Accumulation and Anti-oxidant Enzymes as Biomarkers of Heavy Metal Exposure in *Clarias gariepinus* and *Oreochromis niloticus*

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Abstract This study investigated the accumulation of heavy metals in tissues (gill, muscle and liver) and changes in anti-oxidant enzyme activities of edible fish species as biomarkers of heavy metal exposure using laboratory bioassays. Post juveniles of *Clarias gariepinus and Oreochromis niloticus* were exposed to 10% 96hr-LC_{50} values of $Pb(NO_3)_2$ and $ZnCl_2$ against each species respectively, for 30 days. The two fish species accumulated the heavy metals (Pb and Zn) in their liver, muscle and gill with the highest concentration of the heavy metals in the muscle as observed in results obtained. The biochemical studies showed that the in the level of reduced glutathione (GSH), and the activities of superoxide dismutase (SOD), glutathione-s-transferase (GST) in the liver of fish species reduced significantly ($p < 0.05$) for *C. gariepinus* exposed sublethal concentration of Pb and Zn while catalase (CAT) reduced significantly ($p < 0.05$) for *C. gariepinus* exposed sublethal concentration of Pb. Also, the reduction in GSH, SOD, CAT and GST was significant ($p < 0.05$) for *O. niloticus* exposed to Zn. However, the reduction in GSH, SOD, CAT and GST in *O. niloticus* exposed to Pb was not significant ($p > 0.05$) and the increase in the level of Malondialdehyde (MDA) in liver of the exposed fish species was not significant ($p > 0.05$) after the 30 days exposure period as compared to the control. The need for routine monitoring of aquatic ecosystems integrating field and laboratory studies to assess presence and accumulation of toxic pollutants especially heavy metals in edible fish species and use of antioxidant enzymes as biomarkers of exposures to such pollutants were discussed.

Keywords: *heavy metals, bio- accumulation, biomarkers, antioxidant enzymes, pollution monitoring, aquatic ecosystems, toxic pollutants*

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1. Introduction

Environmental problems have increased exponentially in recent decades mainly because of rapid growth in human population and increased demand for several household materials. While on one hand technological development has improved the quality of life, on the other hand it has created a number of health hazards. The toxic chemicals discharged into air, water and soil get into food chain from the environment. By entering into the biological system they disturb the biochemical processes leading to health abnormalities and in some cases to fetal consequences.

Heavy metals have particular significance with respect to environmental pollution. Apart from their relatively high toxic action they are non-degradable, which makes them highly persistent in the environment, remaining long after emission have ceased. Therefore the continuous emission of heavy metals as wastes; bring about their increase in concentration and redistribution in ecosystem resulting occasionally in pollution and environmental degradation [1].

The use of biomarkers may provide important information which once validated through laboratory studies can provide direct measures of actual effect of heavy metals upon living organisms in the field, thereby overcoming large areas of uncertainty implicit in normal risk assessments [2,3]. Heavy metals accumulated in the tissues of aquatic organisms may catalyze reactions that generate reactive oxygen species (ROS) which may manifest primarily oxidative stress in the exposed organisms. Defensive mechanisms to counteract the impact of ROS are found in many mammalian species including aquatic animals such as fish.

Biochemical effect of ROS is their attack on lipids that leads to the formation of lipid peroxides which can decompose to yield alkanes, ketones and aldehydes. The aldehydes most extensively studied are 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal and malondialdehyde (MDA) [4]. The variety of lipid peroxidation (LPO) by-products can also exert adverse biological effects in exposed organisms [5]. The quantification of the diverse products of peroxidation especially level of MDA is now

being exploited as biomarker of oxidative stress [6]. This quantification has enabled early detection of stress in exposed organisms before lethal or pathological effects are observed. Oxidative damage is counteracted by antioxidant defense systems and repair mechanisms. The antioxidant defense systems comprise of a number of enzymes which act as scavengers of the highly reactive intermediates produced in cells during hydrocarbon metabolism to maintain cell homeostasis [6]. Notable antioxidant enzymes include (a) superoxide dismutase (SOD), which converts superoxides (O_2^-) generated in peroxisomes and mitochondria to hydrogen peroxide; (b) catalase (CAT) which removes the hydrogen peroxide by converting it to water and oxygen; (c) glutathione *S*-transferase (GST); (d) glutathione peroxidase and (e) glutathione reductase all of which are involved in the removal of hydrogen peroxide from the system in conjunction with reduced glutathione (GSH) [6]. The antioxidant defense system is now being increasingly studied because of its potential utility to provide biochemical biomarkers that could be used in environmental monitoring systems [7].

Doherty *et al.*, [8] investigated biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in some selected fishes in Lagos, Nigeria. They found that the trend of accumulation of the metals as follows: Catfish-Cu>Pb>Zn: Tilapia-Cu>Pb>Zn. The concentration of heavy metal in the flesh of the fish from the Lagos Lagoon was higher than that from the reference site for all the metals analyzed. Otitoloju and Olagoke [6] studied lipid peroxidation and antioxidant defense enzymes in *C. gariepinus* as useful biomarkers for monitoring exposure to polycyclic aromatic hydrocarbons and reported a significant increase in MDA with significant decrease in SOD, CAT and GST. The study concluded that the observed reduction in the activities of antioxidant defense enzymes such as SOD, CAT and GST in conjunction with an increase in MDA levels in the gill and liver tissues of test animals exposed to aromatic hydrocarbon compounds were recommended as a good battery of biomarkers for early detection of pollution during biomonitoring programs.

The fish species *Clarias gariepinus* (catfish) and *Oreochromis niloticus* (tilapia) are being widely used as food and feed supplement and are considered as important fishery product. In this study, estimation of bioaccumulation, activity of antioxidant enzymes and lipid peroxidation in *Clarias gariepinus* and *Oreochromis niloticus* was carried out to assess their importance as biological markers of environmental stress related to heavy metals. Heavy metals are major components of untreated industrial effluents frequently discharged into aquatic ecosystems in Nigeria; hence the need to continuously monitor bioaccumulation patterns and early markers of toxic effect in edible fish species.

2. Materials and Methods

2.1. Test Animals (Sources, Acclimatization and Selection) for Bioassays

Fingerlings of *Clarias gariepinus* and *Oreochromis niloticus* of similar age (1 - 2 weeks old) and size (mean snout to tail length: 4 ± 2 cm and mean weight: 15 ± 8 g) were collected from a local fish farm in Isheri, Lagos. Post juveniles of *Clarias gariepinus* and *Oreochromis niloticus* of similar age (6 - 8 weeks old) and size (mean snout to tail length: 16.00 ± 6.00 cm, mean weight: 39.00 ± 11.00 g) were also collected from the same fish farm in Isheri, Lagos. The animals were transported to the laboratory, Zoological Garden, University of Lagos and kept in transparent plastic tanks which contained aerated dechlorinated water (20 litres).

The test animals were allowed to acclimatize to laboratory conditions ($28 \pm 2°C$, R.H $70 \pm 2\%$) for a period of seven days before they were used in the bioassays. During the acclimatization, the animals were fed with 0.5 - 2.0 mm Coppens feed (40g per 100 animals). Water in the holding was changed once every two days to prevent accumulation of wastes and decaying food particles. Feeding was discontinued 24 hours prior to commencement of bio-assays.

2.2. Test Chemicals

The heavy metals investigated in this work were obtained as metallic salts from Ecotoxicology Laboratory, Unilag. The reagents are analytical grades of the following types:

a. Lead as $Pb(NO_3)_2$ (molecular weight 331.21 g, purity 99.5%) and

b. Zinc as $ZnCl_2.4H_2O$ (molecular weight 136.28 g, purity 98%)

These chemicals were manufactured by J.T. Baker, a division of Mallinekrodt Baker Inc.

2.3. General Bioassay Techniques

2.3.1. Single Action Bioassays for Acute Toxicity Testing

Stock solutions of $Pb(NO_3)_2$ and $ZnCl_2$ were prepared by weighing 1g of each salt using a Metler balance, then dissolved in 1 litre of distilled water to make a stock of 1000 ppm each. The resultant stock solutions were serially diluted to obtain solutions of required concentrations. The volume of the treated media (toxicant and dilution water) adopted for each test species were the same.

2.3.2. Measurement of the Physico-chemical Parameters in Test Media

Physico-chemical parameters such as dissolved oxygen, pH, salinity and temperature of the test media were measured at least twice (at the begin and at the end of experiment) for the various bioassays with the aid of digital read out instrument (Jenway product Model 300 series of pH meter, DO meter and Refractometer for salinity).

2.3.3. Acute Toxicity of Heavy Metals against *Clarias gariepinus*

Active catfishes, *Clarias gariepinus* (fingerlings) were randomly assigned to bioassay containers already holding treated or untreated test media. For the series of bioassays, 10 *Clarias gariepinus* were exposed per treatment including untreated control in two replicates (i.e. five animals per replicate) per treatment. In these substantive bioassays, after range finding preliminary trials, test

animals were exposed to graded series of concentrations of each heavy metal compound as follows:

a. Lead as $Pb(NO_3)_2$ against *Clarias gariepinus* at 20, 35, 50, 60, 70 mg/l and an untreated control.

b. Zinc as $ZnCl_2.4H_2O$ against *Clarias gariepinus* at 10, 15, 25, 40, 55 mg/l and an untreated control.

2.3.4. Acute toxicity of Heavy Metals against *Oreochromis niloticus*

Active tilapia, *Oreochromis niloticus* (fingerlings) were randomly assigned to bioassay containers already holding treated or untreated test media. For the series of bioassays, 10 *Oreochromis niloticus* were exposed per treatment including untreated control in two replicates (i.e. five animals per replicate) per treatment. In these substantive bioassays, after range finding preliminary trials, test animals were exposed to graded series of concentrations of each heavy metal compound as follows:

a. Lead as $Pb(NO_3)_2$ against *Oreochromis niloticus* at 10, 15, 20, 25, 30 mg/l and an untreated control.

b. Zinc as $ZnCl_2.4H_2O$ against *Oreochromis niloticus* at 10, 15, 25, 40, 50 mg/l and untreated control.

2.3.5. Assessment of Quantal Response (Mortality)

The animals (*Clarias gariepinus* and *Oreochromis niloticus*) were assumed to be dead when it is observed to be floating motionless on its back and not responding when prodded with a blunt glass rod. Mortality assessments were carried out once every 24 hours over a 96-hour period.

2.4. Bioaccumulation and Biochemical Studies of *Clarias gariepinus* and *Oreochromis niloticus* Exposed to Sublethal Concentrations of Heavy Metal Compounds (Pb and Zn) in Single Metal Bioassay

In this series of experiments, *Clarias gariepinus and Oreochromis niloticus* (post juveniles) were exposed to sublethal concentrations $(0.1 \times 96hr-LC_{50})$ of heavy metal salts $[Pb(NO_3)_2$ and $ZnCO_3]$ for a period of 30 days. *Clarias gariepinus* was exposed to 5.51 mg/l of $Pb(NO_3)_2$ and 3.21 mg/l of $ZnCO_3$ while *Oreochromis niloticus* was exposed to 1.49 mg/l of $Pb(NO_3)_2$ and 1.92 mg/l of $ZnCO_3$ in a single metal bioassays. These metal salts were each made up 10 litres with de-ionized portable water in plastic tanks. Semi-static bioassay procedure was adopted in order to avoid drastic changes in concentration of test media via evaporation and excessive reduction in dissolved oxygen (DO) level. In this semi-static procedure, each test media was changed into a fresh solution at exactly the same concentration of heavy metal salt or untreated control as the case may be, once every three days and transferring the same exposed test animals into the freshly prepared test media over the 30 days period of experimentation. Though, the process was rigorous during the changing of the test media but it was more conservative and feasible than the flow though method. At predetermined time intervals (days 15 and 30), two live juveniles of each species (*Clarias gariepinus and Oreochromis niloticus*) were randomly selected from treated and untreated test media and dissected. The liver, muscle (edible part) and gill were carefully extracted, kept in separate bottles and preserved in the refrigerator ready for digestion (for heavy metal analysis) and homogenization (for biochemical assay).

2.4.1. Bioaccumulation Studies

The digestion method employed was the Agua Agar (wet digestion) as described by Farounbi *et al.* [9]. The heavy metals were determined using Atomic Absorption Spectrophotometer (model 9100 Pye Unicamp) with a hollow for cathode lamp, a gas cylinder and an integrated digitized computer.

2.4.2. Biochemical Studies

2.4.2.1. Homogenizing the Liver Samples for Anti-oxidant Enzymes Assay

The post mitochondria fraction of the liver of each test animal was prepared according to Habbu *et al.* [10]. The organs (liver) were washed in an iced cold 1.15% KCl solution, blotted and weighed. They were then homogenized with 0.1 M phosphate buffer (pH 7.2), before putting the organs each into the mortar; laboratory sand was added to it (acid washed sand) and it was blended together in the mortar with pestle. The resulting homogenate was centrifuged at a speed of 2500 rpm for 15 mins after which it was removed from the centrifuge. The supernatant was decanted and stored at -21°C until spectrophotometric determination of antioxidant enzymes activity using UV-VISIBLE spectrophotometer [10].

2.4.2.2. Determination of Glutathione (GSH) Activity

The reduced glutathione content of the liver as non protein was estimated according to the method described by Sedlak and Lindsay [11]. To the tissue homogenate, 10% TCA was added, centrifuged. 1.0ml of supernatant was treated with 0.5 ml of Ellman's reagent (19.8mg of 5, 5-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm.

2.4.2.3. Determination of Superoxide Dismutase (SOD) Activity

Superoxide dismutase activity was determined by its ability to inhibit auto-oxidation of epinephrine which was estimated by the increase in absorbance at 480 nm as described by Sun and Zigma [12]. The reaction mixture (3 ml) contained 2.95 ml 0.05 M sodium carbonate buffer pH 10.2, 0.02 ml of tissue homogenate and 0.03 ml of epinephrine, 0.005 N HCL was used to initiate the reaction. The reference cuvette contained 2.95 ml buffer, 0.03 ml of substrate (epinephrine) and 0.02 ml of water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 5 minutes.

2.4.2.4. Determination of Catalase (CAT) Activity

Catalase activity was determined by measuring the decrease in absorbance at 240 nm due to the decomposition of hydrogen peroxide (H_2O_2) in a UV recording spectrophotometer. The reaction mixture (3 ml) contained 0.1 ml of tissue homogenate in phosphate buffer (50 mM, pH 7.0) and 2.9 ml of 30 mM H_2O_2 in phosphate buffer pH 7.0. An extinction coefficient for H_2O_2 at 240 nm of 40.0 $M^{-1}cm^{-1}$ was used for the calculation [13]. The

specific activity of catalase was expressed as moles of H_2O_2 reduced per minute per mg protein.

2.4.2.5. Determination of Glutathione-S-Transferase (GST)

The assay is based on the fact that all GST demonstrate a relatively high activity with 1-chlor-2, 4-dinitrobenzene as the second substrate. Consequently, the conventional assay for GST activity utilizes 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. The subsequent conjugation of this substance with reduced glutathione, results in a shift of its absorption maximum to a longer wavelength. The absorption increase at the new wavelength of 340nm provides a direct measurement of enzymatic reaction.

The medium for the estimation was prepared as shown in Table 1 and was allowed to run for 60 secs each time before the absorbance was read against the blank at 340 nm. The temperature was maintained at approximately 31°C. The absorbance was measured using a spectrophotometer.

Table 1. Assay Medium

REAGENT	BLANK	TEST
0.1 M Reduced glutathione (GHS)	30μl	30μl
20 mM CDNB	150μl	150μl
0.1 M Phosphate buffer(pH 6.5)	2.82ml	2.79ml
Sample (Cytosol/microsomes)	-	30μl
Total mixture	3ml	3ml

The extinction coefficient of CDNB = 9.6 $M^{-1}cm^{-1}$
GHS-S-transferase activity = OD/min × 19.6
0.03ml/mg protein = μmole/min/mg protein.

2.4.2.6. Determination of Lipid Peroxidation (LPO)

Malondialdehyde (MDA) an index of lipid peroxidation was determined using the method of Buege and Aust [14]. 1 ml of the super-natant was added to 2 ml of (1:1:1 ratio) TCA-TBA-HCL reagent [0.37% Thioarbituric acid (TBA), 15% Tricarcoxylic acid (TCA) and 0.24 N Hydrochloric acid (HCl)]. The TCA-TBA-HCL reagent was boiled at 100°C for 15 minutes and allowed to cool. Flocculent materials were removed by centrifuging at 3000 rpm for 10 minutes. The supernatant was removed and the absorbance read at 532 nm against a blank. MDA was calculated using the molar extinction coefficient for MDA-TBA-complex of $1.56×10^5$ $M^{-1}cm^{-1}$.

2.5. Statistics

Data collected from the bioaccumulation and biochemical analysis were subjected to descriptive and inferential statistics as follows:

Probit regression analysis based on a computer program (IBM SPSS v20.0) was used to analyze the toxicological dose-response data involving cumulative quantal response (mortality) after 96 hours. The indices of toxicity measurement derived from this analysis were:

LC_{50} = Median Lethal Concentration that causes 50% response (mortality) of exposed organisms.

LC_{95} = Lethal Concentration that causes 95% response (mortality) of exposed organisms.

LC_5 = Lethal Concentration that causes 5% response (mortality) of exposed organisms.

T.F = Toxicity for relative potency measurement i.e ratio of 96hr-LC_{50} of a compound.

$$T.F = \frac{96hr - LC_{50} \text{ of a other chemicals}}{\left(\begin{array}{c} 96hr - LC_{50} \text{ most toxic chemicals} \\ \text{tested against thesame specis} \end{array} \right)} \quad (1)$$

One-way analysis of variance (ANOVA) was used to compare the means of results obtained from bioaccumulation and biochemical analysis and where a significant difference ($p<0.05$) was obtained from the ANOVA, Scheffe multivariate (multiple) comparison was used to detect the source of the difference.

3. Results

3.1. Physicochemical Parameters of the Test Media

The results of the physicochemical parameters of the test media showed that the dissolved oxygen level ranged from 5.8 mg/l (after 2 days of exposure) to 9.7 mg/l (after each change to a clean media). The pH and salinity of the test media remained constant with values ranging from 7.2–7.5 and 2–3 ‰, respectively. The conductivity and total dissolved solids in the test media increased from 0.12 mS/cm and 0.06 mg/l to 0.26 mS/cm and 0.13 mg/l, respectively, over the period of observation.

3.2. Relative Acute Toxicity of Pb [Pb(NO₃)₂] and Zn (ZnCl₂) acting singly against *Clarias gariepinus* and *Oreochromis niloticus*

The 96hr-LC_{50} values of Pb and Zn was found to be 55.12 mg/l and 32.15 mg/l respectively (Table 2) against *C. gariepinus* and 14.93 mg/l and 19.23 mg/l respectively (Table 3) against *O. niloticus*. The ratio of the toxicity factors (T.F) of Zn to Pb when acting singly against *C. gariepinus* was 1.71:1 which implies that Zn was 1.71 times more toxic than Pb against *C. gariepinus* and 1:1.29 against *O. niloticus* which implies that Pb was 1.29 times more toxic than Zn against *O. niloticus.*

Table 2. Relative Acute Toxicity (96hr-LC_{50}) of Pb [Pb(NO₃)₂] and Zn (ZnCl₂) acting singly against *Clarias gariepinus*

Test chemicals	96hr-LC_{50} (95% CL)	SLOPE ± S.E	Probit line equation	D.F	T.F
Pb [Pb(NO₃)₂]	55.12 (68.54-45.98)	0.956±0.402	Y = -2.343 + 0.956x	1	1.71
Zn (ZnCl₂)	32.15 (47.08-22.56)	0.992±0.417	Y = -1.188 + 0.992x	1	1.00

LC = Lethal Concentration, CL = Confidence Limit, D.F = Degree of Freedom, T.F = Toxicity Factor, S.E = Standard Error

Table 3. Relative Acute Toxicity of Pb [Pb(NO₃)₂] and Zn (ZnCl₂) acting singly against *Oreochromis niloticus*

Test chemicals	96hr-LC_{50} (95% CL)	SLOPE±S.E	Probit line equation	D.F	T.F
Pb [Pb(NO₃)₂]	14.93 (19.56-9.17)	0.986±0.490	Y = -1.203 + 0.986x	1	1.00
Zn (ZnCl₂)	19.23 (28.85-7.35)	0.852±0.417	Y = -0.754 + 0.852x	1	1.29

LC = Lethal Concentration, CL = Confidence Limit, D.F = Degree of Freedom, T.F = Toxicity Factor, S.E = Standard Error.

3.3. Bioaccumulation Studies

3.3.1. Bioaccumulation of Pb and Zn in Liver, Muscle and Gill of *Clarias gariepinus* exposed to Sublethal Concentrations of Pb [Pb(NO₃)₂] and Zn (ZnCl₂).

There was differential variability in the rate of bioaccumulation of Pb and Zn in the liver, muscle and gill of *C. gariepinus* exposed to sublethal concentrations of Pb and Zn after 15 and 30 days compared to the control. The variation in the concentrations of Pb in liver of *C. gariepinus* ranged from 0.0065 ± 0.0005 ppm in control to 0.0120 ± 0.0010 ppm and 0.0225 ± 0.0015 ppm in exposed fishes after 15 and 30 days respectively. In muscle: 0.0040 ± 0.0040 ppm in control to 0.0100 ± 0.0000 ppm and 0.0360 ± 0.0250 ppm in exposed fishes after 15 and 30 days respectively. In gill: 0.0045 ± 0.0045 ppm in control to 0.0100 ± 0.0010 ppm and 0.0180 ± 0.0000 ppm in exposed fishes after 15 and 30 days respectively (Table 4). The increasing trend of accumulation of Pb in *C. gariepinus* was muscle > liver > gill after 30 days of exposure. The increase in the level of Pb in the tissues was not significant ($p > 0.05$) after 15 days while it was significant ($p < 0.05$) after 30 days when compared to the control. The variation in the concentrations of Zn in liver of *C. gariepinus* ranged from 0.0170 ± 0.0020 ppm in control to 0.0565 ± 0.0055 ppm and 0.0350 ± 0.0110 ppm in exposed fishes after 15 and 30 days respectively. In muscle: 0.0235 ± 0.0015 ppm in control to 0.0360 ± 0.0040 ppm and 0.0510 ± 0.0170 ppm in exposed fishes after 15 and 30 days respectively. In gill: 0.0195 ± 0.0015 ppm in control to 0.0400 ± 0.0030 ppm and 0.0400 ± 0.0140 ppm in exposed fishes after 15 and 30 days respectively (Table 4). The increasing trend of accumulation of Zn in *C. gariepinus* was muscle > gill > liver respectively after 30 days of exposure. The increase in the level of Zn in the tissues was not significant ($p > 0.05$) after 15 days while it was significant ($p < 0.05$) after 30 days when compared to the control.

Table 4. Bioaccumulation of Pb and Zn in tissues of *C. gariepinus*

SAMPLE		Pb (5.5115mg/l)			Zn (3.2149mg/l)		
		Day 0	Day 15	Day 30	Day 0	Day 15	Day 30
LIVER	Mean±SEM	0.0065±0.0005	0.0120±0.0010	0.0225±0.0015*	0.0170±0.0020	0.0565±0.0055	0.0350±0.0110*
MUSCLE	Mean±SEM	0.0040±0.0040	0.0100±0.0000	0.0360±0.0250*	0.0235±0.0015	0.0360±0.0040	0.0510±0.0170*
GILL	Mean±SEM	0.0045±0.0045	0.0100±0.0010	0.0180±0.0000*	0.0195±0.0015	0.0400±0.0030	0.0400±0.0140*
WHO/FAO Limit				≤0.05			≤5.00

*-significant difference at $p < 0.05$ across rows.

3.3.2 Bioaccumulation of Pb and Zn in Liver, Muscle and Gill of *Oreochromis niloticus* exposed to Sublethal Concentrations of Pb [Pb(NO₃)₂] and Zn (ZnCl₂).

There was differential variability in the rate of bioaccumulation of Pb and Zn in the liver, muscle and gill of *O. niloticus* exposed to sublethal concentrations of Pb and Zn after 15 and 30 days compared to the control. The variation in the concentrations of Pb in liver of *O. niloticus* ranged from not detected (ND) in control to 0.0090 ± 0.0030 ppm and 0.0050 ± 0.0040 ppm in exposed fishes after 15 and 30 days respectively. In muscle: ND in control to 0.0015 ± 0.0015 ppm and 0.0070 ± 0.0040 ppm in exposed fishes after 15 and 30 days respectively. In gill: ND in control to 0.0105 ± 0.0015 ppm and 0.0110 ± 0.0050 ppm in exposed fishes after 15 and 30 days respectively (Table 5). The increasing trend of accumulation of Pb in *O. niloticus* was gill > muscle > liver after 30 days of exposure. The increase in the level of Pb was significant ($p < 0.05$) only in gill after 15 days while it was significant ($p < 0.05$) after 30 days in the liver, muscle and gill of the exposed fishes was when compared to the control. The variation in the concentrations of Zn in liver of *O. niloticus* ranged from 0.0240 ± 0.0030 ppm in control to 0.0475 ± 0.0065 ppm and 0.0410 ± 0.0050 ppm in exposed fishes after 15 and 30 days respectively. In muscle: 0.0210 ± 0.0010 ppm in control to 0.0330 ± 0.0030 ppm and 0.0220 ± 0.0060 ppm in exposed fishes after 15 and 30 days respectively. In gill: 0.0155 ± 0.0025 ppm in control to 0.0335 ± 0.0045 ppm and 0.0395 ± 0.0165 ppm in exposed fishes after 15 and 30 days respectively (Table 5). The increasing trend of accumulation of Zn in *O. niloticus* was liver > gill > muscle after 30 days of exposure. The increase in the level of Zn was significant ($p < 0.05$) only in liver and gill after 15 days while it was significant ($p < 0.05$) after 30 days in the liver, muscle and gill of the exposed fishes was when compared to the control.

Table 5. Bioaccumulation of Pb and Zn in tissues of *O. niloticus*

SAMPLE		Pb (1.4928mg/l)			Zn (1.9230mg/l)		
		Day 0	Day 15	Day 30	Day 0	Day15	Day 30
LIVER	Mean±SEM	ND	0.0090±0.0030	0.0050±0.0040*	0.0240±0.0030	0.0475±0.0065*	0.0410±0.0050*
MUSCLE	Mean±SEM	ND	0.0015±0.0015	0.0070±0.0040*	0.0210±0.0010	0.0330±0.0030	0.0220±0.0060*
GILL	Mean±SEM	ND	0.0105±0.0015*	0.0110±0.0050*	0.0155±0.0025	0.0335±0.0045*	0.0395±0.0165*
WHO/FAO Limit				≤0.05			≤5.00

ND: Not detected; *-significant difference at $p < 0.05$ across rows

3.4. Biochemical studies

3.4.1. Toxic Effects of Sublethal Concentrations of Pb [Pb(NO₃)₂] and Zn (ZnCl₂) on Analyzed Biochemical Parameters in the Liver of *Clarias gariepinus*.

A significant decrease ($p < 0.05$) was recorded in the activity of SOD in the liver of *C. gariepinus* exposed to sublethal concentrations of Pb and Zn after 15 and 30 days as compared to control (***Pb***- 5.84 ± 0.50 in control to 1.83 ± 0.15 and 0.82 ± 0.20 and ***Zn***- 5.84 ± 0.50 in control to 4.47 ± 0.61 and 0.68 ± 0.13 at day 15 and day 30

respectively). The activity of CAT in the liver of the exposed fishes decreased after 15 and 30 days exposure to Pb as compared to control and varied after 15 and 30 days exposure to Zn as compared to control (***Pb***- 22.79 ± 2.38 in control to 15.02 ± 0.91 and 8.06 ± 3.74 and ***Zn***- 22.79 ± 2.38 in control to 26.63 ± 3.20 and 4.96 ± 0.70 at day 15 and day 30 respectively). There was a significant decrease ($p<0.05$) in the activity of GST in the liver of the exposed fishes after 15 and 30 days of exposure to both metals as compared to the control (***Pb***-1.74 ± 0.14 in control to 0.55 ± 0.05 and 0.22 ± 0.06 and ***Zn***- 1.74 ± 0.14 in control to 1.33 ± 0.17 and 0.20 ± 0.04 at day 15 and day 30

respectively). The level of GSH in the liver decreased significantly ($p<0.05$) after exposure to both metals at 15 and 30 days as compared to the control (***Pb***- 0.56 ± 0.03 in control to 0.12 ± 0.02 and 0.10 ± 0.02 and ***Zn***- 0.56 ± 0.03 in control to 0.15 ± 0.11 and 0.08 ± 0.02 at day 15 and day 30 respectively). The level of MDA in the liver increased after 15 and 30 days of exposure to both metals as compared to the control, though not significant at $p>0.05$ (***Pb***- 0.07 ± 0.02 in control to 0.11 ± 0.01 and 0.10 ± 0.01 and ***Zn***- 0.07 ± 0.02 in control to 0.10 ± 0.02 and 0.11 ± 0.01 at day 15 and day 30 respectively) (Table 6).

Table 6. Biochemical Parameters of *Clarias gariepinus* exposed to sublethal concentrations of Pb [Pb(NO₃)₂] and Zn (ZnCl₂)

			SOD U/mg Pro	CAT U/mg Pro	GST U/mg Pro	GSH U/mg Pro	MDA U/mg Pro
Day 0	*Pb*	Mean±SEM	5.84±0.50	22.79±2.38	1.74± 0.14	0.56±0.03	0.07± 0.02
	Zn	Mean±SEM	5.84±0.50	22.79±2.38	1.74± 0.14	0.56±0.03	0.06± 0.02
Day 15	*Pb*	Mean±SEM	1.83±0.1*	15.02±0.91	0.55± 0.05*	0.12±0.02*	0.11± 0.01
	Zn	Mean±SEM	4.47±0.6	26.63±3.20	1.33± 0.17	0.15±0.11	0.10± 0.02
Day 30	*Pb*	Mean±SEM	0.82±0.2*	8.06± 3.74*	0.22± 0.06*	0.10±0.02*	0.10± 0.01
	Zn	Mean±SEM	0.68±0.1*	4.96± 0.70	0.20± 0.04*	0.08±0.02*	0.11± 0.01

*-significant difference at $p<0.05$ across columns

3.4.2. Toxic Effects of Sublethal Concentrations of Pb [Pb(NO₃)₂] and Zn (ZnCl₂) on Analyzed Biochemical Parameters in the Liver of *Oreochromis niloticus*

There was a decrease in the activity of SOD in the liver of *O. niloticus* exposed to sublethal concentration of Pb and Zn after 15 and 30 days exposure to both metals as compared to the control (***Pb***- 4.64 ± 0.12 in control to 4.50 ± 1.38 and 1.06 ± 0.06 and ***Zn***- 4.64 ± 0.12 in control to 4.18 ± 0.19 and 0.62 ± 0.16 at day 15 and day 30 respectively). The activity of CAT in the liver of the exposed fishes increased after 15 days and decreased after 30 days of exposure to both metals as compared to control (***Pb***- 22.00 ± 0.04 in control to 26.65 ± 7.26 and 8.72 ± 0.14 and ***Zn***- 22.00 ± 0.04 in control to 28.08 ± 4.10 and 6.56 ± 0.96 at day 15 and day 30 respectively). There was a decrease in the level of GST in the liver after 15 and 30

days exposure to both metals as compared to the control (***Pb***- 1.74 ± 0.14 in control to 1.34 ± 0.42 and 0.30 ± 0.02 and ***Zn***- 1.74 ± 0.14 in control to 1.26 ± 0.06 and 0.32 ± 0.09 at day 15 and day 30 respectively). A decrease was also recorded in the level of GSH in the liver after 15 and 30 days exposure to both metals as compared to control (***Pb***- 0.59 ± 0.12 in control to 0.35 ± 0.09 and 0.16 ± 0.00 and ***Zn***- 0.59 ± 0.12 in control to 0.44 ± 0.08 and 0.05 ± 0.00 at day 15 and day 30 respectively). However, this decrease in the levels of GSH, SOD, CAT and GST was only significant ($p<0.05$) in the liver of *O. niloticus* exposed to Zn after 30. A non significant increase ($p>0.05$) was recorded in the level of MDA in the liver after 15 and 30 days of exposure to both metals as compared to control (***Pb***- 0.03 ± 0.01 in control to 0.06 ± 0.02 and 0.06 ± 0.01 and ***Zn***- 0.03 ± 0.01 in control to 0.05 ± 0.02 and 0.05 ± 0.01 at day 15 and day 30 respectively) (Table 7).

Table 7. Biochemical Parameters of *Oreochromis niloticus* exposed to sublethal concentrations of Pb [Pb(NO₃)₂] and Zn (ZnCl₂)

			SOD U/mg Pro	CAT U/mg Pro	GST U/mg Pro	GSH U/mg Pro	MDA U/mg Pro
Day 0	*Pb*	Mean±SEM	4.64±0.12	22.00±0.04	1.74±0.14	0.59±0.12	0.03± 0.01
	Zn	Mean±SEM	4.64±0.12	22.00±0.04	1.74± 0.14	0.59±0.12	0.03± 0.01
Day 15	*Pb*	Mean±SEM	4.50±1.38	26.65±7.26	1.34±0.42	0.35±0.09	0.06± 0.02
	Zn	Mean±SEM	4.18±0.19	28.08±4.10	1.26± 0.06	0.44±0.08	0.05± 0.02
Day 30	*Pb*	Mean±SEM	1.06±0.06	8.72± 0.14	0.30±0.02	0.16±0.00	0.06± 0.01
	Zn	Mean±SEM	0.62±0.16*	6.56± 0.96*	0.32± 0.09*	0.05±0.00*	0.05± 0.01

*-significant difference at $p<0.05$ across columns.

4. Discussion

Toxic effects of the test heavy metals on the behaviour of the test organisms was observed during the acute toxicity studies and included loss of balance, weakness, skin bleaching, gasping for air at water surface as air bubbles were seen on the test medium and a thick layer of mucus on the skin of the test animals that died within 96 hours. There was also reduced activity indicated by vertical positioning and reduced mobility of the fishes in the test medium.

The result of the acute toxicity tests indicated that Zn was more toxic than Pb against *C. gariepinus*, while Pb was more toxic than Zn against *O. niloticus*. Saliu and Bawa-Allah [15] who investigated the toxicological effects of sublethal concentrations of lead and zinc salts [Pb(NO₃)₂ and ZnCl₂] on the antioxidant enzymes of post juvenile *C. gariepinus* in laboratory bioassays also reported a higher toxicity of Zn than Pb against *C. gariepinus*. Also, Otitoloju and Don-Pedro [16] who established the toxicity ranking order of heavy metals and sensitivity scales of benthic animals inhabiting Lagos lagoon reported that Zn was more toxic than Pb. The 96hr-LC_{50} values in this study indicated a lesser toxicity of Zn

and Pb against *C. gariepinus* than the earlier work of Saliu and Bawa-Allah [15]. Several factors which include the physico-chemistry (e.g. affinity for organic molecules and solubility) of the heavy metal compound tested, predominant ion in test medium, physico-chemical characteristics of the test medium and mechanisms of action of different heavy metals may result in the observed differential toxicity of the heavy metals. In this study, *O. niloticus* was more susceptible to the tested heavy metals than *C. gariepinus* which indicates that *O. niloticus* may be a more sensitive (less tolerant) species to heavy metal pollution than *C. gariepinus*.

Bioaccumulation occurs as a result of competing rates of chemical uptake and elimination, the latter comprising biotransformation and excretory process [17]. Biotransformation of metals in animal tissues involves the synthesis of low molecular weight protein for example metallothionien in animal tissues which then complex with the metals. The resultant metal-protein complexes may be water-insoluble, so they become sequestrated in lipid or fatty tissues resulting in bioaccumulation but if the metal-protein complexes are water-soluble, then they are excreted resulting in a decrease in the metal burden in the organism [18,19]. In this study, the accumulation of Zn was higher than Pb in tissues of both *C. gariepinus* and *O. niloticus* and this is in contrast to the field assessment carried out by Doherty *et al.* [8] who studied biomarkers of oxidative stress and heavy metal (including Pb and Zn) levels as indicators of environmental pollution in some selected fishes in Lagos, Nigeria. The rate of accumulation of Pb and Zn in *C. gariepinus* was higher than that of *O. niloticus* also in contrast to the field assessment carried out by Doherty *et al.* [8]. This observation implies that the concentration of metals accumulated is directly related to the concentrations of metals prevailing and readily available in the environment which can be affected by physico-chemical parameters of the prevailing medium. Bioaccumulation of heavy metals in animal tissues occurs as a result of competing rates of chemical uptake and excretion whereby a net accumulation is due to a higher rate of uptake than the rate of excretion [20]. The trends of accumulation of Pb and Zn in *C. gariepinus* was muscle > liver > gill and muscle > gill > liver respectively while in *O. niloticus* it was gill > muscle > liver and liver > gill > muscle for Pb and Zn respectively after 30 days exposure. The trend of accumulation may be due to the relative rate of uptake and excretion of the heavy metals [17].

Fishes are largely being used for the assessment of the quality of aquatic environment and as such, can serve as bio-indicators of environmental pollution [21]. Many environmental pollutants including heavy metals are known to induce oxidative stress through generation of ROS (Reactive Oxygen Species) [22]. Elevated ROS level in tissues leads to cellular damage when the rate of its generation surpasses the rate of its decomposition by antioxidant defense systems. Exposure of the test organisms to sublethal concentrations of heavy metals (Pb and Zn) was found to cause an increase in level of lipid peroxides (measured as MDA), indicative of oxidative stress in the exposed animals compared to control. This result is in agreement with findings of Achuba and Osakwe [23], Avci *et al.* [24], Otitoloju and Olagoke [6] who reported an increase in LPO in tissues of fishes exposed to petroleum hydrocarbons. The increase in LPO

is due to an inhibitory effect on mitochondrial electron transport system leading to stimulation in the production of intracellular ROS [25]. The measurements of lipid peroxides levels in plants and animal tissues exposed to different pollutants have been recognized as reliable early warning signal of exposure to environmental stress and integrated to environmental monitoring programs [24,26,27].

Several studies on aquatic organisms have demonstrated the importance of enzymatic antioxidant defenses in protecting cellular systems from oxidative stress induced by xenobiotics [27]. The activity of antioxidant enzymes may be enhanced or inhibited under chemical stress depending on the intensity and the duration of the stress applied, as well as, the susceptibility of the exposed species. In this study, the activity of the enzyme SOD was inhibited in the liver tissues of fishes exposed to the heavy metals (Pb and Zn) after 30 days when compared to the control. This result is in agreement with the findings of Faramobi *et al.* [9], Otitoloju and Olagoke [6], Saliu and Bawa-Allah [15]. However, this inhibition was significant in *C. gariepinus* exposed to Pb and Zn while it was significant in *O. niloticus* exposed to only Zn. The enzyme SOD is known to provide cytoprotection against free radical induced damage by converting superoxide radicals (O_2^-) generated in peroxisomes and mitochondria to hydrogen peroxides. The hydrogen peroxide is then removed from the system by the enzyme CAT, which converts it to water and molecular oxygen (O_2). The inhibition of the enzyme SOD by the test chemicals will, therefore, lead to increased oxidative stress in the tissues as a result of the damaging activities of the superoxide radicals (O_2^-). Furthermore, the inhibition of the enzyme SOD will expectedly result in a reduction in the activity of the enzyme CAT, due to a decrease in H_2O_2 generation from SOD activities [28]. This indeed proved to be the case in this study as there was a reduction in CAT activity in the exposed fishes after 30 days of exposure to the heavy metals when compared to the control. Similar observation of a decrease in CAT activity following an inhibition of the activity of enzyme SOD has been reported by Fatima and Ahmad [26], Otitoloju and Olagoke [6] and Saliu and Bawa-Allah [15]. However, the reduction was significant only on exposure of *C. gariepinus* to Pb and *O. niloticus* to Zn after 30 days.

GST is a cytosolic or microsomal enzyme that catalyses the conjugation of reduced glutathione (GSH) with oxidative products, such as 4-hydroxyalkenals (membrane peroxides) and/or base propenals, resulting from DNA oxidative degradation [29]. It also plays an important role in protecting tissues from oxidative stress [30,31]. In this study, the activity of GST was reduced in the liver tissues of fishes exposed to the heavy metals after 30 days when compared to control. This result is in agreement with the findings of Otitoloju and Olagoke [6] and Saliu and Bawa-Allah [15]. However, the inhibition was significant in *C. gariepinus* exposed to Pb and Zn while it was significant in *O. niloticus* exposed to only Zn. Impairment in antioxidant enzymes will produce an imbalance between pro and antioxidant systems causing the formation of toxic hydroxyl radicals, with direct consequences on the cell integrity and cell functions itself [32]. The level of GSH activity in this study was also reduced in the fishes exposed to the heavy metals after 30 days when compared to control. This result is in agreement with the findings of

Saliu and Bawa-Allah [15] who reported a decrease in the level of GST-GSH activity in the liver of post-juvenile *C. gariepinus* exposed to Pb(NO$_3$)$_2$ after 30 days. However, the reduction was significant in *C. gariepinus* exposed to Pb and Zn while it was significant in *O. niloticus* exposed to only Zn.

Recent investigations of changes in antioxidant defenses has shown that they can be used as biomarkers of oxidative stress by various pollutants in aquatic organisms [33,34,35]. Bocchettii *et al*., [36] have also confirmed the role of antioxidant enzymatic systems under polluted environmental conditions and their importance in sensitive ecotoxicological studies. The use of biochemical responses as biomarkers during environmental monitoring programs is derived from the basis that a toxic effect manifests itself at the subcellular level before it becomes apparent at higher levels of biological organization. The measurement of these biochemical responses may therefore serve to improve the assessment of biologically significant exposures to toxic chemicals and enhance ability to assess the risk of effects of pollutants on the health and survival of toxicant-exposed populations.

5. Conclusion

This study reports a significant decrease in anti-oxidant defense system (GSH, SOD and GST) of *C. gariepinus* exposed to Pb and Zn, a significant decrease in CAT of *C. gariepinus* exposed to Pb, a significant decrease in anti-oxidant defense system (GSH, SOD, CAT and GST) of *O. niloticus* exposed to Zn, and an apparent increase in MDA in both test organisms in conjunction with increasing bioaccumulation of the heavy metals (Pb and Zn). These can serve as biomarkers for early detection of pollution during biomonitoring programs. There is a need for extensive evaluation and comparison of data obtained from field studies and those obtained from laboratory studies because there are some environmental factors that are at play in the field which cannot be replicated under laboratory conditions, and which may also have significant effects on oxidative defense response of organisms to persistent levels of high concentrations of heavy metals. Evaluation of field observations viz laboratory data will also help to establish the usefulness of biomarkers as indicators of environmental pollution. Furthermore, studies integrating the study of biomarkers and bioaccumulation patterns in aquatic organisms will serve as a better tool for monitoring environmental pollution and improving ecological risk assessment associated with heavy metals in aquatic ecosystems.

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