Scholars Academic Journal of Biosciences (SAJB)
Abbreviated Key Title: Sch. Acad. J. Biosci.
ISSN 2347-9515 (Print)
ISSN 2321-6883 (Online)

A Unit of Scholars Academic and Scientific Society, India
www.saspublishers.com

Anatomy

Micromorphological Study of Clarias Gariepinus' Vital Organs: An Ecotoxicological Study of Commercial Fish Farm in Ogbogoro, Rivers State, Nigeria

Paul John Nwolim*, Ogbilikana Prince Sampson
Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, University of Port Harcourt, Port Harcourt, Nigeria

Original Research Article

***Corresponding author**
Paul John Nwolim

Article History
Received: 03.07.2018
Accepted: 11.07.2018
Published:30.07.2018

DOI:
10.36347/sajb.2018.v06i07.011

Abstract: The study is an ecotoxicological evaluation of commercial fish farm in Ogbogoro (OGB), Rivers State Nigeria using micromorphological assessment of Clarias gariepenus, and African Aquaculture Centre (ARAC) as a the reference site. The sampling involved harvesting of table-sized fish: twenty fishes from OGB and ten fishes from ARAC). The micro-morphological assessment involved the determination of the qualitative and semi-quantitative analysis of the gills, Liver and kidney of the harvested fishes. Histological assessment of gills, liver and kidney showed almost same prevalence in lesion in OGB as compared to ARAC. The major histological alterations observed were circulatory disturbances (CD) which includes hyperaemia, haemorrhage, and vacuolation. Regressive change (RC) which includes necrosis and progressive change (PC). The Histological Alteration Index or Fish Index (FI) of OGB (14.7) is slightly greater than ARAC (14.6). It was concluded therefore that OGB fish farm was moderately polluted.

Keywords: Micromorphology, Ecotoxicology, Necrosis, Vacuolation and Hyperaemia.

INTRODUCTION

Fish farming is becoming an interesting business for most farmers. This is because there is a ready market for it and the Government has not considered regulating the growth of fish farms in the nation at the moment. This has allowed people who are not well informed about fish farming to join the business, thereby producing low quality fishes that are not healthy for human consumption. The fact remains that the health of these farms will directly impact on the consumers and in turn the nation[1-3].

Aim

This study was done to assess the micro-morphological features of the fish organs to ascertain their level of health in Ogbogoro and ARAC fish farms.

Significance of study

This study would provide baseline data for food consumption regulatory agencies or environmental pollution control agency as a precursor for Fish Consumption Advice or Environmental Alert. It will also provide commercial fish farmer scientific evidence of the pollution status of their fish ponds.

Scope of study

Circulatory disturbances (CD) (hyperaemia, haemorrhage, and vacuolation), Regressive change (necrosis and progressive change) in vital organs (Gills, Livers and Kidneys).

Experimental Area (Ogbogoro Commercial Fish Farm)

Ogbogoro is a community, located in Obior Akpor Local Government Area of Rivers state. It is bounded by, Choba, Rumuekini, Emohua and Diobu. The geographical coordinates are, 4º 50¹ 48¹¹North, 6º 55¹ 50¹¹ East in DMS- Degrees Minutes Seconds or 4.8451ºN, 6.9290ºE, [in decimal degrees] [4].

The pond is sited in a swampy area which is prone to flood during the mid-raining season. It is located far from residential building. Aside the fishing section in the establishment, there are sections for piggery and poultry farming, although no animals were in them as of the time of my harvest of fishes for my experiment. The pond is divided into units according to fig. 2.2. Each of this units has nothing less than 200 fishes in them. The fishes in each unit vary in size/age ranging from, hatchling, fingerling, nursery, table size.

Water regulation

According to koi food, the ideal regime for water changes is 10% per week or 20% per two weeks or 50% every six weeks. The percentage of water changed depends on the level of haze or cloudy appearance seen on the water, which is sign of high organics aquamed.com, 2000. 75% - 80% water must be changed at least twice per year. In Ogbogoro, the pond water is changed every two weeks, completely, although varies depending on the level of pollution of the water [5].

Feeding

Commercial fish feeds are concentrated, and if too much is eaten, it will pass through the fish only partly digested and then pollute the water. If feeding as a supplement to natural foods in the pond, two or three times a week will be ample [6]. If excess food goes uneaten, it should be netted out to avoid pollution and feed less the next time. Ogbogoro commercial fish pond, feed their fishes during the day with fish feeds like coppens.

Reference Area (ARAC)

African Regional Aquaculture Centre (ARAC) was chosen as the control site. It is situated at the training centre, Omuihuechi, Aluu in Ikwere Local Government Area of Rivers State. Most of the activities in the centre include research, training, and development of sustainable aquaculture options, in sub Saharan African. It covers an area of 81 hectares of land.

It's a centre of excellence that focuses on multidisciplinary approach to user-driven aqua cultural research, development and training in sub-Saharan Africa geared towards sustainable fish production in the region.

STUDY SPECIES

African catfish (Clarias gariepinus) is one of the most important primary treatments for tropical cultured fish due to high growth rate, high stocking-density capacities, and high resistance to poor water quality and oxygen and considered as a model for Eco toxicological studies.

African catfish (Clarias gariepinus)

Natural Distribution

They are found throughout Africa and the Middle East, and live in freshwater lakes, rivers, and swamps, as well as human-made habitats, such as oxidation ponds or even urban sewage systems. The African sharp tooth catfish was introduced all over the world in the early 1980s for aquaculture purposes, so is found in countries far outside its natural habitat, such as Brazil, Vietnam, Indonesia, and India[7].

Habitat

It is a nocturnal fish like many catfish. It feeds on living, as well as dead, animal matter. Because of its wide mouth, it is able to swallow relatively large prey whole. It has been known to take large water birds such as the common moorhen. It is also able to crawl on dry ground to escape drying pools. Further, it is able to survive in shallow mud for long periods of time, between rainy seasons. African catfish sometimes produce loud croaking sounds, not unlike the voice of the crow [8].

Natural spawning

Spawning mostly takes place at night in the shallow, inundated areas of the rivers, lakes and streams. Courtship is preceded by highly aggressive encounters between males. Courtship and mating takes place in shallow waters between isolated pairs of males and females. The male lies in a U-shape curved around the head of the female, held for several seconds. A batch of malt and eggs is released followed by a vigorous swish of the female's tail to distribute the eggs over a wide area. The pair usually rests after mating (from seconds up to several minutes) and before resume mating [9-10].

Parental care for ensuring the survival of the catfish offspring is absent except by the careful choice

of a suitable site. Development of eggs and larvae is rapid, and the larvae are capable of swimming within 48–72 hours after fertilization [11-15].

MATERIALS AND METHOD

Phases of Study

There are two phases of the study- phase one and two *Phase One (Preliminary Study):* The experimental site was inspected and vital information gotten as questions were asked. Such questions included: the number of fishes contained in the pond, type and frequency of fish feed used, treatment administered to fish in poor health condition, mode and frequency of changing the water content of the pond. These questions were asked in order to obtain necessary information about the experimental site.

A sample fish was harvested and taken to the African Regional Aquaculture Centre for identification by a taxonomist.

Phase Two (Sampling of Fish)

According to Institute of Veterinary Research and Food Security, Tirana, Albania, The European standards for fish sampling in lakes determined the sampling protocols and methodology developed in the course of fish and fishery monitoring for Prespa lakes. The sampling procedure was based on stratified random sampling.

Control Sample

Control fishes were harvested from ARAC, this was done by first collecting some water content of the pond into a plastic container which would contain the fishes from the control site to the laboratory. The essence was so that the original aquatic habitat of the fishes will remain the same after harvesting as it was before. Failure to do this would have led to alteration of the fish habitat by that distorting the internal morphology of the fishes. Thereafter, the remaining water content of the pond was drained, then with the aid of a seine; ten table-sized cat fishes were harvested and put into the plastic container in which there was exactly the same water content of the pond.

Experimental Sample

Experimental fishes were harvested following similar procedure used for the control. Twenty table-sized cat fishes were harvested from the pond

MICRO-MORPHOLOGICAL ASSESSMENT

20 table-sized fish were harvested from Ogbogoro commercial fish pond and 10 table sized fish were also harvest from ARAC fish pond and organs of interest (Gill, Liver, and Kidney) were harvested for histological assessment. This assessment involved the microscopic study of the tissues and was divided into two qualitative and semi quantitative.

Tissue Preparation

This assessment involved histological processes in order to view the cells of harvested organs with the aid of a light microscope, these processes are as follows:

STEP 1 (PITHING)

This is done by positioning the pointer end of a knife above its brain. Push it down quickly into the brain cavity of the fish.

Locate the spot marked on the head, cut through with a sharp knife. STEP 2 (RESECTION) Cut a slit along the belly of the catfish all the way to the anal fin.This is done with the aid of a dissecting kit (i.e., a scalpel and a scissors to be precise) to harvest the organs of interest from the sample fish while the heart of the fish is still beating.

STEP 3 (FIXATION)

This was done by immersing the organs in 10% formal saline (10mls formaldehyde in 90mls of water) after excision. Fixation is the process of preserving biological tissues from decay, therefore preventing putrefaction or autolysis. Some types of chemical fixatives are: formaldehyde, glutaraldehyde, alcohol, and acetone. Formaldehyde was used. This is because….

The formalin solution slowly penetrated the tissues, caused them to harden and preserved the tissues. They were left in the fixative for about 24hours to allow the fixative penetrate into every part of the tissue.

STEP 4 (DEHYDRATION)

The tissue samples were dehydrated to remove their water content. Alcohol was used. Dehydration is commonly carried out by immersing specimen in different grades of alcohol of increasing concentrations until 100 % (absolute) alcohol. In this step, the alcohol penetrates the tissue quickly and the water is replaced with alcohol.

Normally, a series of increasing concentrations is used in the following sequence [33] alcohol 50%, 70%, 90%, 95%, 100% for 6 hours each.

STEP 5 (CLEARING)

The alcohol used for dehydration of the tissue had to be cleared off the tissues using xylene. A process called clearing.

STEP 6 (IMPREGNATION)

After clearing, the internal spaces in the tissues were filled with molten paraffin wax. A process called impregnation. A typical wax is liquid at 60°C and can be infiltrated into tissues at this temperature then allowed to cool to 20°C where it solidifies to a

consistency that allows sections to be consistently cut [31].

STEP 7 (EMBEDDING)

The tissue samples which had been thoroughly infiltrated with wax were formed into "tissue blocks" which could be clamped into a microtome for sectioning. This step was carried out using an embedding mould which was filled with molten wax and the specimen placed into it. The specimen was carefully orientated in the mould because its placement would determine the "plane of section", an important consideration in both diagnostic and research histology. It should be noted that, if tissue processing is properly carried out, the wax blocks containing the tissue specimens are very stable and represent an important source of archival material.

In theory and in practice the paraffin blocks that will be easiest to section contain relatively homogenous tissue of uniform soft consistency (such as kidney), which, when infiltrated with wax, have a consistency similar to that of solidified wax alone (not containing tissue). Tissues of a dense or fibrous nature or a specimen where both hard and soft tissue are present in discrete layers can pose more of a challenge because parts of them are not so well supported by the solidified wax. Differential shrinkage of the various elements in these blocks during fixation and processing contributes to the problems that might be experienced when they are being sectioned[32].

STEP 8 (SECTIONING)

The first step to sectioning is trimming; this is to reduce the excess solid paraffin wax in which the tissue was embedded. It is done to expose tissue before sectioning to ensure fine thin sections, trimming is done by setting the microtome machine to10- 20 microns. This was done to the already embedded tissues, thereafter the tissues were sectioned at 3-5 microns. Sectioning tissues is a real art and takes much skill and practice. It is important to have a properly fixed and embedded block or much artifact can be introduced in the sectioning. Common artifacts include tearing, ripping, "venetian blinds", holes, folding, etc. Once sections are cut, they are floated on a warm water bath that helps remove wrinkles. Then they are picked up on a glass microscopic slide.

The glass slides are then placed in a warm oven for about 15 minutes to help the section adhere to the slide. If this heat might harm such things as antigens for immunostaining, then this step can be bypassed and glue-coated slides used instead to pick up the sections.

STEP 9 (STAINING)

The whole process must be reversed in order to get the paraffin wax out of the tissue and allow water soluble dyes to penetrate the sections. Therefore, Routine H&E (haematoxylin and eosin) was done using the following procedures:

- The tissues were dewaxed in xylene 1 and 2.
- Then hydrated in descending grades of alcohol and brought to water.
- They were stained in haematoxylin for 25-30minutes, brought to water.
- Differentiation in 1% alcohol was done thereafter, rinsed in water immediately.
- The slides were further rinsed in 1% ammonia water, rinsed in water, and then stained in eosin for 2mins.
- The slides were placed in the oven to dry.

STEP 10 (MOUNTING/COVER SLIPPING)

The stained section on the slide must be covered with a thin piece transparent plastic or glass to protect the tissue from being scratched, to provide better optical quality for viewing under the microscope, and to preserve the processed tissues. Therefore the stained tissues on the slides were covered using a plastic coverslip.

SEMI QUANTITATIVE HISTOLOGICAL ASSESSMENT

This is a qualitative assessment protocol that is use to quantify histopathological alterations observed in the sections of each of the organ. A qualitative histopathological assessment was done using CX31 Olympus light microscope. Tissue sections were scanned on 400x magnification. The result were semi-quantitatively assessed using part of a scoring system [25] modified from the protocol by [30]. In brief, the tissue samples were assessed by identifying histopathological alteration in terms of reaction patterns including:

- Circulatory disturbance
- Regressive changes
- Progressive changes
- Inflammatory responses
- Neoplasia; if identified, the alteration was given an importance factor which represents the potential of the alteration to affect fish health: 1 (alteration is reversible; 2 (alteration is reversible if the stressor is neutralised); 3 (alteration is irreversible). A score value, representing the occurrence of the alteration throughout the tissue was also assigned: 0 (absent), 2 (mild), 4 (moderate), and 6 (severe). The score value and the importance factor for each alteration were multiplied and these results for all the alterations identified in a single organ were then summed to give an organ index per fish. Thus, 3 organ indices were calculated: gill index; Liver index; Kidney index. These organ indices were calculated for each sample fish. A mean of each organ index was calculated for each sample group (experimental and control group) and was used to compare the same organs between the groups.

- The sum of the three indices per fish yielded a total fish index value. This index indicates the combined histological response of the sampled organs for the individual fish. A mean fish index was calculated for the total sample group per species.
- Mathematical calculations of lesion indices:

Where org= organ; rp= reaction pattern (constant); alt= alterations; a= score value; w = importance factor.

- **Organ index:** The organ index (Iorg) represents the extent of damage to an organ. It allows for comparison of the extent of damage of the same organ in different individuals and is calculated as follows:

$I_{org} = \sum_T \sum_{alt} (a_{org\ rp\ alt} \times w_{org\ rp\ alt})$.

- **Total fish index**

The fish index (Ifish) signifies a measure of the overall health status based on the lesions observed. It is also possible to compare individuals as the Ifish for each fish is calculated the same way:

$\mathbf{I}_{fish} = \Sigma_{org} \Sigma_{rp} \Sigma_{alt} (a_{org\ rp\ alt} \times w_{org\ rp\ alt})$.

Furthermore, a modified classification system by [27] based on a scoring scheme by Zimmerli *et al*. [29] was employed to evaluate the degree of histological changes. This classification system is based on the calculated mean organ index values.

Class 1 (index value <10): Slight histological alterations.

Class 2 (index value 10-25): Moderate histological alterations.

Class 3 (index value 26-35): Pronounced alterations of organ tissue.

Class 4 (index value >35): Severe alterations of organ tissue.

RESULTS

Micro-morphological assessment of the gills

Qualitative histological assessment results showed that circulatory disturbances (CD) and regressive changes (RC) were identified in gill tissue. Figure 1 rpresent the microscopic photograph of the gill tissue for *C. gariepinus*. CD included hyperemia, haemorrhage, vacuolation and epithelial lifting while RC included architectural and structural alterations of epithelial cells were so high with samples from experimental site (Figure 1: b-f) and necrosis. Structural changes in the form of fusion of secondary lamellae and fusion of adjacent lamella were also noted (Figure 1: a-d). Secondary lamella with lamella hyperaemia was identified more in fish specimens from Ogbogoro while there was no necrosis identified in ARAC specimens.

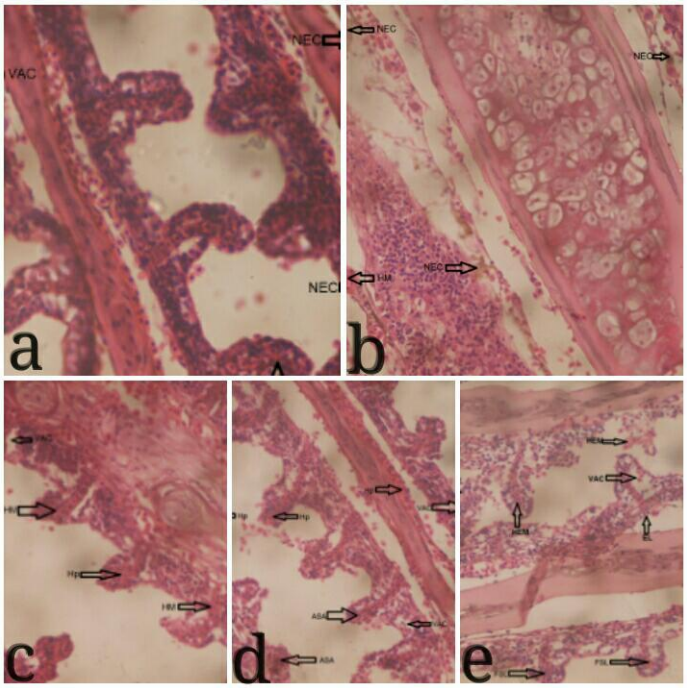

Fig-1: (a-e) Gills microscopic structures {H and E, x400}, (a-b) indicating Vacoulation (VAC) and necrosis (NEC). (C-d) Hyperemia (Hp) and Haemorrahge (HM). (e). Fusion of secondary lamella (FSL)

Table-1: The percentage prevalence of Gills micro-morphology of fishes harvested from OGBOGORO and ARAC

Alteration	Prevalence (%)	
	OGBOGORO (n=20)	ARAC (n=10)
Circulatory Disturbance (CD)		
Haemorrhage	11.25	16.4
Vacuolation	23.75	28.4
Hyperaemia	13.75	7.5
Regressive Change (RC)		
Structural alterations	32.5	38.8
Necrosis	7.5	0
Progressive Change (PC)		
Epithelial Lifting	11.25	8.96
Average % Prevalence	16.67	16.68

Percentage prevalence for each alteration is calculated by the total number of each alteration divided by the overall sum of the organ alterations, multiplied by 100

MICRO-MORPHOLOGICAL ASSESSMENT OF LIVER

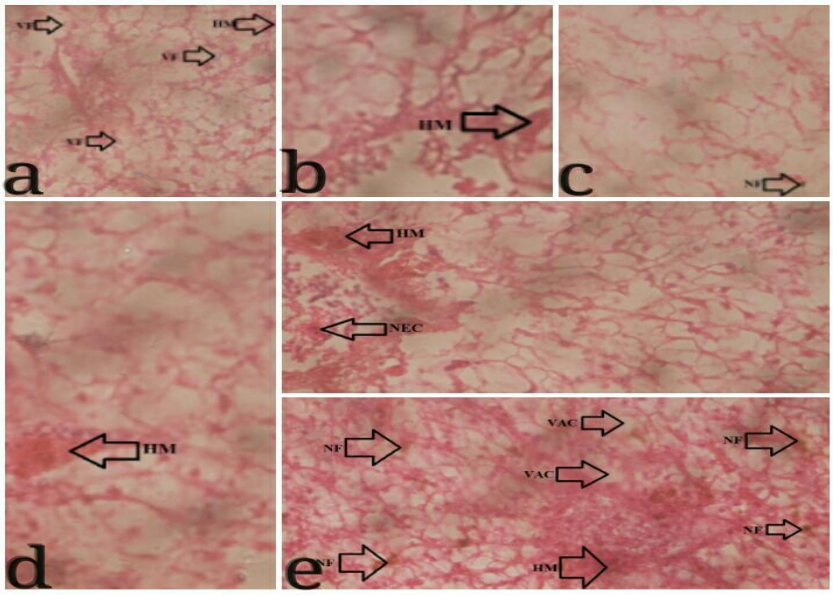

Fig-2: (a-e) Liver microscopic structures {H and E, x400 (a) Control showing Hemorrhage (HM) and vacuolat (b). Haemorrahge (HM) (c) Necrotic foci (NF). (d & e). Vacuolation(vac), Hemorrhage and Necrotic foci.

A variety of histological alterations were identified in the liver tissue (Figure 2: a-e). These alterations included vacuolated hepatocytes, necrosis, haemorrhage, foci of cellular alteration (FCA) which includes vaculated and necrotic foci. Vacuolated hepatocytes and haemorrhage were more prominent in fish specimen from Ogbogoro. Percentage prevalence of FCA and RC were not so different between ARAC and Ogbogoro (table 2)

Table-2: The percentage prevalence of Liver of fishes harvested from OGBOGORO and ARAC

Alteration	Prevalence (%)	
	OGBOGORO (n=20)	ARAC (n=10)
Circulatory Disturbance (CD)		
Haemorrhage	25	20.75
Vacuolation	46.25	37.74
Regressive Change (RC)		
Necrosis	11.25	15.09
Foci of Cellular Alteration (FCA)		
Vacuolated Foci	7.5	62.5
Necrotic Foci	10	16.98
Average % Prevalence	20	20

MICRO-MORPHOLOGICAL ASSESSMENT OF KIDNEY

Histological alterations noted in the kidney tissue were only circulatory disturbances (CD) regressive changes (RC). These alterations included vacuolation, structural alterations and MMCs (Figure 3: a-e). All the noted alterations and their prevalence percentage are represented in table 3.

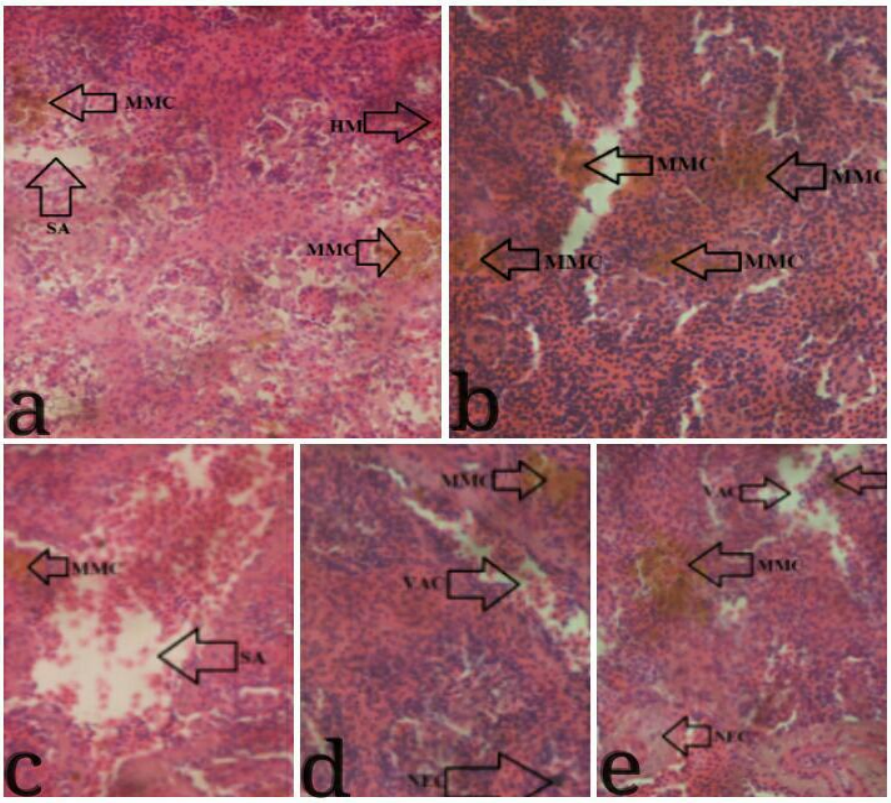

Fig-3: (a-e). Kidney microscopic structure {H and E, x400}

- Showing hemorrhage, melanomacrophage centers (MMC)
- Showing MMCs
- Showing structural alterations (SA)
- Showing necrosis (NEC) and lipid vacuolations (Vac)
- Showing MMCs, VAC, and NEC

Table-3: The percentage prevalence of Kidney histopathology of fishes harvested from OGBOGORO and ARAC

Alteration	Prevalence (%)	
	OGBOGORO (n=20)	ARAC (n=10)
Circulatory Disturbance (CD)		
Haemorrhage	8.77	0
Vacuolation	19.29	14.29
Regressive Change (RC)		
Necrosis	15.79	19.05
Structural Alteration	29.82	28.57
Melano-Macrophage Centers	26.32	38.09
Average % Prevalence	19.99	20

Table-4: Mean organ indices and mean fish index of the semi-quantitative histopathological assessment of C. glariepinus from ARAC AND OGBOGORO.

MEAN INDEX	ARAC	OGBOGORO
Gill	16	18.4
Liver	16.4	11.1
Kidney	11.6	14.6
Mean Fish Index	14.6	14.7

Table-5: The statistical analysis of organ index (Gill, Liver and Kidney)

Groups	Tissue	N	Mean	Std. Deviation	df	F	Sig.
ARAC	Gill	10	16.0000	3.77124		0.770	0.388
OGBOGORO		20	18.4000	8.17184	1		
	Liver						
ARAC		10	11.6000	11.46201		.656	.425
OGBOGORO		20	14.6000	8.51253	1		
	Kidney						
ARAC		11	14.9091	8.26383		1.721	.200
OGBOGORO		20	11.1000	7.44029	1		

The analysis showed that there was no significant difference on comparison between groups.

DISCUSSIONS

GILLS

Histological alterations in varying degrees were identified in gills. These were mostly circulatory disturbances and regressive changes. Circulatory disturbances are related to pathological conditions of blood and tissue fluid flow. Epithelial lifting in focal areas was noted in both fish species. Epithelial lifting is characterized by detachment of epithelial cells due to the outflow of serous fluids into the interstices of gill tissue [26]. This alteration has been observed in various other studies [10-15].

Epithelial lifting may be a defence mechanism of fish in response to toxicants. The lifting up of epithelium increases the distance through which toxicant has to travel to reach the blood stream [14-16].

Structural alterations in the form of lamella fusion were also identified. Fusion of lamellae is the result of hyperplasia of undifferentiated gill epithelial cells. According to Mallat [17] lamella fusion could be protective in that it diminishes the amount of vulnerable gill surface area. This alteration has previously been identified in fish exposed to pesticides Fish [17-21] and polluted streams [26-27].

LIVER

Histological alterations observed were structural alterations such as hypertrophy; circulatory disturbance such as vacuolation, necrosis of hepatic tissue and haemorrhage. It suggests that bioaccumulation of copper in liver tissues caused serious pathological damages to the hepatocytes, which led to lipidoses of the sinusoids and space of Disse [15]. In the current study circulatory disturbances, regressive changes, and focal cellular alterations were identified. The histological responses in the liver were mostly associated with circulatory disturbances and regressive changes including vacoulation, necrosis, and increase in FCA.

Focal cellular alteration was also identified in both fish species in the study [18-22] have previously reported the occurrence of this alteration.

KIDNEY

The kidney alterations showed more of necrosis and melanomacrophage centers (mmc). These alterations were mostly associated with progressive and regressive changes, which could the reason for changes in blood pressure passing through the kidneys and thus affecting the kidney function [23-25]. These were mainly regressive changes and included necrosis, degeneration as well as increase in MMCs.

Necrotic changes were also observed in fish kidney exposed to various chemicals by various authors [20-24]. Alterations such as necrosis could lead to functional problems ultimately leading to the death of fish.

Increase in MMCs was again observed in some fish specimen in the current study. Vinodhini and Narayanan, [26] noted the presence of macrophages in exposed to heavy metals. Bernet [6] also obtained similar results to that of the current study in C. gariepinus from a polluted site.

Melanomacrophage centers were a common occurrence in the kidneys of both species. It has been suggested that MMCs are a normal characteristic in fish tissue [28] but an increase in the number or size of these structures can be as a result of a number of factors, including toxicant exposure [25-27] and possibly age.

CONCLUSION

In conclusion, it suggests that the fishes from Ogbogoro commercial fish farms were moderately contaminated and the level of distortion is not yet lethal as condition factor and health assessment index still indicate that the fishes were normal.

ETHICAL APPROVAL

Ethical clearance was obtained from the University of Port Harcourt Research Ethics Committee before commencement of the study.

ACKNOWLEDGMENTS

The authors are grateful to the Head of Department and staff of the Department of Anatomy, Faculty of Basic Medical Sciences, University of Port Harcourt, for the use of their facilities and equipment

for this study and the managers of the experimental site where this study was carried out.

CONFLICT OF INTEREST

We write to declare that there is no conflict of interest

REFERENCES

1. Donohue JM, Abernathy CO. Exposure to inorganic arsenic from fish and shellfish. InArsenic Exposure and Health Effects III 1999 (pp. 89-98).
2. Akiyoshi H, Inoue A. Comparative histological study of teleost livers in relation to phylogeny. Zoological science. 2004 Aug;21(8):841-50.
3. Alina Bradford. Pollution facts and types of pollution. 2015.
4. Allison TA, Paul CW. Histological based biomonitoring: a baseline ecotoxicological evaluation of New-Calabar River using Chrysichthys nigrodigitatus. Int J Environ Poll Res. 2014;2(3):17-41.
5. Anoop KR, Sundar KSG, Khan BA & Lal S. Common Moorhen *Gallinula chloropus* in the diet of the African catfish *Clarias gariepinus* in Keoladeo Ghana National Park,India. Indian Birds. 2009; 5(2):22-23.
6. Bernet D, Schmidt-Posthaus H, Wahli T, Burkhardt-Holm P. Evaluation of two monitoring approaches to assess effects of waste water disposal on histological alterations in fish. Hydrobiologia. 2004 Aug 1;524(1):53-66.
7. Cengiz EI. Gill and kidney histopathology in the freshwater fish Cyprinus carpio after acute exposure to deltamethrin. Environmental Toxicology and Pharmacology. 2006 Sep 1;22(2):200-4.
8. Cengiz EI, Ünlü E. Histopathological changes in the gills of mosquitofish, Gambusia affinis exposed to endosulfan. Bulletin of environmental contamination and toxicology. 2002 Feb 1;68(2):290-6.
9. Crick FH, Orgel LE. Directed panspermia. Icarus. 1973 Jul 1;19(3):341-6.
10. Dickens CW, Graham PM. The South African Scoring System (SASS) version 5 rapid bioassessment method for rivers. African Journal of Aquatic Science. 2002 Jan 1;27(1):1-0.
11. Froese, Rainer and Pauly, Daniel, eds. "*Clarias gariepinus*" in Fish Base. March 2014 version.2014.
12. Hinton B and Lauren J. Integrative Histopathology Approaches to detecting effect of Environmental Stressors on Fishes. Am. Fish. Soc. Sym. 1990; 81-51-66
13. Huggett RJ. Biomarkers: biochemical, physiological, and histological markers of anthropogenic stress. CRC Press; 2018 Jan 18.
14. Jiraungkoorskul W, Upatham ES, Kruatrachue M, Sahaphong S, Vichasri-Grams S, Pokethitiyook P. Histopathological effects of Roundup, a glyphosate herbicide, on Nile tilapia (Oreochromis niloticus). Science Asia. 2002;28:121-7.
15. Kotsanis N, Iliopoulou-Georgudaki J. Arsenic induced liver hyperplasia and kidney fibrosis in rainbow trout (Oncorhynchus mykiss) by microinjection technique: a sensitive animal bioassay for environmental metal-toxicity. Bulletin of environmental contamination and toxicology. 1999 Feb 1;62(2):169-78.
16. Lara-Ortíz T, Riveros-Rosas H, Aguirre J. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in Aspergillus nidulans. Molecular microbiology. 2003 Nov;50(4):1241-55.
17. Mallatt J. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Canadian Journal of Fisheries and Aquatic Sciences. 1985 Apr 1;42(4):630-48.
18. Maltby L, Naylor C. Preliminary observations on the ecological relevance of the Gammarusscope for growth'assay: effect of zinc on reproduction. Functional Ecology. 1990 Jan 1:393-7.
19. Morgan M, Tovell PW. The structure of the gill of the trout, Salmo gairdneri (Richardson). Zeitschrift für Zellforschung und mikroskopische Anatomie. 1973 Jun 1;142(2):147-62.
20. Olaleye VF, Adewumi AA. African Journal of Agricultural Research. 2001); 6(6):1282-1285.
21. Ortiz JB, De Canales MLG and Sarasquete C. Histopathological changes induced lindane (gamma-HCH) in various organs of fishes. 2003; *Sci.* 67 (1), 53-61.
22. Roberts RJ. *Fish pathology*. WB. Saunders: Harcourt Publishers Ltd. Skelton P. (2001*). A Complete Guide the Freshwater Fishes of Southern Africa.* Struik Publishers. 2001.
23. Skelton PH. A complete guide to the freshwater fishes of southern Africa. Struik; 2001.
24. Skidmore JF, Tovell PW. Toxic effects of zinc sulphate on the gills of rainbow trout. Water Research. 1972 Mar 1;6(3):217-IN4.
25. Marchand MJ, Van Dyk JC, Pieterse GM, Barnhoorn IE, Bornman MS. Histopathological alterations in the liver of the sharptooth catfish Clarias gariepinus from polluted aquatic systems in South Africa. Environmental Toxicology: An International Journal. 2009 Apr;24(2):133-47.
26. Vinodhini R, Narayanan M. Heavy Metal Induced Histopathological Alterations in Selected Organs of the Cyprinus carpio L.(Common Carp). International Journal of Environmental Research. 2009 Jan 1;3(1).
27. Van Dyk JC, Marchand MJ, Pieterse GM, Barnhoorn IE, Bornman MS. Histological changes in the gills of Clarias gariepinus (Teleostei: Clariidae) from a polluted South African urban aquatic system. African Journal of Aquatic Science. 2009 Dec 1;34(3):283-91.

28. Leknes IL. Melano-macrophage centres in the liver of platyfish, Xiphophorus maculatus, Poeciliidae: Teleostei. Zoology. 2004 Sep 16;107(3):201-4.
29. Morgenthaler M, Schweizer E, Hoffmann-Röder A, Benini F, Martin RE, Jaeschke G, Wagner B, Fischer H, Bendels S, Zimmerli D, Schneider J. Predicting and tuning physicochemical properties in lead optimization: amine basicities. ChemMedChem: Chemistry Enabling Drug Discovery. 2007 Aug 13;2(8):1100-15.
30. Bernet D, Schmidt H, Meier W, Burkhardt-Holm P, Wahli T. Histopathology in fish: proposal for a protocol to assess aquatic pollution. Journal of fish diseases. 1999 Jan;22(1):25-34.
31. Seeman-Lodding H, Häggmark S, Jern C, Jern S, Johansson G, Winsö O, Biber B. Systemic levels and preportal organ release of tissue-type plasminogen activator are enhanced by PEEP in the pig. Acta anaesthesiologica scandinavica. 1999 Jul;43(6):623-33.
32. Clayden EC. Practical section cutting and staining.1971.
33. Gurney PC, inventor; Leica Biosystems Melbourne Pty Ltd, assignee. Device and method for cross-referencing. United States patent US 9,691,043. 2017 Jun 27.