation as brownish-yellow granules and area of inflammation were observed. Ethylbenzene: Severe area of lesion, necrosis, pigment, inclusion bodies, irregular shaped nucleus, vacuolization, bile stagnation and area of inflammation were observed. Xylene: Area of inflammation, vacuolization, spotted structures melano- macrophages and bile stagnation were observed.

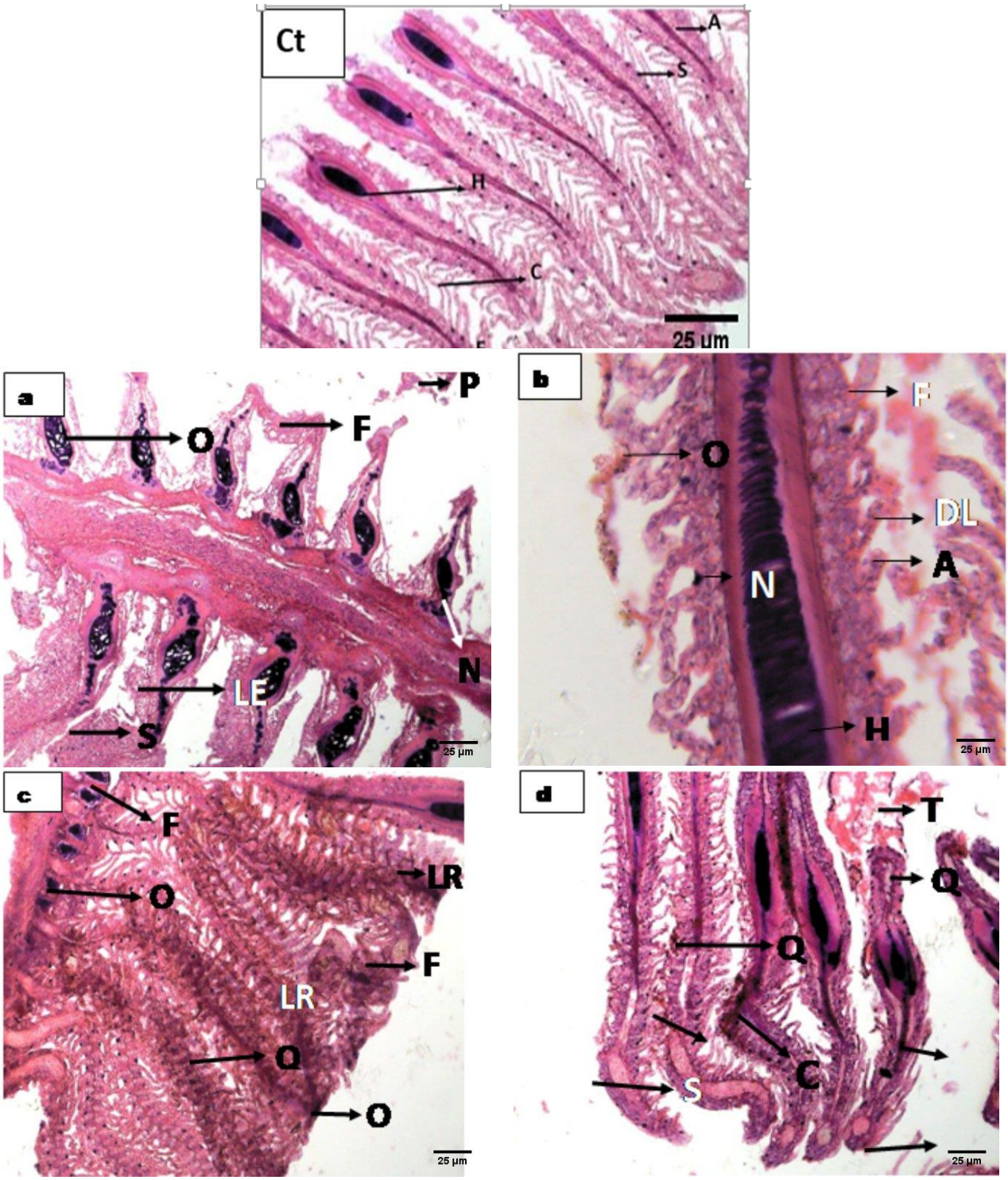

Fig. 1 - Gill histology of African Catfish (*Clarias gariepinus*)- Ct: Control shows normal structure of gill arch (A), filament (C), ceratobrachial bone of the arch (H), and nucleus (S). Exposed catfishes to BTEX ($1/10^{th}$ 96 hr LC_{50}) for 60 days a: Benzene, b: Toluene, c: Ethylbenzene, d: Xylene show evidence loss of both primary and secondary lamellae (S), fused secondary lamellae (F), Severe area of lesion (LE), Necrosis (N), Pigment (O), Inclusion bodies (P), Deformation of secondary lamellae (LR), Atrophy of lamellae (A), Necrosis (N), Nuclear abnormality (Q), Decreased length of lamellae (DL) and Curved primary lamellae (C). Connective tissue (T). Haematoxylin & Eosin stain, x400.

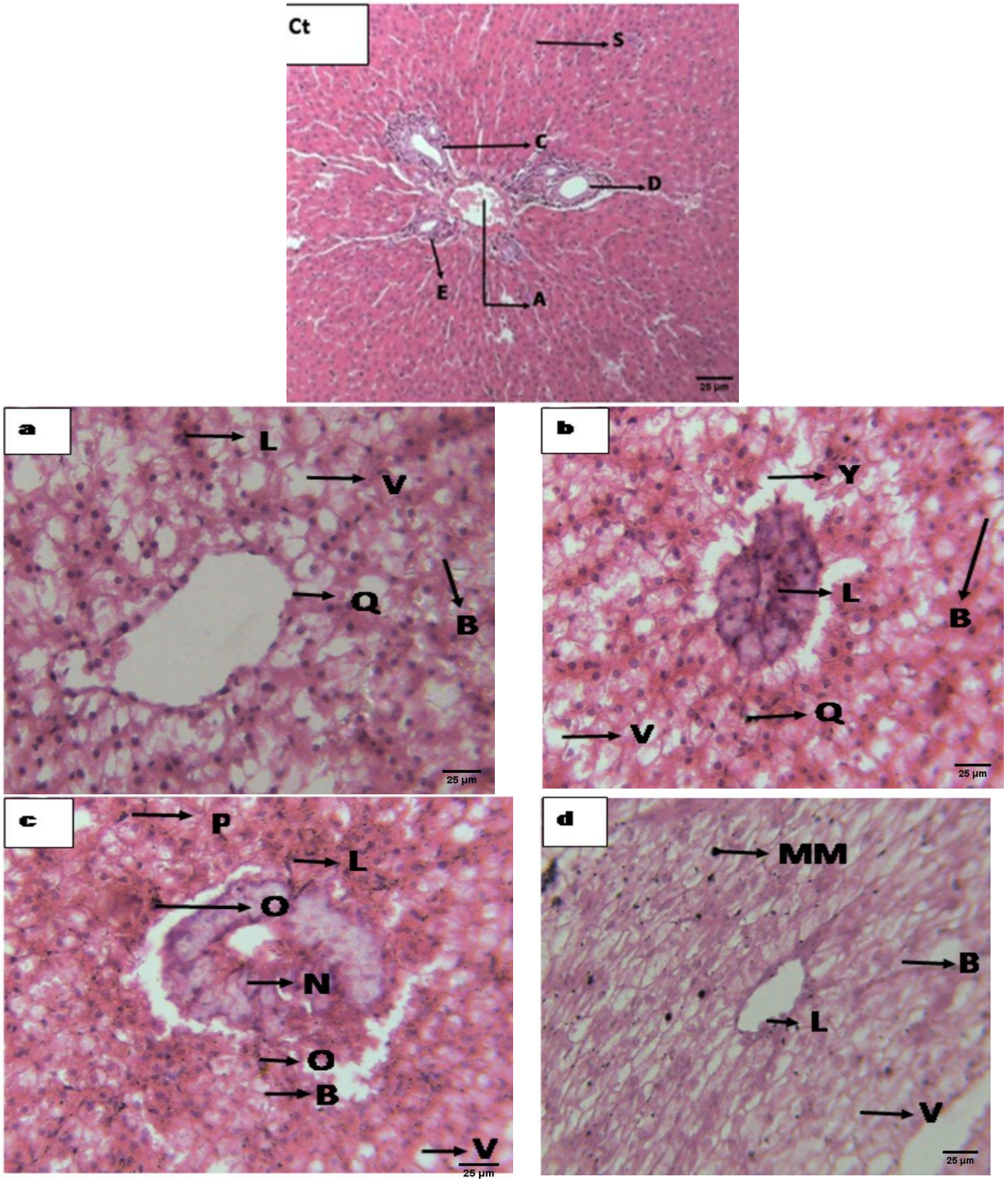

Fig. 2 - Histology of liver of the African Catfish (C*larias gariepinus*). Ct: Control-had normal structural features including central vein (A), bile duct(C), hepatic vein (D), hepatic artery (E) and hepatocytes. Exposed catfishes to BTEX (1/10th 96 hr LC_{50}) for 60 days a: Benzene, b: Toluene, c: Ethylbenzene, d: Xylene show evidence of cellular degeneration CD, Vascular congestion (VC), Inflammatory cells (IF), Fibrous layer (FL), Wrinkling of oocyte membrane (W), Contraction of Oocyte (C), Vascular degeneration (VD) and contraction of ovum (CO), Cytoplasmic vacuolation (CV), Lesion (L) and Pigmentation (P). Haematoxylin & Eosin stain, x400.

3.2.3 Histopathological studies in gonads of *Clarias gariepinus*

Control: Histopathological examination of the gonad of the control and exposed groups of fish is shown in the photomicrographs. The structure of the normal gonads of female fish C*larias gariepinus* consisting of different developmental stages namely oogonia and primary oocytes (Fig. 3). No changes were observed in the control animals.

3.3 Hispathological changes in the gonads of C*larias gariepinus* exposed to 1/10th 96 hr LC_{50} high sublethal concentration of BTEX compounds after 60 days

Benzene: Moderate area of lesion and necrosis with wrinkling of oocyte membrane, deformed oocyte, contraction of oocytes and melano-macrophages, were observed. Toluene: Wrinkling of oocyte membrane, deformed oocyte, vacuolated oocyte, shrinkage of nucleus and macrophages, were observed. Ethylbenzene: Wrinkling of oocyte, deformed oocyte, vacuolated oocyte and melano-macrophages, were observed. Xylene: Moderate area of lesion, necrosis with wrinkling of oocyte membrane, deformed oocyte, contraction of oocyte, shrinkage of nucleus, were noted (Fig. 3).

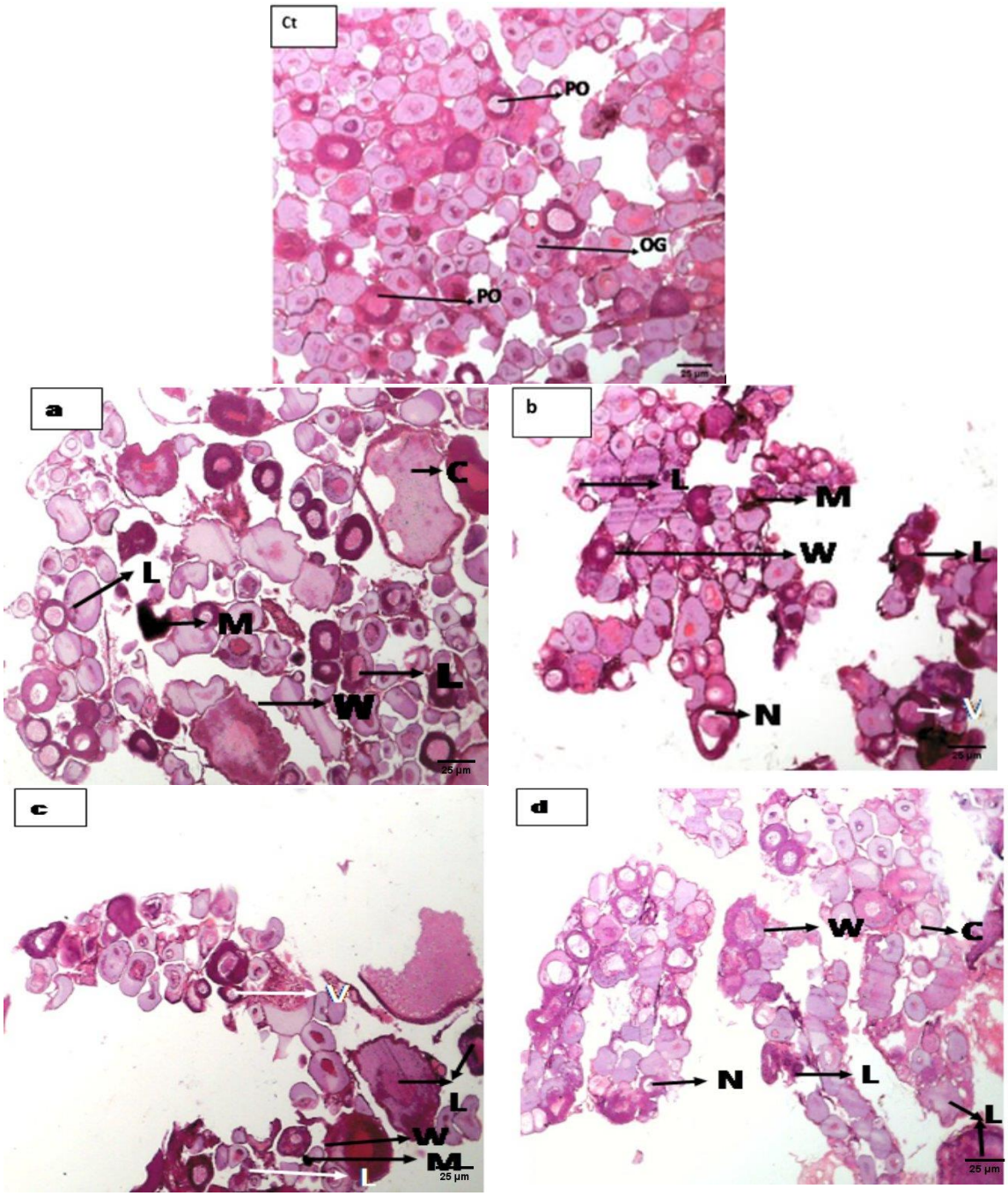

Fig. 3 - Histology of the gonad of the African catfish (*Clarias gariepinus*). Ct: Control showing large number of Oogonia (OG) and primary Oocytes (PO) with no malignant, no necrosis, no lesion, no pigment and inclusion bodies seen. Exposed catfishes to BTEX (1/10th 96 hr LC_{50}) for 60 days a: Benzene, b: Toluene, c: Ethylbenzene, d: Xylene showing evidence of wrinkling of oocyte membrane (W), Deformed oocyte (L), Contraction of oocytes (C), Macrophages (M), and Shrinkage of nucleus (N). Haematoxylin & Eosin stain, x400.

3.4 Identified histopathological biomarkers in *Chrysichthys nigrodigitatus* and *Tilapia Zillii* obtained from the wild in different locations of the Lagos lagoon.

The histopathological alterations seen in the gonad of *C. nigrodigitatus* and *T. zillii* from different locations of the Lagos lagoon (Fig. 4). The pathological findings include aggregation of inflammatory cells, vascular congestion, cellular degeneration and wrinkling of oocyte membrane. Contraction of ovum, cytoplasmic vacuolation, lesion and pigment were also seen in the gonads. The histopathological alterations noticed in the gills of *C. nigrodigitatus* and *T. zillii* from different locations of the Lagos lagoon (Fig. 5). Cellular degeneration, fusion of secondary lamellae, pigment, inflammation, loss of secondary lamellae, necrosis, inclusion bodies, cytoplasmic vacuolation were observed in the gills. Fig. 6 shows the histopathological alterations seen in the liver of *C. nigrodigitatus* and *T. zillii* from different locations of the Lagos lagoon. The pathological changes observed were vascular congestion and degeneration, cellular degeneration, inflammation.

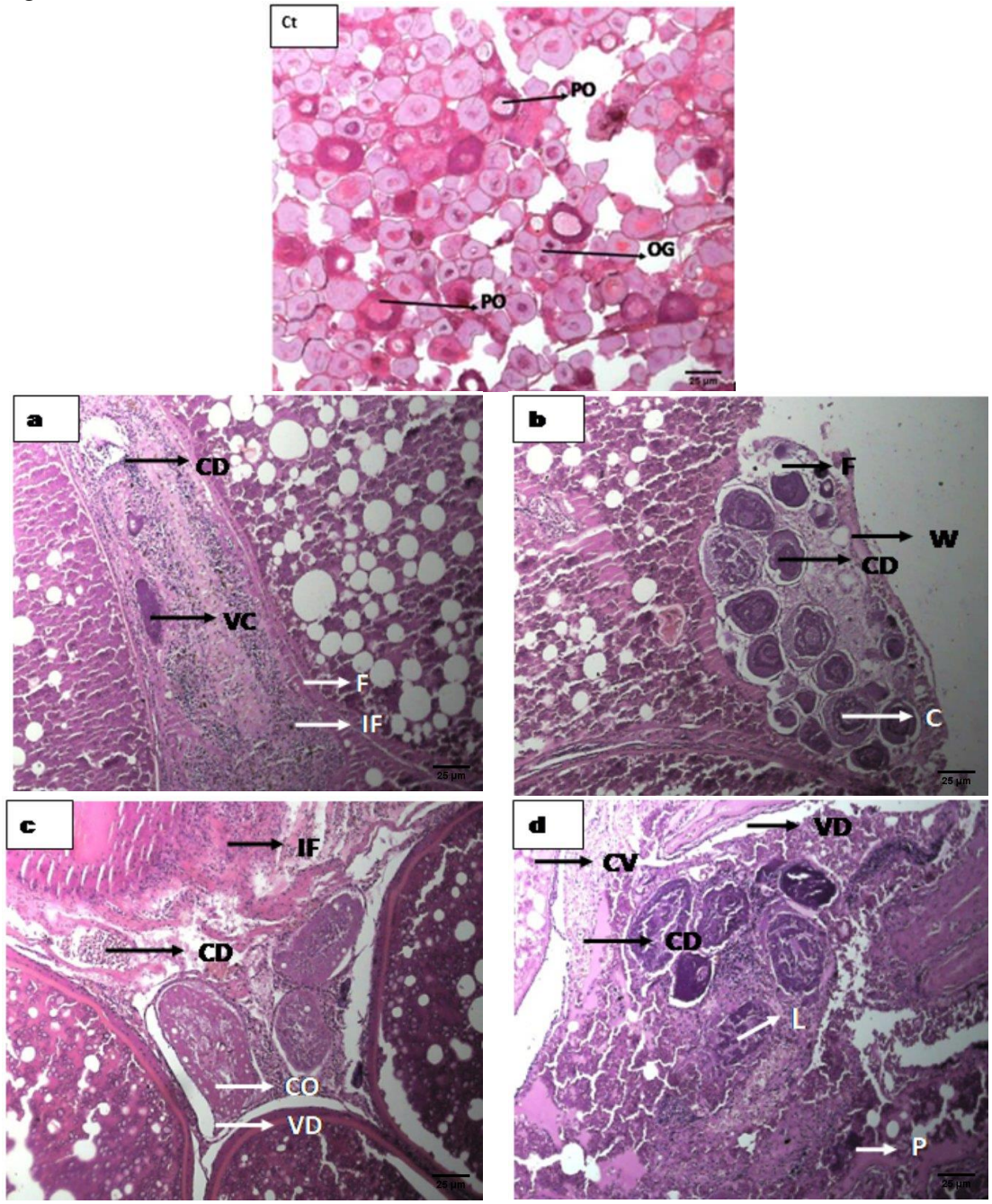

Fig. 4 - Histology of the gonad of the African catfish (*Clarias gariepinus*). Ct: Control showing large number of Oogonia (OG) and primary Oocytes (PO) with no abnormalities. a-d: Gonads of Wild Catfishes from the Lagos Lagoon showing cellular degeneration CD, Vascular congestion (VC), Inflammatory cells (IF), Fibrous layer (FL), Wrinkling of oocyte membrane (W), Contraction of Oocyte (C), Vascular degeneration (VD) and contraction of ovum (CO), Cytoplasmic vacuolation (CV), Lesion (L) and Pigmentation (P). H & E stain, x400.

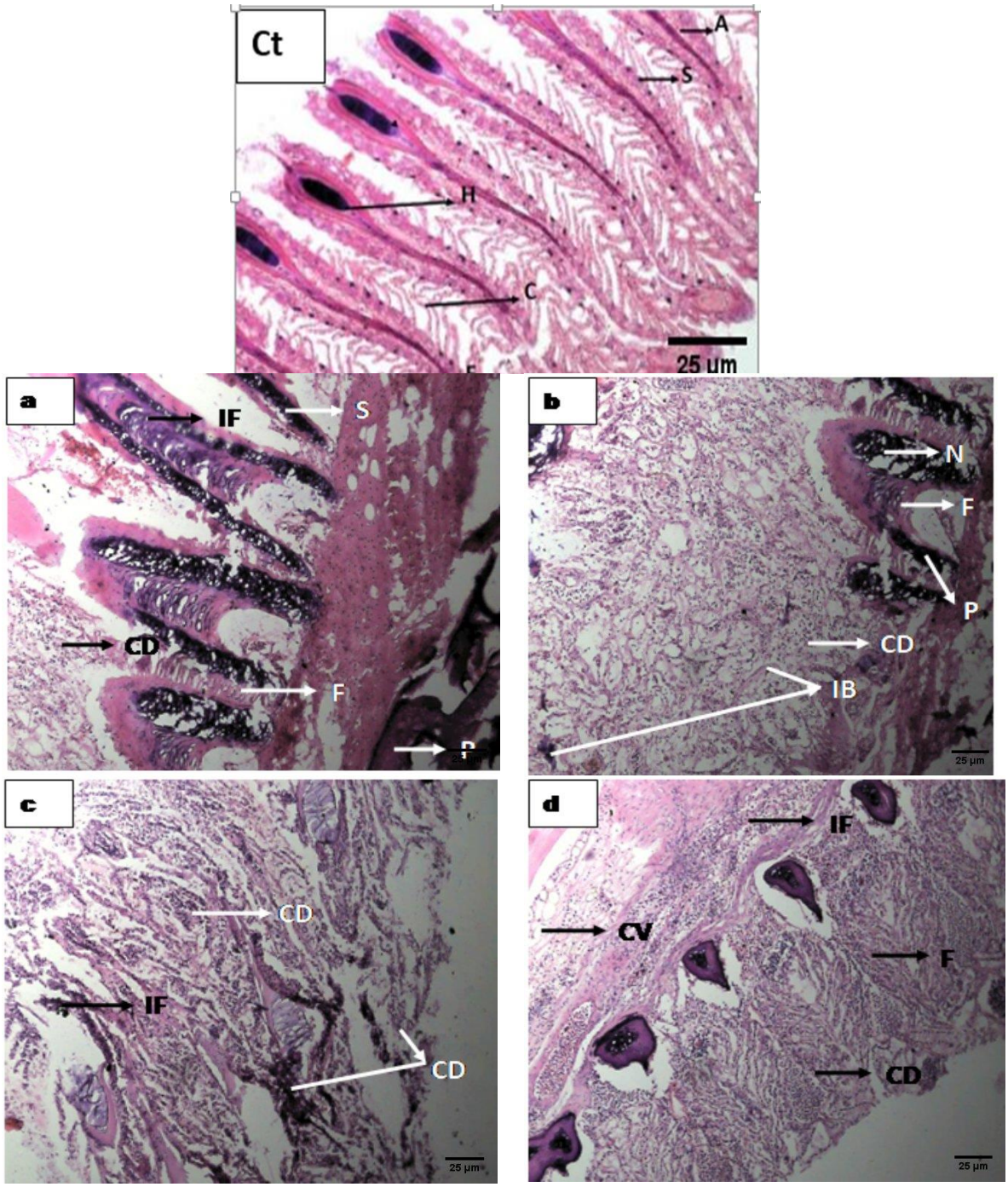

Fig. 5 - Gill histology of African Catfish (*Clarias gariepinus*)- Ct: Control shows normal structure of gill arch (A), filament (C), ceratobrachial bone of the arch (H), and nucleus (S). a-d: Gills of Wild Catfishes showing evidence of cellular degeneration (CD), Fusion of secondary lamellae (F), Pigment (P), Inflammation (IF), Loss of secondary lamellae (S), Necrosis (N), Inclusion bodies (IB) and Cytoplasmic vacuolation (CV). H & E stain, x400.

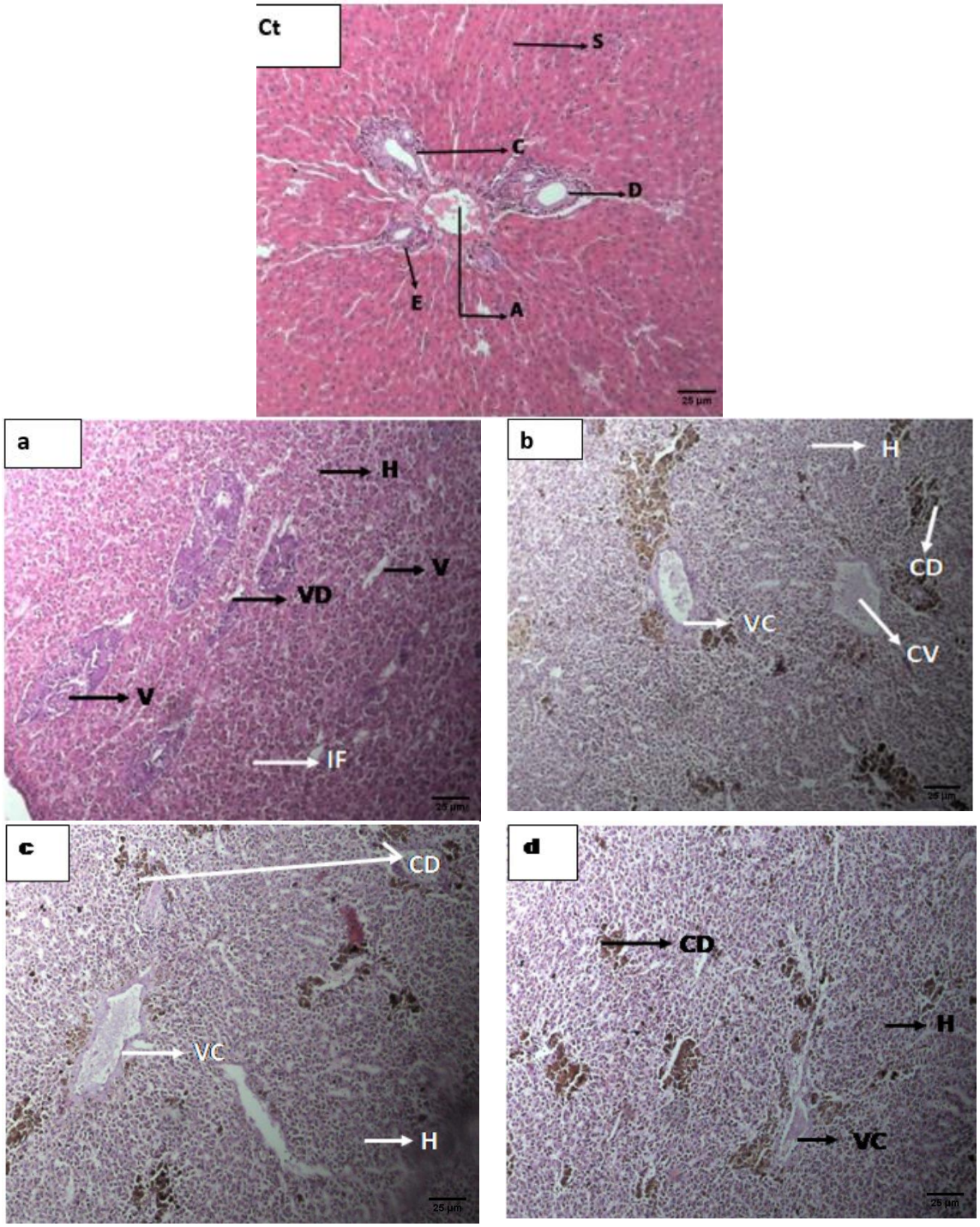

Fig. 6 - Histology of liver of the African Catfish (C*larias gariepinus*). Ct: Control-had normal structural features including central vein (A), bile duct(C), hepatic vein (D), hepatic artery (E) and hepatocytes. a-d: Liver of Wild Catfishes showing evidence of vascular congestion (VC), Vascular degeneration (VD), Inflammation (IF), Vein (V), Hepatocytes H, Cellular degeneration (CD), Central vein (CV). H & E stain, x400.

In the laboratory toxicological evaluations, ethylbenzene was found to be the most lethal when tested against *C. gariepinus*. This result is similar to the 96 hr LC_{50} report from the findings of Barron *et al.*, **[12]**, ethylbenzene was the most toxic chemical to *Pimephales promelas*, a freshwater fish. On the basis of the characteristics of the different compounds, the high toxicity of ethylbenzene can be due to their low vapour pressure, 9.5 mm Hg, which resulted in high concentration in the media (water). Results from ecotoxicology data can be used in the establishment of regulatory standards for water quality for BTEX compounds in developing countries which will preserve organisms in water bodies. BTEX compounds are the water-soluble fractions of petroleum products, as a result they are readily available to aquatic animals.

Histopathological changes were observed in the organs exposed to the BTEX compounds. Fish gills are very efficient as indicators of the quality of water in an aquatic environment **[13]** mainly because of their large surface area and external position. The report of this study agrees with the findings of López-López, and Sedeño-Díaz **[14]**. The outcome of physiological and biochemical changes in an organism can manifest as histological alterations and these represent reliable biomarkers of exposure to toxicants **[15]**. The concentration of chemicals and time of exposure determines the severity of the damage to the gills **[16]**. Therefore, the histopathological alterations noted in the fish tissues in this study, are the effect of pollutants at the histological level. Liver histopathology and macrophage aggregates which have been used by the United States National Marine Fisheries Services, is being proposed by the WHO-FAO to be included in monitoring programmes in other countries **[17, 18]**. The changes in the liver observed in this study such as inflammation, nuclear abnormality, irregular shaped hepatocytes, and increased nuclear vacuolation have been associated with fish exposed to hydrocarbons **[19, 20, 21].** The liver necrosis observed can be a response to the hydrocarbon. Increased vacuolisation of the hepatocytes as a signal of degenerative process that suggests metabolic damage, possibly related to exposure to contaminated water **[20].**

The hepatic tissue in this study was observed to be spotted and dark coloration were observed. Melanomacrophages are known by their dark pigments and are the spotted structures observed in this study. Melano-macrophages are pigment (melanin) containing cells and are associated with severe inflammatory lesions. In a state of environmental stress, melano-macrophage centres increase in size and they have been proposed as efficient indicators for water quality **[22]**. Similar effects in gonads observed in this study have been noticed by several authors including **[23, 24].** Tyor and Pahwa **[25]** observed higher incidence of atretic and deformed oocytes in fish collected from river Yamuna in Delhi region, India. *Tilapia guineensis* exposed to sublethal concentrations of parateq produced deformed oocytes **[26]**. Melanomacrophages were observed in the ovaries of *Corbicula fluminea* exposed to Polychlorinated biphenyl suggesting that the melanomacrophages probably serve as the "clean-up crew" which are involved in innate immunity and usually associated with atretic oocytes **[27]**. The reproductive success of fish can be threatened following reduced quality of gametes as a result of pollution. Histological alterations of gonads of fish resulting from environmental pollutants have been researched to be an effective biomarker tool in environmental monitoring **[28]**. Similar findings to results from this study on the effects of pollutants on fish have been reported **[29, 30]**. The information provided in this study reveals that fish histopathology could be used as biomarkers to provide details on the effects of hydrocarbons on fish health.

4. Conclusion

This study revealed obvious histological alterations in the gill, liver and gonad of African Catfish (C*larias gariepinus*) after exposure to sub-lethal concentrations of benzene, toluene, ethylbenzene and xylene (BTEX). Our finding also revealed some histopathological alterations in the gonad, gill and liver of *C. nigrodigitatus, T. zillii* and C. *gariepinus.* The histopathological changes observed in the selected wild fish species, *C. nigrodigitatus, T. Zillii* and *C.gariepinus* confirmed their relevance as useful biomarkers of hydrocarbon.

References

[1] Sogbanmu, T. O., Nagy, E., Phillips, D. H., Arlt, V. M., Otitoloju, A. A., & Bury, N. R. (2016). Lagos lagoon sediment organic extracts and polycyclic aromatic hydrocarbons induce embryotoxic, teratogenic and genotoxic effects in Danio rerio (zebrafish) embryos. *Environmental Science and Pollution Research*, *23*(14), 14489-14501.

[2] Sogbanmu, T. O., Osibona, A. O., Oguntunde, O. A., & Otitoloju, A. A. (2018). Biomarkers of toxicity in Clarias gariepinus exposed to sublethal concentrations of polycyclic aromatic hydrocarbons. *African journal of aquatic science*, *43*(3), 281-292.

[3] Doherty, V. F., & Otitoloju, A. A. (2013). Monitoring of soil and groundwater contamination following a pipeline explosion and petroleum product spillage in Ijegun, Lagos Nigeria. *Environmental monitoring and assessment*, *185*(5), 4159-4170.

[4] Varjani, S. J. (2017). Microbial degradation of petroleum hydrocarbons. *Bioresource Technology*, *223*, 277-286.

[5] Sogbanmu, T. O., Nagy, E., Phillips, D. H., Arlt, V. M., Otitoloju, A. A., & Bury, N. R. (2016). Lagos lagoon sediment organic extracts and polycyclic aromatic hydrocarbons induce embryotoxic, teratogenic and genotoxic effects in Danio rerio (zebrafish) embryos. *Environmental Science and Pollution Research*, *23*(14), 14489-14501.

[6] Santana, M. S., Sandrini-Neto, L., Neto, F. F., Ribeiro, C. A. O., Di Domenico, M., & Prodocimo, M. M. (2018). Biomarker responses in fish exposed to polycyclic aromatic hydrocarbons (PAHs): Systematic review and meta-analysis. *Environmental pollution*, *242*, 449-461.

[7] Javed, M., Ahmad, I., Usmani, N., & Ahmad, M. (2016). Studies on biomarkers of oxidative stress and associated genotoxicity and histopathology in Channa punctatus from heavy metal polluted canal. *Chemosphere*, *151*, 210-219.

[8] An, Y.J. (2004). Toxicity of Benzene, Toluene, Ethylbenzene and Xylene (BTEX) mixtures to *Surghum bicolor* and *Cucumis sativus. Bulletin of Environmental Contamination and Toxicology.* 72: 1006-1011.

[9] Kundu, D., Mondal, S., Dutta, D., Haque, S., & Ghosh, A. R. (2016). Accumulation and contamination of lead in different trophic levels of food chain in sewage-fed East Kolkata Wetland, West Bengal, India. *Int J Env Tech Sci*, *2*, 61-68.
[10] Hamza-Chaffai, A. (2014). Usefulness of bioindicators and biomarkers in pollution biomonitoring. *International Journal of Biotechnology for Wellness Industries*, *3*(1), 19-26.
[11] Otitoloju, A.A. (2000). Joint action toxicity of Heavy metals and their bioaccumulation by benthic animals of the Lagos lagoon. PhD thesis. University of Lagos. 229pp
[12] Barron, M. G., Lilavois, C. R., & Martin, T. M. (2015). MOAtox: A comprehensive mode of action and acute aquatic toxicity database for predictive model development. *Aquatic Toxicology*, *161*, 102-107.
[13] Fonseca, A. R., Fernandes, L. S., Fontainhas-Fernandes, A., Monteiro, S. M., & Pacheco, F. A. L. (2016). From catchment to fish: impact of anthropogenic pressures on gill histopathology. *Science of the Total Environment*, *550*, 972-986.
[14] López-López, E., & Sedeño-Díaz, J. E. (2015). Biological indicators of water quality: The role of fish and macroinvertebrates as indicators of water quality. In *Environmental indicators* (pp. 643-661). Springer, Dordrecht.
[15] Wang, L., Al-sawafi, A. G. A., & Yan, Y. (2017). Biochemical Changes in the Tissues of Zebrafish (Danio rerio) Exposed to Organophosphorous Pesticides and Heavy Metal Cadmium. *International Journal of Environmental Science and Development*, *8*(10).
[16] Vergilio, C. S., CEV, C., & Melo, E. J. T. (2018). Accumulation and histopathological effects of mercury chloride after acute exposure in tropical fish Gymnotus carapo. *Journal of Chemical Health Risks*, *2*(4).
[17] Paschoalini, A. L., Savassi, L. A., Arantes, F. P., Rizzo, E., & Bazzoli, N. (2019). Heavy metals accumulation and endocrine disruption in Prochilodus argenteus from a polluted neotropical river. *Ecotoxicology and environmental safety*, *169*, 539-550.
[18] Camargo, M. M., & Martinez, C. B. (2007). Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream. *Neotropical Ichthyology*, *5*(3), 327-336.
[19] Haque, S., Mondal, S., Kundu, D., & Ghosh, A. R. (2017). Effect of multiple polycyclic aromatic hydrocarbons (PAHs) on liver of three teleostean fishes Labeobata, Labeorohita and Cirrhinusmrigala in Burdwan Loco Tank, Burdwan, West Bengal, India. *Austin Environ. Sci.*, *2*(1), 1017.
[20] Sumi, N., & Chitra, K. C. (2017). Histopathological alterations in gill, liver and muscle tissues of the freshwater fish, Pseudetroplus maculatus exposed to fullerene C60. *International Journal of Fisheries and Aquatic Studies*, *5*(3), 604-608.
[21] Ahmed, Y. H., Bashir, D. W., Abdel-moneam, D. A., Azouz, R. A., & Galal, M. K. (2019). Histopathological, biochemical and molecular studies on the toxic effect of used engine oil on the health status of Oreochromis niloticus. *Acta histochemica*, *121*(5), 563-574.
[22] Steinel, N. C., & Bolnick, D. I. (2017). Melanomacrophage centers as a histological indicator of immune function in fish and other poikilotherms. *Frontiers in immunology*, *8*, 827.
[23] Tjahjaningsih, W., Pursetyo, K. T., & Sulmartiwi, L. (2017, February). Melanomacrophage centers in kidney, spleen and liver: A toxic response in carp fish (Cyprinus carpio) exposed to mercury chloride. In *AIP Conference Proceedings* (Vol. 1813, No. 1, p. 020012). AIP Publishing.
[24] Sayed, A. H., & Younes, H. A. (2017). Melanomacrophage centers in Clarias gariepinus as an immunological biomarker for toxicity of silver nanoparticles. *Journal of microscopy and ultrastructure*, *5*(2), 97-104.
[25] Tyor, A. K., & Pahwa, K. (2017). Pollutants induced oxidative stress, DNA damage and cellular deformities in Clarias gariepinus (burchell), from river Yamuna in Delhi region, India. *Bulletin of environmental contamination and toxicology*, *99*(1), 33-38.
[26] Akpu, V.I. and Chinda, A.C. (2009). Gonad histology in post fingerlings *of Tilapia guineensis* exposed to parateq. *Revista UDO Agricola*. 9 (3): 672-680
[27] Lehman, D.W., Levine, J.F. and Law, J.M. (2007). Polychlorinated Biphenyl exposure causes gonadal atrophy and oxidative stress in *Corbicula fluminea clams. Toxicologic Pathology. 35*: 357-365.
[28] Solé, M., Mañanós, E., & Blázquez, M. (2016). Vitellogenin, sex steroid levels and gonadal biomarkers in wild Solea solea and Solea senegalensis from NW Mediterranean fishing grounds. *Marine environmental research*, *117*, 63-74.
[29] Adeogun, A. O., Onibonoje, K., Ibor, O. R., Omiwole, R. A., Chukwuka, A. V., Ugwumba, A. O., ... & Arukwe, A. (2016). Endocrine-disruptor molecular responses, occurrence of intersex and gonado-histopathological changes in tilapia species from a tropical freshwater dam (Awba Dam) in Ibadan, Nigeria. *Aquatic Toxicology*, *174*, 10-21.
[30] Ngcobo, S. (2017). *The suitability of estrogen and androgen bioassays for the measurement of endocrine activity in different water matrices* (Doctoral dissertation, University of Pretoria).