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Investigation of Selected Heavy Metals, Histopathological and Oxidative Stress Responses of Nile Tilapia, *Oreochromis niloticus* in Water from the Hadejia-Nguru Wetlands Nigeria.

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Abstract
Hadejia-Nguru wetland has been known to be a productive area of Agricultural engagements and fisheries activities allowing for intensive farming and fishing activities, effluents from agricultural activities, sewage, and chemical seepages enter this water body. This study investigated the levels of several heavy metals Mercury (Hg), Lead (Pb), Cadmium (Cd),Chromium (Cr), and Aluminum (Al) in O. niloticus tissues (gills, liver, muscle) collected from five sampling sites in the study area. Tissue histology and the presence of antioxidant enzymes revealed the degree of tissue damage and stress in fish. The results for heavy metals showed concentrations in the order Hg>Cr>Pb>Al>Cd, which are higher than the maximum residue limits recommended by FAO and WHO. Superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), and reduced glutathione (GSH) activities were observed at high concentrations in gills, liver, and muscle. Liver of the fish showed a high level of SOD concentration of 18.3µg/ml, followed by a concentration of 16.93 µg/ml in the gills, and CAT with a concentration of 1.48 U/ml was observed in the liver, MDA was highest in the liver with a concentration level of 1.08n/mol and there was no significant difference between MDA concentrations in liver and other organs ($P < 0.05$). Reduced glutathione levels of 18.36 µg/ml was seen in the liver of the fish. Histopathology of the select organs i.e. gills, liver and muscle cells showed that the organs of the fish were affected by the level of toxicity in the Hadejia-Nguru environment. The presence of metal toxicity, antioxidant enzymes, and tissue damage in fish is an indication of contamination and serves as a means for monitoring the safety of freshwater organisms.

Keywords: Antioxidants, Cichlid fish, Contaminants, Freshwater wetlands, Toxicity,

INTRODUCTION
Contamination levels in fish are of great interest because of the hazardous risk associated with it especially to humans who consume them. The Hadejia-Nguru wetland is a vast land of Agricultural activities and fisheries known for its richness which allows for intensive farming and fishing activities, it covers a massive expanse of land covering an area of 3,500Km2 (Abubakar *et al*., 2015) This water receives varying level of waste which are discharges of agricultural, municipal, residential or industrial waste products and when contamination in water occurs in high concentration it can be a serious threat because of their toxicity, long persistence and bioaccumulation in fish. Heavy metals are commonly found in natural waters

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and some are essential to living organisms as these metals gain entrance contamination and accumulation sets in. The continuous accumulation of pollutants affects the aquatic organisms (Maruf *et al.*, 2021). The nile tilapia(*Oreochromis niloticus*) is a very important fish in connection to the ecology of tropical and sub-tropical regions and it is considered as the most popular species of the freshwater bony fish in Africa. Rate of bioaccumulation of heavy metals in aquatic organisms depends on their ability to digest and absorb the metals from their environment. Essential metals of the likes of Copper (Cu),Iron (Fe),Manganese (Mn),Nickel (Ni) and Zinc (Zn) are basic requirement for biochemical and physiological functions in the body of fish, while Mercury (Hg), Chromium (Cr), Lead (Pb), Cadmium (Cd) are non-essential with no particular biological role (Saleh *et al*.,2016) hence the latter are among the classified metals of public health significance, they accumulate in the food chain and become carcinogenic with other adverse risks to health of humans as a result of bioaccumulation over time. Many works have been carried out on contamination by heavy metal with their corresponding impact on tissue and biochemical formation, Ochuwa, 2017, observed genotoxic and histopathological changes in *C. gariepinus* exposed to heavy metals, Dooa *et al*., 2011 recorded changes in *O. niloticus* exposed to doses of lead acetate, Udibia, *et al.*, 2014 studied the dispersion of Lead, Copper and Zinc in the muscles of C. gariepinus across River Galma and fish farms in Zaria. Amal and Gad, 2012, reported accumulation of some heavy metals and biochemical alterations in muscles of O. niloticus from River Nile.

Monitoring of contamination levels is necessary as it provides important information about contamination levels (Maurya *et al*., 2018).Oxidative stress biomarkers commonly used in monitoring aquatic ecosystems include superoxide dismutase (SOD), catalase, reduced glutathione, and the lipid peroxidation biomarker malondialdehyde (Gharred *et al*., 2015, Ochuwa *et al*., 2017). Oxidative stress causes cell damage, often associated with tissue damage and structural damage to organs. Histopathological examination provides useful information about changes in tissue architecture that occur due to external influences. The purpose of this work is to assess the effects of heavy metals on inducing body changes in fish. Metals such as mercury, lead, cadmium, chromium and aluminum are known to be harmful to health even at low concentrations. Heavy metal accumulation leads to the production of antioxidants and histopathological changes in fish tissue (Ochuwa, 2017).

MATERIALS AND METHOD

Study area

Hadejia-Nguru Wetland (HNW) lies between latitudes 120°10°N and 130°N, and longitudes 100°15°E and 110°30E. It is located in the semi-arid region of Nigeria. The area of wetlands is about 3,500 km2. The terrain of the area is predominantly low-lying on the northeastern side, with limited local relief in the south and west. Precipitation patterns at the Nguru-Hadejia Wetlands (NHW) have not been stable over the years, but for the most part starts in June and last through September. The vegetation consists mainly of Sudanese savannah, including transitional savannah from northern Guinea and Sahelian savannah on the southern-northern border, respectively Abubakar *et al.* (2015).

Site A: Hadejia barrage dam (Kalgwai)
Site B: Kirikasanma
Site C: Maikintari
Site D: Nguru lake
Site E: Dagona

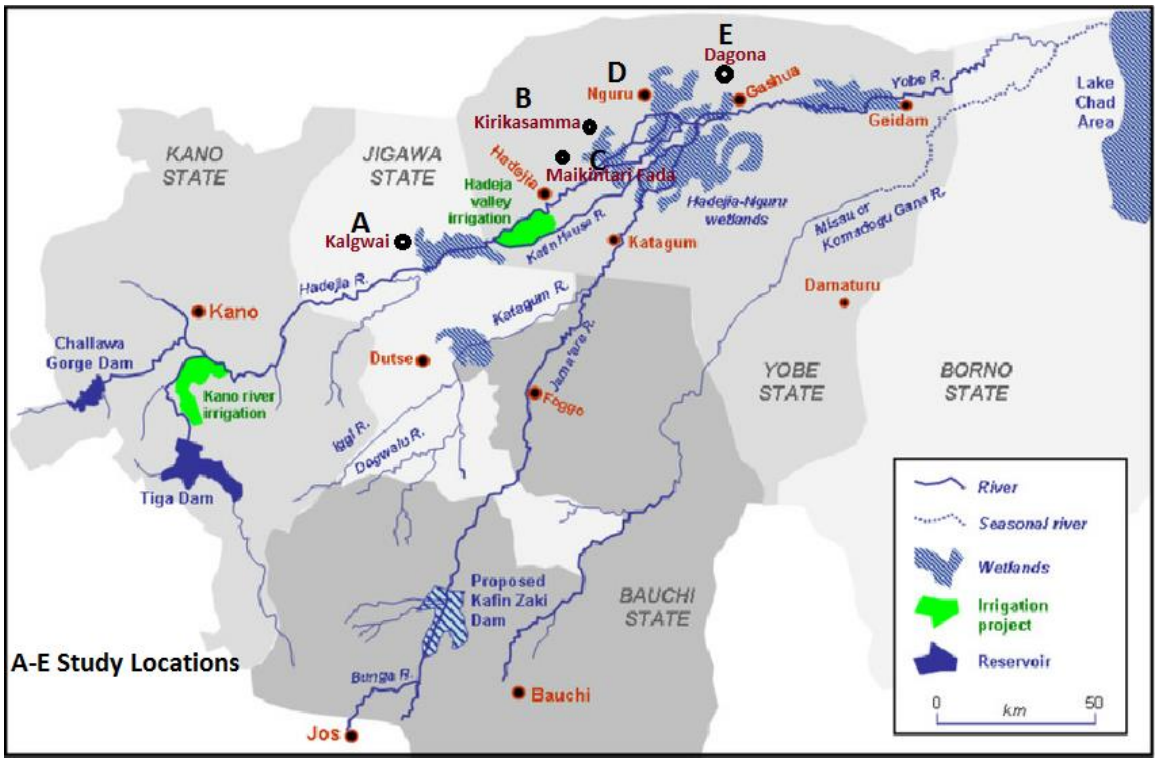

Figure 1: Map of Hadejia-Nguru wetland showing the sampling sites

Fish sampling and analysis: Fish samples (*O. niloticus)* were captured from five sampling sites through the services of hired fishermen and collected bimonthly throughout the study period. A total of 140 fish were used in the experiment. Fish samples were taken in the early morning at 06:00. They were then transported in ice-cold containers to the Laboratory at the Federal University Dutse for dissection and analysis. Control fish were obtained from Rumbun Kifi Fish Farm, Modobbi Road, Dutse, Jigawa. Fish were dissected to remove gills, liver and muscle and stored in a −40 °C refrigerator for further experiments.

Heavy metal analysis

Analysis was performed according to the wet method used by Tyokumbur (2016) Musa and Imam (2021). Dissected gills, livers, and muscles were removed, oven-dried at a temperature of 105 °C to constant weight, and the dried samples were pulverized in a porcelain mortar before crushing. To digest the samples, powdered muscle, gills, and liver were homogenized and powdered samples of concentrated nitric acid and hydrogen peroxide (1: 1) v/v were placed in 250 mL round-bottom flasks and 10 mL each. of HNO_3 (65%) and H_2O_2 (30%) were added to react the contents of the flask. The contents of the flask are heated in a hood on a heating mantle to a melting temperature of 130°C to reduce the volume to 3-4 mL, the digested sample is allowed to cool, filtered into an Erlenmeyer flask, and the filtered sample is added then transferred to a 50ml volumetric flask. The concentrations of Cd, Al, Cr, Pb, and Cd were determined using an atomic absorption spectrophotometer (Buck Scientific Model 230) at the Department of Soil Science, Ahmadu Bello University Zaria. The same was done with water and sediment.

Measurement of Markers of Oxidative Stress

Liver samples were analyzed for markers of oxidative stress: superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and lipid peroxidation (malondialdehyde MDA). These were determined according to the analytical method described by Achuba *et al.*,(2014). Of the isolated gills, liver and muscle 0.5g were separated and homogenized with 10ml of ice-cold

0.05M phosphate buffer pH 7.0 containing 1% (w/v) Triton X-100, excess butylated hydroxyl toluene (BHT) and a few crystals of protease inhibitor, phenylmethylsulfonyl fluoride using an MSE blender immersed in ice. Triton X-100 solubilizes membrane-enclosed organelles while BHT prevents in vitro oxidation of lipid during homogenization. The extract was centrifuged at 7000g for 20 min (40C). The supernatant (S1) was used for the determination of lipid peroxidation by the method of Hunter *et al.,* (1963) as modified by Gutteridge and Wilkins (1982).

Catalase Extraction and Assay

Catalase activity was determined according to Beers and Sizer (1952) by measuring the decrease in H_2O_2 concentration at 240 nm absorbance. An extinction coefficient of 40 M-1 cm-1 for H_2O_2 (Abel, 1974) was used for calculations.

Extraction and Assay of Superoxide Dismutase (SOD)

The resulting supernatant was used to assay superoxide dismutase (SOD) activity based on its ability to inhibit the oxidation of epinephrine by superoxide anions (Aksnes and Njaa, 1981) Enzyme activity was analyzed with an SP 1800 UV/VIS spectrophotometer.

Extraction and Assay of Glutathione (GSH)

Tissue samples were prepared by washing twice with PBS, 0.1 g sample was placed in homogenizer, add 1 ml reagent (keep tissue to reagent ratio constant). Centrifugation was performed at 8000 x g for 10 minutes at 4°C, and the supernatant was placed at 4°C, with complete ice grinding (liquid nitrogen improved the grinding effect). The spectrophotometer was then preheated for 30 minutes and adjusted to a wavelength of 412 nm with distilled water.

Histopathological Examination

Gill, liver, and muscle specimens were prepared, and a small portion of the gill arches, liver lobes, and muscle specimens were removed, fixed in 10% formal saline, dehydrated in ascending alcohols, and treated with toluene and infiltrated with molten paraffin wax. Microtone sections were stained with the hematoxylin and eosin staining technique, examined with a Leica DM 750 microscope and photographed with a Leica ICC 50HD camera (Roberts, 2001; Auwioro, 2010)

Statistical Analysis

The generated data were analyzed using the Social Science Statistical Package (SPSS) version 25. All results are expressed as mean ± standard deviation and data were analyzed using one way analysis of variance (ANOVA). Significant differences between contaminated sites and controls were determined at a 5% confidence level ($P < 0.05$) using Duncan's multiple test range.

RESULTS

Heavy Metals

Results from heavy metal concentration in the gills of *O. niloticus* is presented in figure 1. The result showed that the concentration of Cr was highest in site B with a value of 101.55mg/kg and was followed by the concentration value of 50.15mg/kg of Cr in site C. Concentration of Cr in the gills of the fish from site E was 77.00mg/kg. Heavy metal concentration in the liver of fish is shown in figure 2, highest concentration of Hg was seen in site D with a value 98.75mg/kg followed by a value of 92.15mg/kg of Hg in site E. Concentration of Al was highest in site A with a value 30.15mg/kg followed by site A with a mean concentration value

of 15.50mg/kg. The highest liver concentration of Cr was recorded in the liver of site B with a value of 91.25mg/kg. Figure 3 showed the average mean concentration of the five heavy metals in the fish muscles, highest concentration value of 95.15mg/kg in Hg was seen in site B followed by Hg concentration in site E and D with values of 90.15mg/kg and 90.00mg/kg respectively. Cadmium (Cd) concentration was highest in site B with a value of 2.55mg/kg followed by 2.00mg/kg in site A. The concentration of Pb showed a level of 38.15mg/kg in site E and lowest Pb concentration of 0.55mg/kg was seen in site B.

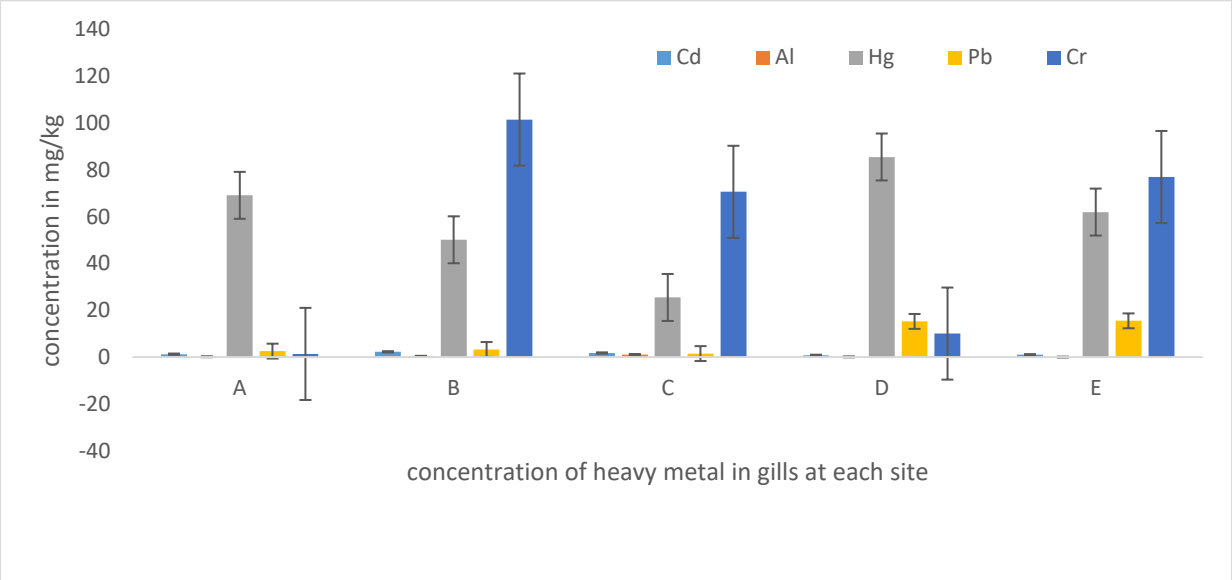

Figure 1: Mean concentration of heavy metal in fish gills in sampling sites

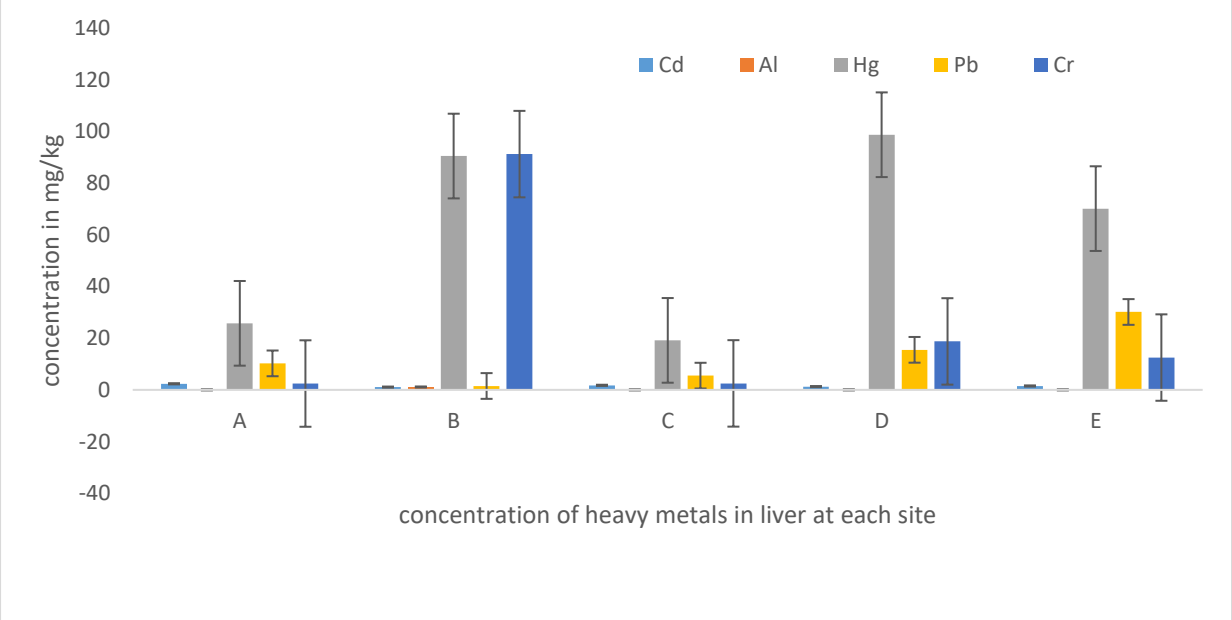

Figure 2: Mean concentration of heavy metal in fish liver in sampling site

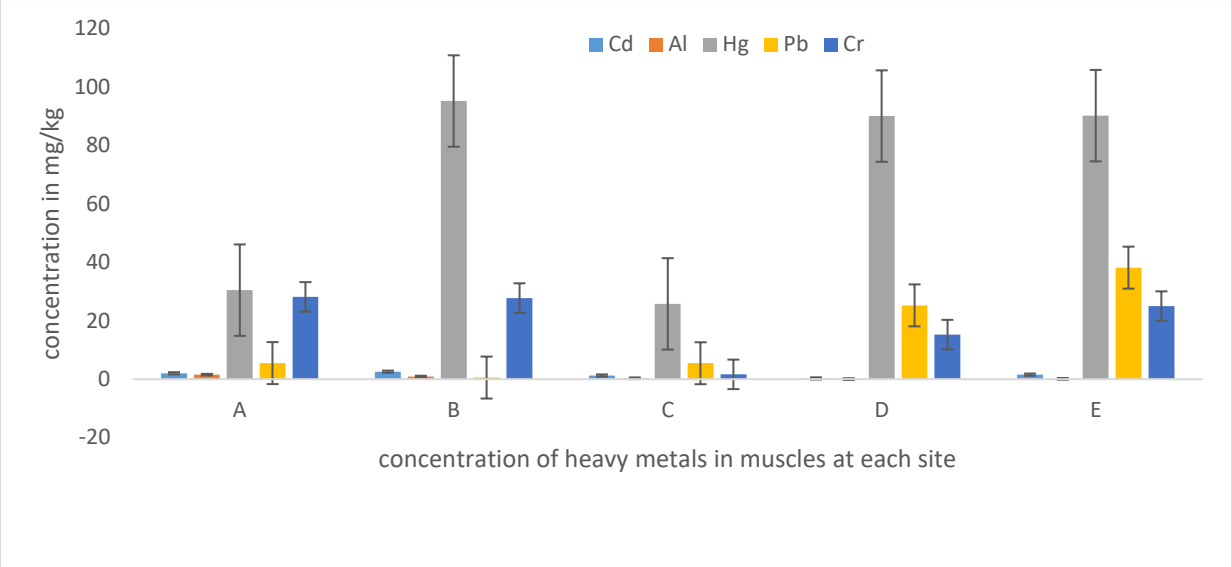

Figure 3: mean concentration of heavy metal in fish muscles in sampling site

Oxidative stress biomarkers

Activities of oxidative stress enzymes were analyzed in gills, liver, and muscle of *O. niloticus* from the five sampling stations. The measured oxidative stress enzymes included superoxide dismutase (SOD), Glutathione (GSH), Catalase (CAT) and lipid peroxidation (malondialdehyde). Table 1 showed concentration of SOD in the fish tissues, gill from site A showed the highest values of 3.00U/ml followed by SOD concentration of 2.23U/ml. SOD concentration of liver was highest in site D with a value of 4.10U/ml. Muscle tissue showed values of 10.55U/ml of SOD, the highest SOD concentration was recorded in the muscle of *O. niloticus* with a value of 10.55U/ml of SOD. Reduced glutathione (GSH) levels in the organs of fish is showed in Table 2, fish gills in site A showed values of 11.67µg/ml followed by 15.33 µg/ml in site E while liver samples from site E gave the highest values of 18.36 µg/ml and lowest liver concentration of GSH was seen in site A with a value of 0.58 µg/ml. The muscle concentration had the overall highest value of GSH of 18.30 µg/ml. Catalase(CAT) concentration in fish is shown in Table 3, with the gills of the fish having a value of 1.48U/ml from site E, liver concentration was 1.12 U/ml in site B followed by 0.83 U/ml in site D. Values of 1.05 U/ml was recorded in the muscle of the fish in site E. Catalase concentration was low in fish samples with a moderate correlation between values gotten in the different organs of fish. Lipid peroxidation (malondialdehyde) is presented in Table 4, MDA in the gills showed concentration of 0.79n/mol in site D, 0.82n/mol in site B, 0.78n/mol in site E, 0.75n/mol in site C and 0.57n/mol in site A, there was no significant difference($P<0.005$) in concentration of MDA in the gill of *O. niloticus*.Liver concentration showed values of 1.08n/mol in site C, 0.78n/mol in site A, 0.78n/mol in site D and 0.53n/mol in site E with no significant difference ($P<0.005$) in the liver concentrations. Muscle concentration showed values of 0.63n/mol in site E of 0.60n/mol in site A.

Table 1: Superoxide dismutase levels in *O. niloticus* organs from HNW (in U/ml)

O. niloticus		**SITE**				
Organ/Tissue	Control	A	B	C	D	E
Gills	0.22 ± 0.02^{a}	3.00 ± 0.05^{d}	ND	2.23 ± 1.59^{d}	0.82 ± 0.55^{c}	0.32 ± 0.13^{b}
Liver	0.04 ± 0.01^{a}	0.66 ± 1.13^{b}	4.10 ± 0.90^{b}	1.02 ± 0.02^{b}	6.70 ± 3.52^{d}	3.03 ± 1.05^{c}
Muscle	ND	2.20 ± 0.20^{c}	10.55 ± 0.26^{d}	3.05 ± 0.00^{c}	0.78 ± 0.38^{b}	0.50 ± 0.10^{a}

Values are mean ± SD of single fish species determinations from 5 points in HNW.
ND = Unrecognized

Table 2: Reduced Glutathione levels in *O. niloticus* from HNW (in µg/ml)

O. niloticus	Sites					
Organ/Tissue	Control	A	B	C	D	E
Gills	1.83±0.03[a]	16.93±5.87[d]	3.62±1.48[a]	11.67±5.77[c]	7.33±0.30[b]	15.33±4.76[d]
Liver	0.22±0.01[a]	0.51±0.43[a]	3.35±0.69[b]	4.32±0.13[b]	15.30±7.05[c]	18.36±10.35[d]
Muscle	ND	13.34±7.85	6.93±4.16[a]	18.30±2.93	8.60±4.84[b]	6.97±2.80[a]

Values are mean ± SD of single fish species determinations from 5 points in HNW.
ND = Unrecognized

Table 3: Catalase levels in *O. niloticus* organs from HNW (in U/ml)

O. niloticus	**SITE**					
Organ/Tissue	Control	A	B	C	D	E
Gills	ND	ND	1.03±0.78[b]	0.32±0.29[b]	0.27±0.24[a]	1.48±0.50[b]
Liver	ND	0.19±0.02[a]	1.12±0.17[c]	0.66±0.57[b]	0.83±0.06[b]	0.25±0.22[a]
Muscle	ND	0.63±0.55[b]	0.77±0.68[c]	0.83±0.58[c]	0.35±0.05[a]	1.05±0.09[d]

Values are mean ± SD of single fish species determinations from 5 points in HNW.
ND = Unrecognized

Table 4: Malondialdehyde levels in *O. niloticus* organs from HNW (in n/mol)

O. niloticus	**SITE**					
Organ/Tissue	Control	A	B	C	D	E
Gills	ND	0.57±0.06[a]	0.82±0.74[b]	0.75±0.26[b]	0.79±0.03[b]	0.78±0.65[b]
Liver	0.35±0.03[a]	0.78±0.65[b]	0.77±0.56[b]	1.08±0.01[c]	0.78±0.09[b]	0.53±0.50[a]
Muscle	0.21±0.02[a]	0.60±0.53[b]	0.35±0.06[a]	0.32±0.24[a]	0.35±0.06[a]	0.63±0.55[b]

Values are mean ± SD of single fish species determinations from 5 points in HNW.
ND = Unrecognized

Histopathological Examination

Microscopic histopathological examination of various organs of the nile tilapia showed some damage/changes in gills, liver and muscle affected by the presence of heavy metals in the Hadejia Nguru wetlands. Plates 1, showed the changes seen in the organization of the Gill filaments it showed detrimental effects such as cell proliferation, clubbing, hyperplasia and fusion of lamellar cells, loss of secondary lamellae and inflammatory cells. Hepatocyte clogging and degeneration were observed in the tissues observed. There was hepatocyte degeneration and erythrocyte distortion, and hepatocyte vacuolization. (Plate 2, A, B, C, D and E) Prior to examination tissues showed no evidence of tissue damage, color change, odor and texture changes observed prior to examination, all the tissues were consistent. Muscle tissue degeneration and vacuolated blood vessels were observed (Plate 3, A, B, C, D and E)

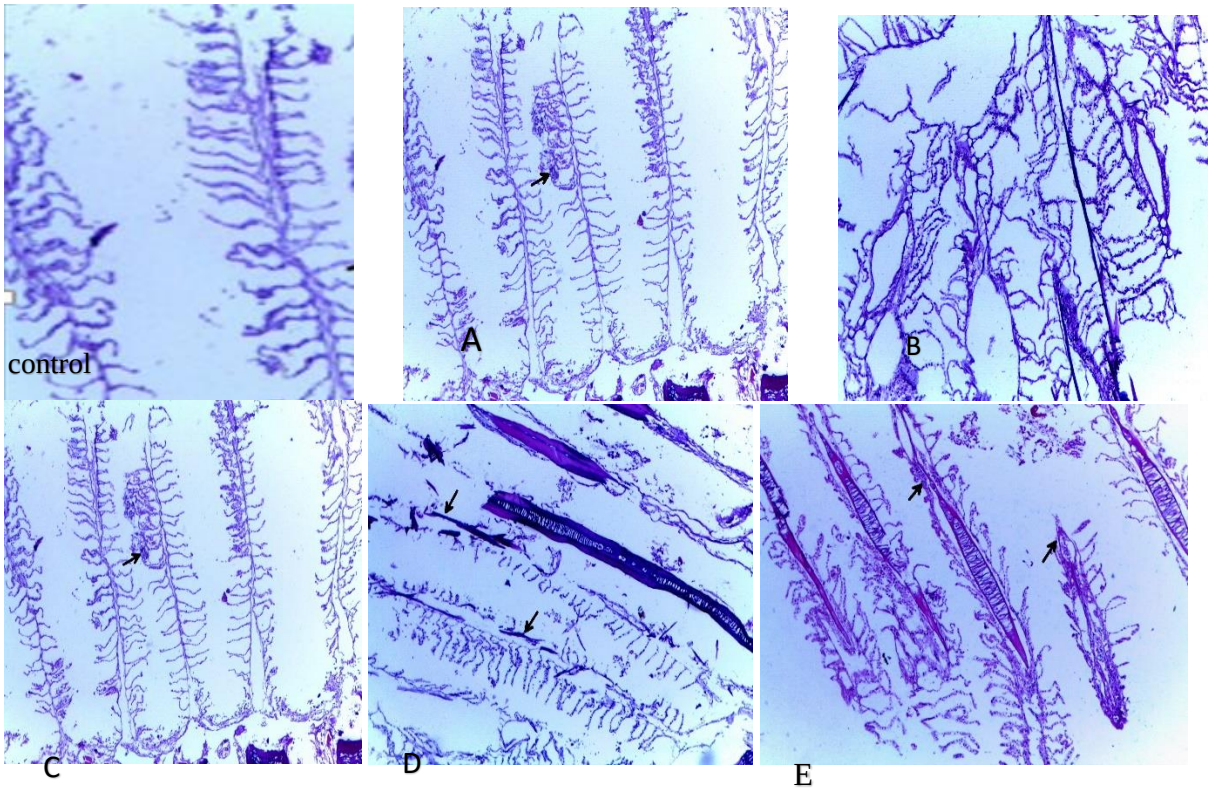

Plate 1: Gills histology of O. *niloticus (Sites* A,B,C,D and E) control showed normal gill filaments, cell proliferation and clubbing of lamella(A), lamellar cell hyperplasia and inflammation of cell(B)Desquamation of secondary lamella (C), inflammatory cells(D) loss and fusion of secondary lamella (E) **(Stain uptake H&E X100)**

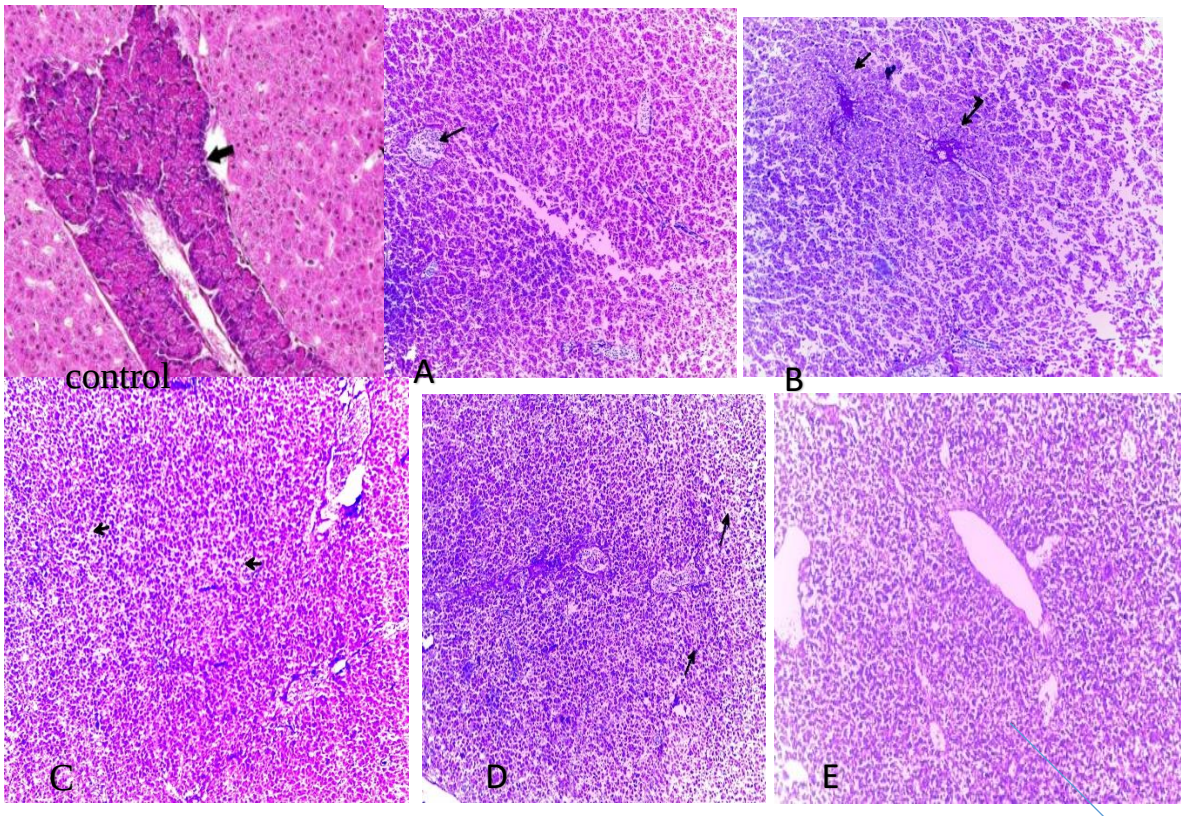

Plate 2: Liver histology of *O. niloticus*. Normal hepatocytes cell seen in control fish, swollen hepatocytes with vacuolated cytoplasm in A. distortion of bile duct and RBC in B, vacuolation of hepatocytes in C, dilated blood sinusoids in D and vacuolation and slightly dilated blood sinusoids in E. **(Stain uptake H&E X100)**

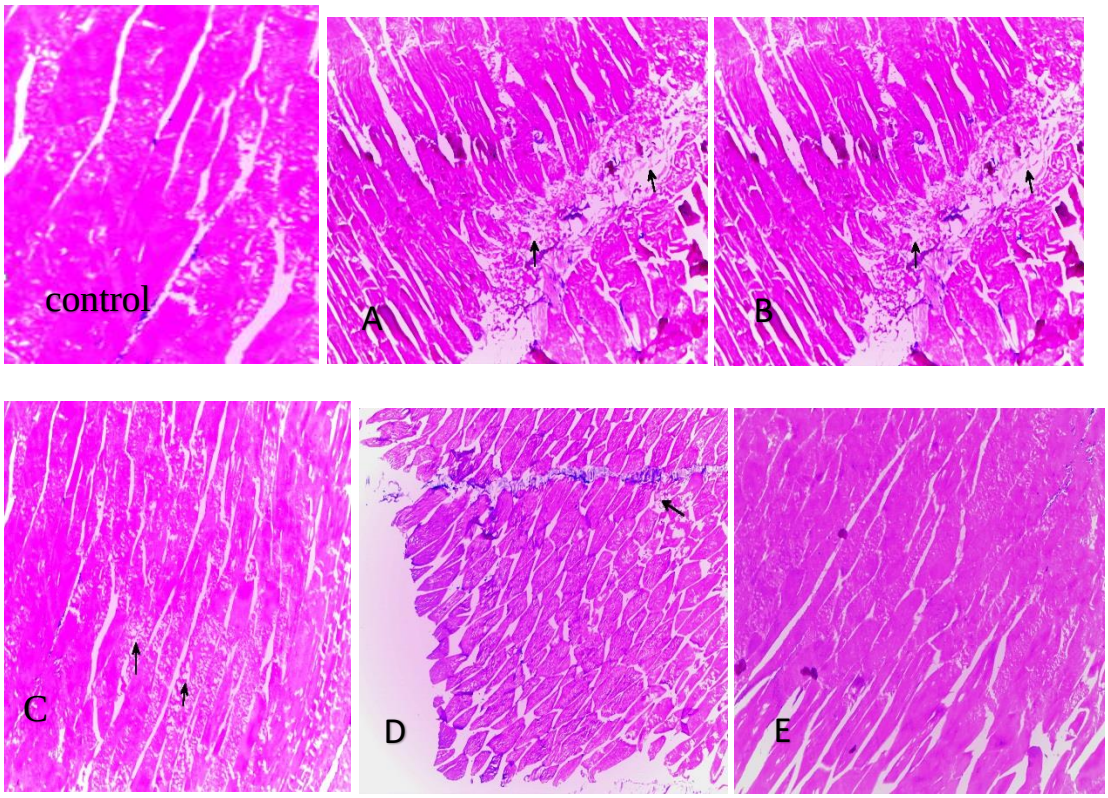

Figure 19: Histology of muscle cells *O. niloticus* (Sites A, B, C, D and E) normal muscle histology I control fish, sites A to E showed vacuolated blood vessels. **(Stain uptake H&E X100)**

DISCUSSION

Contamination levels observed in this study was high exceeding the basic optimal level s for heavy metal. High concentrations of mercury and chromium does result from human activities. High amounts of Cr and Hg recorded in fish tissues can be attributed to the level of pollution entering into the water body, human activities ranging from mining, fertilization and chemicals loaded with this heavy metals. Cr enters water bodies through effluents discharged from tanneries, textiles, metal finishing, and agricultural chemicals (Authman *et al*., 2015) Histological observation seen in his studies agrees with that of Ackermannn (2008) who observed histological alterations in *Oreochromis mossambicus* liver as a result of exposure to sublethal Cr. Deformity and abnormal body axis have been observed in the face of Cr exposure in fish (Virk and Sharma, 2003). Mercury (Hg) is found at trace amounts in unpolluted water (Authman *et al*., 2015) High concentration of mercury in the study agrees with the findings of Gochfield (2003) who asserted that fish tissues are sensitive indicators of aquatic pollution and have a high mercury bioaccumulation capacity for both organic and inorganic forms. Low mercury concentrations reduce sperm viability in fish, reduce egg production, and impair viability of developing eggs. Lead is a toxic metal that occurs naturally in aquatic ecosystems through anthropogenic activities such as metal-based mining, lead paint, and gasoline, it is a persistent heavy metal which has been characterized as a priority hazardous substance (Sfakianakis, 2015). Toxicity of Pb in liver has been known to cause varying body disorders in fish especially in the histology of tissues. Hepatic vacuolization, hepatic cirrhosis, necrosis, shrinkage, parenchyma degeneration and increase in sinusoidal spaces are observable characteristics of Pb exposure. The limits for lead residues recommended by WHO (1999) and FAO (2004) are between 0.3 mg/kg and 0.01 mg/kg in food. Even at low levels of concentration Pb can affect reproduction and fish health. Farombi *et al*., (2007) observed similar trends in the liver and kidney *C. gariepinus* from the Ogun River. Cd concentrations in fish were higher than acceptable limits, and Cd concentrations in this

study were similar to those reported by Hashim *et al*. (2014) who observed concentrations in fish higher than recommended permissible limits in a study conducted in the Keratan River, Malaysia.

High amount of oxidative stress enzymes observed in this study can be attributed to the presence of metallic pollutants and this was observed by Souid *et al*., 2013, who observed a similar trend in the rise of oxidative stress enzymes in several tissues of *Sparus aurata.* Metals are important inducers of oxidative stress in aquatic organisms promoting formation of reactive oxygen species through redox cycling, while metals without redox potential impair antioxidant defences, especially that of thiol-containing antioxidants and enzymes (Sevcikova *et al*., 2011). Superoxide dismutase (SOD), redox-sensitive thiol compounds GSH, CAT, and MDA were observed in organs at increased proportions. A possible explanation for this increase detected in fish organs is the result of the presence of heavy metals in the water, and it is possible that the accumulation of heavy metal residues causes the production of superoxide anions, leading to the induction of transforming SOD. It converts superoxide radicals to H_2O_2 and catalytically scavenges the SOD superoxide radical, which appears to be a key factor in oxygen toxicity (Musa and Imam, 2021). GSH showed increased levels in all samples and GSH is known to be a substrate for the activity of GST (glutathione peroxidase) .The highly detected increase in GSH formation suggests an adaptive and protective mechanism of this biomolecule against oxidative stress caused by heavy metal residues, consistent with the results of Farombi *et al*. (2007). Fish and their environment and such contaminants (heavy metals) can penetrate the thin epithelium of fish. Catalase activity was present in lower concentrations in some of the sampling sites but showed appreciable amount in some of the sites. As reported by Dautremepuits *et al*. (2004) increases in CAT and SOD activity are usually observed in the face of environmental contaminants. The amount of CAT reduction observed in this study is comparable to that reported by Stanic *et al*., (2005) who claimed that low amount of CAT Activity can be attributed to superoxide radical overshadowing ability. Significantly higher levels of lipid peroxidation in all organs observed indicated an accumulation of heavy metals in the organs, with increasing metal concentrations leading to higher levels of antioxidants, as can be seen from the results and in some cases damage in DNA, proteins and lipids. Pandey *et al*. (2003).

One of the organs more susceptible to toxic chemicals/pollutants due to direct contact with the environment is the gills, the absorption of toxic substance through the gills has been one of the effective means of measuring the effect of aquatic pollutants into water bodies (Pandey *et al*., 2008, Khan *et al*., 2011) From results obtained there was a structural organization in the control group of fish however, the exposed fish to the metal pollutants showed hypertrophy and the clubbing of the epithelial cells. Doaa and Hanan, 2013 observed similar trend in the histology of gills, liver and ovaries of nile Tilapia (*Oreochromis* niloticus) fish exposed to lead and other metallic pollutants. Toxicity in metals interferes with vital functioning's and physiology which includes respiratory (Doaa and Hanan, 2013) Lifting and hyperplasia of lamellar epithelium can be interpreted as defense responses in fish as these changes in the gills increase the distance across which waterborne irritants must diffuse to reach the blood stream (Pandey *et al*., 2008) Oxygen deficiency as a result of gill toxicity has been described as the most common cause of cellular degeneration in gill filaments. Among abnormalities noticed in the liver are the degeneration of hepatocytes, the high alterations in the liver can be attributed to its functions of detoxification and accumulation of toxic elements in its cells, this is consistent with the findings of Ekeanyanwu *et al*. (2015) where concentrated levels of heavy metals were found in the liver of fish from Oguta lake. The concentration level of metals in the liver can cause a lot of alterations in the liver histology (Doaa and Hanan, 2013). Fish

muscle showed many deleterious changes due to heavy metal toxicity, but the changes were more pronounced in gills and liver than in muscle, indicating that heavy metal concentrations were higher in gills and liver

CONCLUSION

The toxic effects of heavy metals in *O. niloticus* showed that these metals can induce early responses in the fish as evidenced by alterations at both the structural and biochemical levels affecting the well-being of the exposed fish, increasing its susceptibility to varying types of disease. The fish biomarkers are necessary for the monitoring of environmentally induced alterations in order to assess the impact of heavy metals on fish. It is therefore recommended that there should be an effective monitoring and treatment should be carried out on all kinds of waste waters, sewage and agricultural waste before being discharged into the water body. However, fish tested from the sampling points are not suitable for human consumption due to high contamination level observed in them.

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