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Biomarkers of Oxidative Stress and Histopathological Studies in Fish from Ibaka and Ifiayong Rivers, Akwa Ibom State, Nigeria

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Abstract: Ibaka and Ifiayong rivers are located in Akwa Ibom State, within the Niger Delta region of Nigeria. They serve as important sources of fish to the people in this locality. Ibaka River is subject to perturbations arising from oil exploration activities whereas Ifiayong River is free from oil exploration, but maybe susceptible to agricultural runoff and waste discharges. To evaluate the extent of heavy metal contamination of these Rivers and associated oxidative stress, the concentrations of copper, zinc, cadmium and lead were determined in the liver, kidney, heart and gills of silver catfish, *Chrysichthys nigrodigitatus*, from these sources. The activities of superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and malondialdehyde (MDA) levels were also determined. Isolated organs were examined for histopathological changes. Tissue levels of Cu and Zn were found to be high and significantly higher ($p<0.05$) in fish from Ibaka River than those from Ifiayong River. Low levels of Cd and Pb were detected in the organs of fish and the concentrations of these elements were significantly higher ($p<0.05$) in fish from Ibaka River. Activities of antioxidant enzymes and malondialdehyde levels were also significantly higher in fish from Ibaka River. Histopathological changes in fish organs were also more severe in fish from Ibaka River. These results indicate heavy metal contamination and oxidative stress in fish from these Rivers. However, there is need for a more comprehensive assessment of all possible pollutants in these rivers to better understand the extent of contamination and the development of strategies for intervention.

Key words: Antioxidant enzymes · *Chrysichthys nigrodigitatus* · Heavy metals · Ibaka · Ifiayong · Oxidative stress · Silver catfish

INTRODUCTION

Oxidative stress is a condition that prevails when there is an imbalance between the production of reactive oxygen species (ROS) and antioxidant defences [1]. In the marine environment, substances reported to generate ROS include heavy metals, pesticides, fertilizers, accidental oil spills and runoff from coastal areas [2, 3]. ROS may also be generated endogenously in living cells from various sources but is most prominent in the mitochondrial respiratory chain [4]. The increased production of ROS causes oxidation of proteins, lipids and nucleic acids with devastating consequences on cellular biochemistry [5].

Heavy metals that accumulate in the tissues of fish catalyse reactions that elaborate ROS and induce oxidative stress [3]. Many Rivers in Nigeria are reported to be polluted by heavy metals [6- 9]. Oxidative stress in fish caused by heavy metal contamination in some Nigerian Rivers has also been documented [10, 11].

The assessment of histopathological changes in animal tissues is an invaluable tool in the determination of environmental pollution [12, 13]. It serves as an early warning sign of injury to cells, tissues and organs [14].

Ibaka (Mbo) and Ifiayong Rivers are located in the Niger Delta Region of Akwa Ibom State, Nigeria. These

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Rivers are important sources of silver catfish (*Chrysichthys nigrodigitatus*) which are widely consumed by the populace in this geographical area. Whereas Ibaka River is subject to perturbations arising from oil exploration activities [15], Ifiayong River, on the other hand, is free of oil exploration but may be susceptible to fertilizer runoff and waste discharges.

The present study was, therefore, carried out to assess the levels of some heavy metals (Zn, Cu, Cd and Pb) in liver, heart, kidney and gills of Chrysichthys nigodigitatus obtained from these two Rivers. The extent of oxidative stress in the fish samples was determined by measurement of malondialdehyde formation and antioxidant enzyme activities as biomarkers. Histopathological studies were also carried out on the isolated organs.

MATERIALS AND METHODS

The Study Area: Ibaka (Mbo) River is one of the major Rivers in Akwa Ibom State, Nigeria. It lies within latitude 40° 39' North and longitude 8° 19' East of the south-eastern Nigerian coastline. The River plays a prominent role in the fisheries resources of Akwa Ibom State [15]. On the other hand, Ifiayong River is located at latitude 5° 03' North and longitude 8° 01' East. Whereas Ibaka river is subject to perturbations from oil exploration activities [15], Ifiayong River is free from developmental activities but may be subject to agricultural runoff and waste discharges.

Fish Samples: Silver catfish (*Chrysichthys nigrodigitatus*) was bought directly from fishermen at Ibaka beach and Ifiayong beach. They were transported to the laboratory in ice-cold containers (0-4°C). The samples were authenticated by Dr J. P. Udoh, Department of Fishery and Aquaculture, University of Uyo, Uyo. The fish samples were frozen in dry ice and stored at -20°C. The fishes (10 from each location) weighing between 200 and 650 g were dissected and the liver, heart, gills and kidney were removed and washed in ice cold 1.15% potassium chloride (KCl) solution, blotted and weighed. They were then homogenized in four volumes of homogenizing buffer (50 mM Tris HCl mixed with 1.15% KCl and pH adjusted to 7.5) using a laboratory mortar and pestle to which acid washed sand was added. The resulting homogenate was centrifuged in a Sigma 301 centrifuge at 6,000 rpm for 20 minutes. The supernatant was decanted and stored at -20°C until analysis.

Heavy Metal Analysis: All fresh tissues were dried in an oven at 105°C for 24 hours to constant weight and milled with a mortar and pestle. The samples were transferred into dry labelled plastic containers and stored in a desiccator until required for digestion. A procedure similar to that described by Poldoski [16] was used to digest the samples. This involved digesting 1 g portion of the ground samples with 10ml HNO_3/2ml HClO4 and heating on a hot plate for one hour. After complete digestion, the residue was dissolved and diluted with 0.2%v/v HNO3 to 20 ml. The metals (Zn, Cu, Cd, Pb) were determined by using atomic absorption spectrophotometer(Bulk Scientific 205).

Estimation of Total Protein: Total protein was estimated by the method of Lowry *et al*. [17] using bovine serum albumin as standard.

Assay of Superoxide Dismutase: Superoxide dismutase (SOD) activity was measured using an assay kit (Cayman, MI, USA) according to manufacturer's instructions. The assay kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidases and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical HNO_3(SOD Assay Kit, Cayman chemical, Ann Arbor, MI).

Assay of Glutathione Reductase: The Cayman Chemical GR Assay Kit measures GR activity by measuring the rate of oxidation of NADPH to NADP+. The oxidation of NADPH to is accompanied by a decrease in absorbance at 340 nm which is directly proportional to the GR activity in the sample (GR Assay Kit, Cayman Chemical, Ann Arbor, MI).

Assay of Glutathione Peroxidase: GPx activity was determined using BIOXYTECH GPx-340TM Assay kit. The assay measures GPx indirectly by a coupled reaction with glutathione reductase (GR). Oxidised glutathione (GSSG) produced by reduction of hydroperoxide by GPx is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm which is directly proportional to the GPx activity in the sample (GPX Assay Kit, Cayman Chemical, Ann Arbor, MI).

Assay of Malondialdehyde (MDA): This was carried out according to Soon and Tan [18]. 1.0 ml of sample was combined with 2.0 ml of TCA-TBA-HCl reagent and

thoroughly mixed. The mixture was heated for 15 minutes in a boiling water bath. After cooling, the flocculent was removed by centrifugation at 1000g for 10 minutes. Absorbance of the supernatant was determined at 535 nm against a blank containing all the reagents except the sample. The MDA concentration in the sample was calculated using extinction co-efficient of 1.56 x 10-5 M^{-1} CM^{-1}.

Histopathological Analysis: At necropsy, tissue samples were extracted and fixed immediately in 10% buffered formal saline processed to paraffin wax by passing through ascending grade of alcohol, clear in xylene, infiltrated using paraffin wax, embedded, mounted, sectioned at 3-5 µm using microtome (rotary) and subsequently stained with haematoxylin and eosin technique and observed under light microscope for histopathological changes.

Statistical Analysis: Data were expressed as mean ± standard deviation (SD) and analysed with the SPSS 18.0 software. Independent T-test was used for the evaluation of measurement data between the fishes from Ibaka and Ifiayong rivers. P values <0.05 were regarded as statistically significant. Analysis of Variance (ANOVA) and Games-Howell comparison test was used to compare oxidative stress biomarkers data at all tissues in the same location.

RESULTS

Concentration of Heavy Metals: The values obtained for the concentration of heavy metals in various tissues of C. nigrodigitatus are presented in Fig. 1- 4. Heavy metals were accumulated to varying extent in the tissues. The order of concentration of metals in the tissues of fish bought at Ibaka beach was: Zn - liver > kidney > heart > gills; Cu- liver > heart > kidney > gills; Pb - Kidney > gills > heart > liver; Cd - Kidney > gills > liver > heart while that of Ifiayong was: Zn - liver > heart = gills > kidney; Cu - liver = heart > kidney > gills; Pb - liver > kidney > gills > heart; Cd - gills = kidney > heart > liver.

Antioxidant Enzymes/Malondialdehyde: The activity of SOD was significantly higher (P<0.05) in the kidney and liver of fish from Ibaka than Ifiayong Rivers (Fig. 5). There was no significant difference (P<0.05) in SOD activity in gills and heart of fish from both Rivers. Fig. 6 is a comparison of activity of GR in fish from Ibaka and

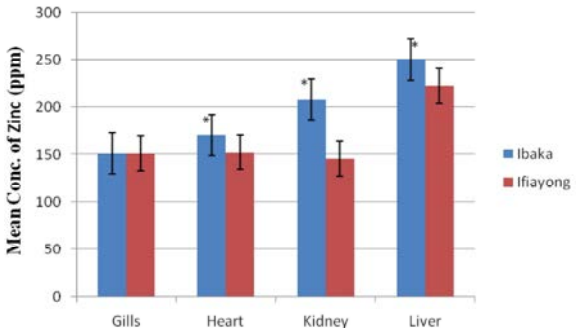

Fig. 1: The mean concentration of Zinc in liver, kidney, gills and heart of Chrysichthys nigrodigitatus from Ibaka and Ifiayong Rivers. Values are mean ± S.D (n=10). *significantly different from Ifiayong (P< 0.05)

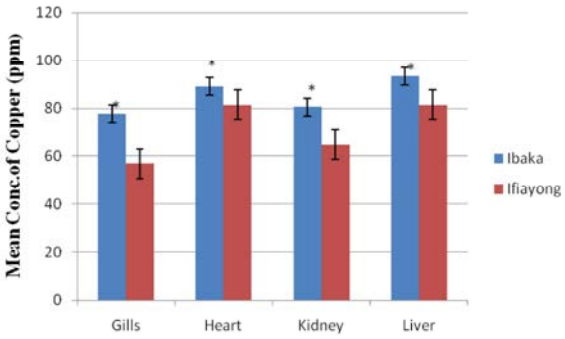

Fig. 2: The mean concentration of Copper in liver, kidney, gills and heart of Chrysichthys nigrodigitatus from Ibaka and Ifiayong Rivers. Values are mean ± S.D (n=10). *significantly different from Ifiayong (P< 0.05)

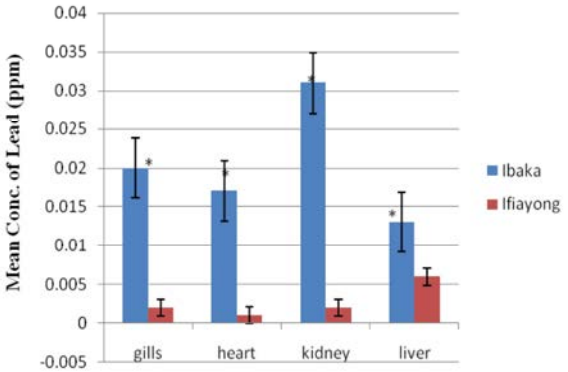

Fig. 3: The mean concentration of Lead in liver, kidney, gills and heart of Chrysichthys nigrodigitatus from Ibaka and Ifiayong Rivers. Values are mean ± S.D (n=10). *significantly different from Ifiayong (P< 0.05)

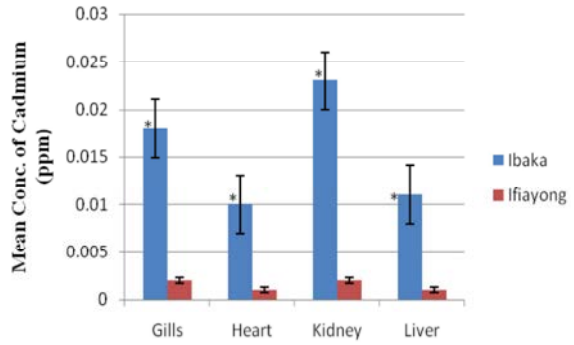

Fig. 4: The mean concentration of Cadmium in liver, kidney, gills and heart of Chrysichthys nigrodigitatus from Ibaka and Ifiayong Rivers. Values are mean ± S.D (n=10). *significantly different from Ifiayong (P< 0.05)

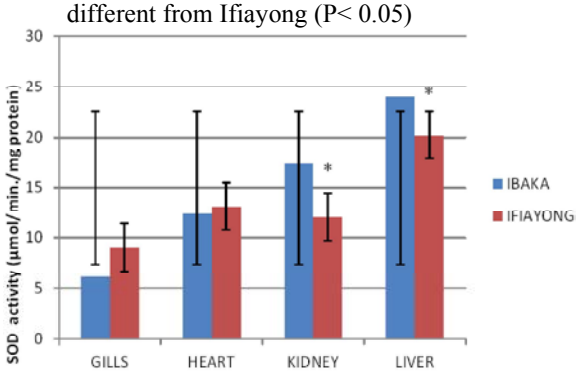

Fig. 5: The activities of superoxide dismutase in liver, kidney, gills and heart of Chrysichthys nigrodigitatus from Ibaka and Ifiayong Rivers. Values are mean ± S.D (n= 10) fishes. *significantly different from Ifiayong (P< 0.05).

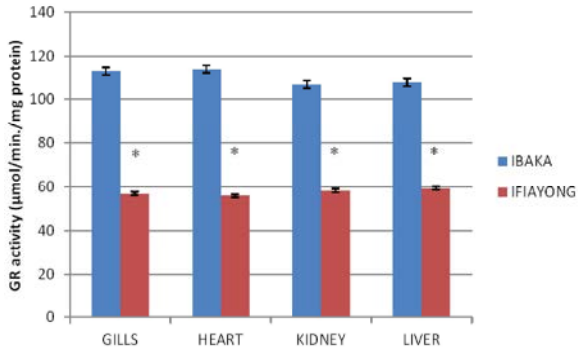

Fig. 6: The activities of glutathione reductase in liver, kidney, gills and heart of Chrysichthys nigrodigitatus from Ibaka and Ifiayong Rivers. Values are mean ± S.D (n=10) *significantly different from Ifiayong (P< 0.05)

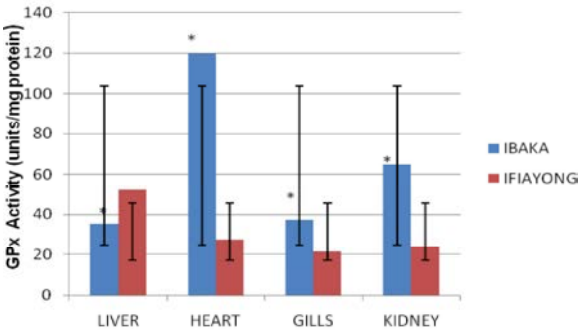

Fig. 7: The activity of glutathione peroxidase in liver, kidney, gills and heart of Chrysichthys nigrodigitatus from Ibaka and Ifiayong Rivers. Values are mean ± S.D (n=10). *significantly different from Ifiayong (P< 0.05)

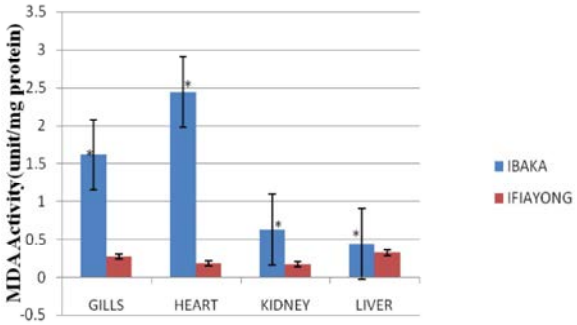

Fig. 8: The concentration of MDA in liver, kidney, gills and heart of Chrysichthys nigrodigitatus from Ibaka and Ifiayong Rivers. Values are mean ± S.D of 10 fishes. *significantly different from Ifiayong (P< 0.05)

Ifiayong. GR activity was significantly higher (P<0.05) in all the organs of fish from Ibaka than Ifiayong River. Fig. 7 shows GPx activity in fish fromIbaka and Ifiayong. GPx was significantly elevated at Ibaka than Ifiayong in the heart, gills and kidney but there was no significant difference in the liver. Fig. 8 shows that the concentration of MDA in fish caught from Ibaka and Ifiayong Rivers. This result indicates that concentration of MDA was significantly increased in fish from Ibaka in all the organs.

Histopathology: The results for the histopathological studies carried out on the various organs are shown in Plate 1-8.

Liver: The liver of fish from Ibaka River showed areas of severe metal accumulation, hypertrophy, vacuolization, pyknotic nuclei, vascular congestion

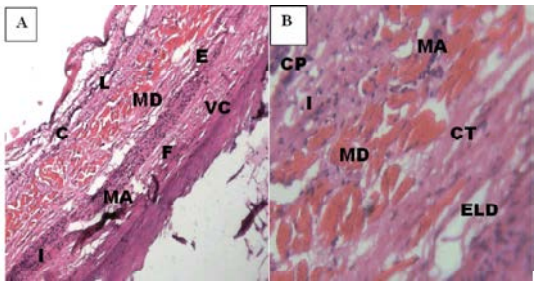

Plate 1: Histologic photomicrographs of gill of Chrysichthys nigrodigitatus from Ibaka River at magnification A(x100) and B(x400) stained with Haematoxylin and Eosin (H & E) technique

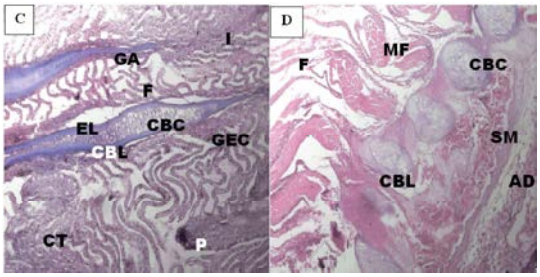

Plate 2: Histologic photomicrographs of gill of Chrysichthys nigrodigitatus from Ifiayong River at magnification C(x100) and D(x400) stained with H & E technique.

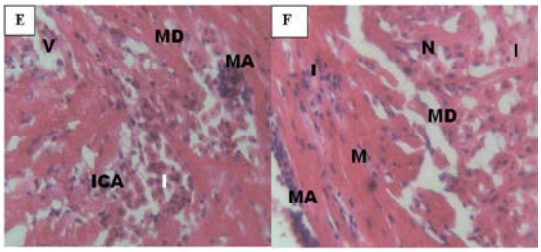

Plate 3: Histologic photomicrographs of heart of Chrysichthys nigrodigitatus from Ibaka River at magnification E(x100) and F(x400) stained with H & E technique

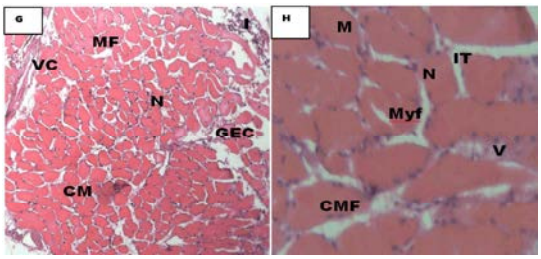

Plate 4: Histologic photomicrographs of heart of Chrysichthys nigrodigitatus from Ifiayong River at magnification G(x100) and H(x400) stained with H & E technique

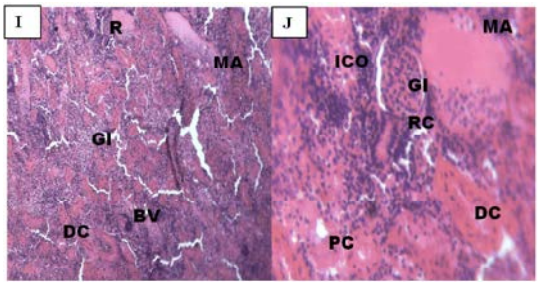

Plate 5: Histologic photomicrographs of kidney of Chrysichthys nigrodigitatus from Ibaka River at magnification I(x100) and J(x400) stained with H & E technique.

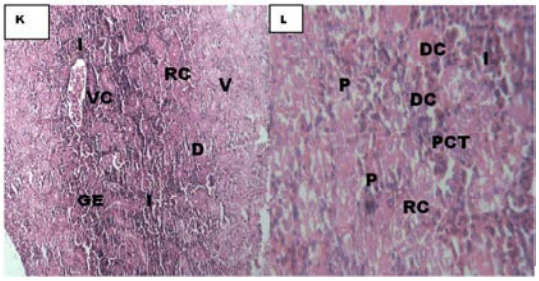

Plate 6: Histologic photomicrographs of kidney of Chrysichthys nigrodigitatus from Ifiayong River at magnification K(x100) and L(x400) stained with H & E technique.

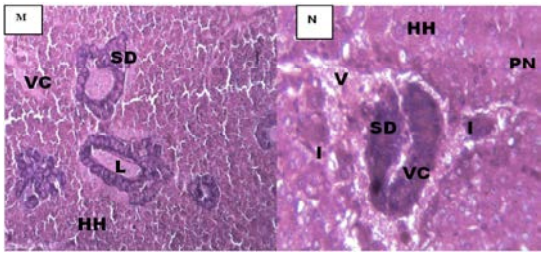

Plate 7: Histologic photomicrographs of liver of Chrysichthys nigrodigitatus from Ibaka River at magnification M(x100) and N(x400) stained with H & E technique

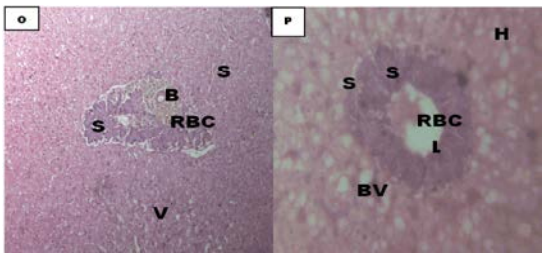

Plate 8: Histologic photomicrographs of liver of Chrysichthys nigrodigitatus from Ifiayong River at magnification O (x100) and P(x400) stained with H & E technique.

with inflammation and inclusion bodies. The organs of fish from Ifiayong showed only moderate alterations.

Gills: The gills of fish from Ibaka River showed abnormal area of metal accumulation, cell proliferation with ceratobranchial arch, connective tissue distortion, disruption of epithelial cells, fusion of primary lamellae, vacuolization, inflammation, vascular congestion and cellular degeneration whereas the gill of C. nigrodigitatus from Ifiayong River showed area of slight metal accumulation, inflammation and fusion of primary lamellae.

Kidney: There was moderate metal accumulation in the kidney of fish from Ibaka River, abnormal area of cell proliferation, glomerular inflammation, dystrophic changes and inter-corpuscular oedema against the background of inflammatory cells while the kidney of fish from Ifiayong River showed slight area of metal accumulation, cellular pattern containing renal corpuscle, pigment, distal and proximal convoluted tubule with pyknotic nuclei and inflammation.

Heart: The heart of *C. nigrodigitatus* from Ibaka River showed moderate accumulation of metals, abnormal area of cell proliferation with thickening of muscle bundle and filament, intramuscular oedema and dystrophic changes against the background of inflammatory cells. However, the fish from Ifiayong River showed slight area of metal accumulation, nuclear spreading, vacuolization and marked thickening of the muscle bundles with dystrophic changes.

DISCUSSION

The present study was carried out to evaluate the levels of heavy metals, some biomarkers of oxidative stress and histopathology of the gill, heart, kidney and liver of *Chrysichthys nigrodigitatus* from Ibaka and Ifiayong Rivers located in the Niger Delta region of Nigeria. The results showed that there were significant differences ($p<0.05$) in the mean concentrations of Cu and Zn in all the organs from both locations. Other studies [7,10,19] have also reported high mean concentrations of Cu and Zn in sites considered polluted. However, the pattern of variation of Cd and Pb in various organs of the fish was not quite distinct.

Copper (Cu) is an essential element required for the synthesis of haemoglobin [20]. It is an important component of the enzyme cytochrome oxidase found in the mitochondrial respiratory chain [21] as well as Cu-Zn SOD, an antioxidant enzyme [22]. High levels of Cu is toxic to aquatic animals with adverse effects manifested as reduced growth, shorter lifespan, reproductive problems, reduced fertility and behavioural changes [24]. High intake of Cu, particularly Cu^{2+} can also be harmful to man causing anaemia and damage to the pancreas and kidneys [24]. The mean concentrations of Cu in all the organs of the fish at the two locations were above the permissible levels of 0.5 ppm [25,26]. This is an indication of severe contamination of the fish; hence, consumers may be at risk Cu toxicity.

Zinc (Zn) plays an important role in the biological functions of various proteins and enzymes [27]. Despite its essentiality, Zn has been reported to be toxic at high concentrations [28].It is reported to suppress the absorption of copper and iron [29]. In the present study, high concentrations of Zn were detected in all organs of *C. nigrodigitatus* at Ibaka and Ifiayong Rivers. The highest mean concentration of Zn was found in the liver followed by the kidney and gills. Javed and Usmani [30] have also reported high levels of Zn in the liver (233.3 ± 0.96 ppm) and kidney (275.0 ± 3.15 ppm) of *C. punctatus*. They also observed high levels of Zn in liver (459.4 ± 1.11 ppm) and kidney (350.0 ± 2.28 ppm) of *L. rohita*. The accumulation of Zn in the liver of fish has been attributed to the fact that liver is the primary organ for storage and detoxification [31,32]. Zn is also reported to show a very high affinity for the kidneys [33]. The permissible limits for Zn set by WHO [34] is 40 ppm, hence the levels of Zn observed in various organs of fish in this study is of public health concern.

Cadmium (Cd) is regarded as a non-essential element that normally gains entry into the aquatic environment through industrial discharges, agricultural and mining activities [35]. The toxic effects of Cd in fish include growth retardation [36], disturbance in bone metabolism and inhibition of absorption of essential amino acids [37] as well as damage to beta cells of the pancreas accompanied by a decline in the capacity to secrete insulin [38]. In the present study, low levels of Cd (0.001-0.013 ppm) were observed in the organs of fish. Nyirenda *et al*. [39] had also reported low levels of Cd (0.0023-0.013 ppm) in organs of fish from Modinola dam, South Africa. Other authors reported much higher concentrations of Cd in organs of fish from polluted waters. For instance, Farombi *et al*. [10] obtained high concentrations of Cd (0.25-2.10 ppm) in *Clarias gariepinus* caught in Ogun River, Nigeria; Ashraf [40], reported mean Cd concentration of 0.41 ± 0.16 ppm in

kidney of *Epinepletus microdon* fish from the Arabian Gulf,while Kalay *et al*. [41] reported Cd level of 1.98 ppm in liver of Mallus barbatus from the coastal region of Mediterranean sea. The permissible limit of Cd set by international organizations is 0.005 ppm [26,42]. The levels of Cd obtained in all the organs of fish analysed the present study were below the permissible limit. Hence, there is no danger of Cd toxicity from the consumption of fish from Ibaka and Ifiayong Rivers.

Lead (Pb) is a trace element that does not play any useful function in living organisms [43]. Sources of lead contamination of aquatic environment include the oil refinery, printing, battery and paint industries [43]. In animals and men, Pb is reported to exert adverse effects on the central and peripheral nervous system [44], haematopoietic system [45], cardiovascular system [46], kidneys [47], liver [48] and the reproductive system [49]. In fish, the biological effects of sub-lethal concentrations of Pb include delayed embryonic development, inhibition of growth, enzyme inhibition and kidney dysfunction [50]. The mean concentration of lead in the present study ranged between 0.01-0.03 ppm in fish from Ibaka River and between 0.003-0.005 ppm in fish from Ifiayong river. The kidney was seen to accumulate the highest mean concentration of lead followed by gills, heart and liver in both locations. In all the organs, there were significant differences between the two locations ($p<0.05$). The permissible limit of Pb is 0.29 ppm [26,26,50].Our present findings correlates with the study of Falco *et al*. [52], who reported lead concentration in various edible marine species to vary from 0.002 to 0.21 ppm. Other authors have reported high mean concentrations of Pb in various organs of fish. For instance, Ashraf [40] found levels of lead in *Epinepletus microdon* in the range of 2.61 ± 1.26 ppm in kidney and 3.19± 2.03 ppm in heart.

Superoxide dismutase (SOD) activity was found to increase significantly in the kidney and liver of *Chrysichthys nigrodigitatus* from Ibaka River than Ifiayong River ($p <0.05$). The gills had lower SOD activity in both locations. The increase in SOD activity in the liver and kidney may be a response to oxidative stress associated with heavy metal accumulation [33, 53]. Within the fish samples, SOD activity was highly significant with liver having the highest activity of SOD. The present finding is in line with the work of Farombi *et al*. [10] and Doherty *et al*. [11], who reported elevation of SOD in all organs of fish except the gills due to heavy metal toxicity.

Glutathione reductase (GR) activity was increased significantly in the liver, kidney, gills and heart of fish caught at Ibaka than Ifiayong Rivers. Within the Ifiayong location, there was no significant difference in GR activity in the organs, whereas in Ibaka, there was significant difference in GR activity in all the organs of fish. GR is known to catalytically reduce oxidized glutathione in order for GSH/GSSG ratio to be maintained. An increase in GR activity portrays an increase in oxidative stress which requires more GSH to be produced to counter the effect of the stress. Pandey *et al*. [54] reported that fish from polluted river site possessed higher activities of glutathione reductase (GR) in liver and gills.

Glutathione peroxidase (GPx) activity increased significantly in all the organs of *Chrysicthys nigrodigitatus* from Ibaka River than Ifiayong River ($p<0.05$). GPx activity was highly significant in the kidney and heart with heart having the highest GPx mean activity. This may be attributed to the accumulation of heavy metals in these organs [54]. Lenartova *et al*. [55] investigated antioxidant enzymes in fish as biomarkers of river pollution and observed that total GPx activity was 1.8 fold higher in fish from the polluted site.

Malondialdehyde (MDA) was significantly increased in all the organs of *Chrysicthys nigrodigitatus* caught in Ibaka River when compared to that of Ifiayong River. The heart and gills had the highest MDA concentration. This is in agreement with the findings of Farombi *et al*. [10] in which the levels of metals and biomarkers of oxidative stress were elevated in *Clarias gariepinus* from Ogun River, Nigeria. The increase in lipid peroxidation observed in the present study may, therefore, be attributed to the accumulation of heavy metals especially Zn and Cu in the organs of this fish.

Histopathological studies of the gills, kidney, liver and heart of *Chrysichthys nigrodigitatus* indicated cellular abnormalities which was more severe in fish from Ibaka River compared to Ifiayong. The abnormal features seen include inflammation, gill arch distortion, vascular congestion or occlusion, vacuolization and cellular degeneration which could be attributed to the accumulation of metals and oxidative stress. Similar alterations have also been observed in the gills [56], liver [57] and kidney [58] of fish from other sources.

CONCLUSION

This is the first biomarker study of Ibaka and Ifiayong Rivers assessing heavy metal levels, enzymatic antioxidants (SOD, GR and GPx), MDA and histopathological changes in four different Organs (liver, gill, muscle, heart and kidney) of *Chrysicthys nigrodigitatus*. This study has demonstrated that Ibaka

River is more contaminated than Ifiayong River with respect to the concentration of Cu and Zn. This might have precipitated the observed oxidative stress and histopathological changes in fish organs. However, there is a possibility that other pollutants, which were not assessed in the present study, could also have contributed to this observation. Hence, there is need for a more comprehensive investigation involving an assessment of all possible marine pollutants so as to place in proper perspective, the extent of pollution of these Rivers.

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