Oxidative Stress Markers (Malondialdehyde, Catalase, Superoxide Dismutase, Glutathione-Peroxidase, and Glutathione Reductase) and Metallothionein Activities of *Papyrocarpus Afer*

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ABSTRACT

The contamination of water bodies has become a serious issue in Nigeria today, as xenobiotics entering the aquatic environment exert their effects through redox cycling. The effect of these pollutant can be assessed using malondialdehyde (MDA), antioxidant enzymes such as (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GXP), glutathione reductase (GR)) and metallothionein (MT) as biomarkers in fish from Banegbe River. Our results showed Significant ($p < 0.05$) elevations in MDA, and the antioxidant enzymes (SOD, CAT, GXP, GR and MT) activities from middle-stream, down-stream and up-stream when compared to control (from Orogodo river). But the fish from control II (Ekpan pond) also had a significant ($p < 0.05$) elevation in these enzyme activities when compared to the fish from control I, except for the fat content of the fish that had a Significant ($p < 0.05$) decrease in its activities in the same pattern.

The elevation in MDA and the enzymes indicates the presence of pollutants in the river and oxidative damage in fish, especially around the middle-stream environment. In conclusion, responses to the pollutants can be monitored using the antioxidant system as a useful tool in early warning in an aquatic environment.

Keywords: oxidative stress, Banegbe river, xenobiotics, antioxidant enzymes, metallothionein

INTRODUCTION

Aquatic ecosystems are inhabited by a wide range of organisms, from simple prokaryotes to more complex vertebrates. These environments also benefit humans directly by supplying essentials like water and food, as well as opportunities for transportation, economic activities, and recreation. Sadly, these ecosystems often serve as repositories for numerous chemical and physical agents, many of which are toxic substances known as xenobiotics, causing ongoing harm to the natural world. Pollutants originate from various sources, including fossil fuel combustion, industrial wastewater, sewage treatment facilities, agricultural activities, and non-point source runoff. A significant number of these environmental contaminants can accumulate in the bodies of aquatic organisms, leading to detrimental effects linked to oxidative stress (Winston *et al*., 1991).

Oxidative stress is a redox disequilibrium in which the pro-oxidant/antioxidant balance is shifted in favour of the pro-oxidants, a phenomenon related to the aerobic nature of cellular metabolism, in which O_2 reduction is a major event. The latter proceeds through electron transfer reactions due to the electronic structure of O_2 in the ground state, with the generation of reactive oxygen species (ROS). It also includes both antioxidant defenses as well as oxidative damage, which is a common effect in organisms exposed to xenobiotics in their environment. xenobiotics can generate reactive oxygen species, including superoxide anion (O_2·-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and singlet oxygen (O_21), which in turn are responsible for cell and tissue damage associated with different pathologic processes, including mutagenesis and carcinogenesis

Oxidative stress refers to a state of redox imbalance where the ratio of pro-oxidants to antioxidants is tipped in favor of pro-oxidants. This situation is tied to the aerobic nature of cellular metabolism, where the reduction of O2 is a key step. This reduction occurs through electron transfer processes due to the electronic configuration of O_2 in its ground state, leading to the formation of reactive oxygen species (ROS). Additionally, this encompasses

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both the body's antioxidant mechanisms and the oxidative damage that frequently occurs in organisms that encounter xenobiotics in their environment. Xenobiotics can produce reactive oxygen species such as superoxide anion ($O_2 \cdot -$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and singlet oxygen (O_2^1), which are ultimately responsible for cellular and tissue harm linked to various pathological conditions, including mutagenesis and carcinogenesis.

The antioxidant defense system (ADS) of organisms provides a means of dealing with oxidative stress and includes several enzymes and vitamins (Binet *et al.*, 1998). A primary role of the antioxidant defense system is protecting cellular compounds from ROS (Reactive Oxygen Species) (Kelly *et al.*, 1998).

Normally In aerobic cells, reactive oxygen species (ROS) including Primary oxidants such as superoxide anions ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO\bullet$) and secondary oxidants such as hydroperoxides and peroxyl radicals of biomolecules, (Veldela and Fernanda, 1988) are generated during normal metabolism, particularly as a result of oxidative metabolism in mitochondrial membranes. Thus, the reactive oxygen species (ROS) can be removed to protect the organisms from oxidative damage (Livingstone, 2001). To prevent oxidation-induced damage, caused by these reactive species, living organisms have evolved numerous defense mechanisms to counteract their harmful effects. The detoxication of ROS is a major requirement of aerobic life (Sies, 1986), which is accomplished via several enzymatic and non-enzymatic antioxidant mechanisms that are available in different cell compartments.

Studies of the stress response related to oxidative stress in aquatic organisms have been used as a source of important information or provide biochemical biomarkers that could be used as tools for monitoring the quality of the environment (Valavanidis *et al*., 2006).

According to Sogbanmu and Otitoloju (2004), the use of biomarkers in environmental monitoring confers significant advantages over traditional chemical measurements because measured biological effects can be meaningfully linked to environmental consequences so that environmental concerns can be directly addressed. The activity of antioxidants may be increased or inhibited under chemical stress, depending on the intensity and duration of stress applied as well as the susceptibility of the exposed species. (Vander Oost *et al*., 2003). The antioxidant enzymes tend to respond differently to various chemical compounds; therefore, the activity of an individual antioxidant enzyme cannot serve as a general marker of oxidative damage. Hence, multiple antioxidant values are often measured together to indicate the total oxyradical scavenging capacity, and this has been observed to provide a greater indication value (Regoli *et al.*, 2002). The antioxidant enzymes that make up the antioxidant defense system are expected to be intrinsically linked and dependent upon the activity of one another.

Metallothionein (MT) comprises a group of low molecular weight proteins found in the cytosol, characterized by their highly conserved cysteinyl residues. These residues enable MT to bind, carry, and store various essential metals (such as Zn and Cu) along with non-essential heavy metals (including Cd and Hg) through thiolate bonding (Isani and Carpene, 2014). Furthermore, it is widely recognized that MT serves as a part of the antioxidant system and plays a role in neutralizing free radicals and reactive oxygen species (Viarengo *et al.*, 2000). Consequently, MT is regarded as a marker for assessing environmental degradation and as a specific measure of toxic, carcinogenic, and mutagenic substances in biological materials (Verlecar *et al.*, 2006). Among the aquatic species, the fish is the major target of xenobiotic contamination. Fish are largely being used for the assessment of the quality of the aquatic environment, and as such can serve as bioindicators of environmental pollution. The study looked at the health of fish from the Banegbe River using antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase), metallothionein, MDA, and fat content (and MDA to show the effect of pollutants received by the river through different industrial effluents.

MATERIALS AND METHODS

Study site

Fish samples were collected from different stations (Fig. 1) just like from Joy *et al*., 2023 in Banegbe River; Latitude 5 º 14N and longitude 5º 22E, Latitude 5º 28N and longitude 5º 10E, and Latitude 5 º 43N, longitude 5º 14E and latitude 5º 30N and longitude 5º 44E.

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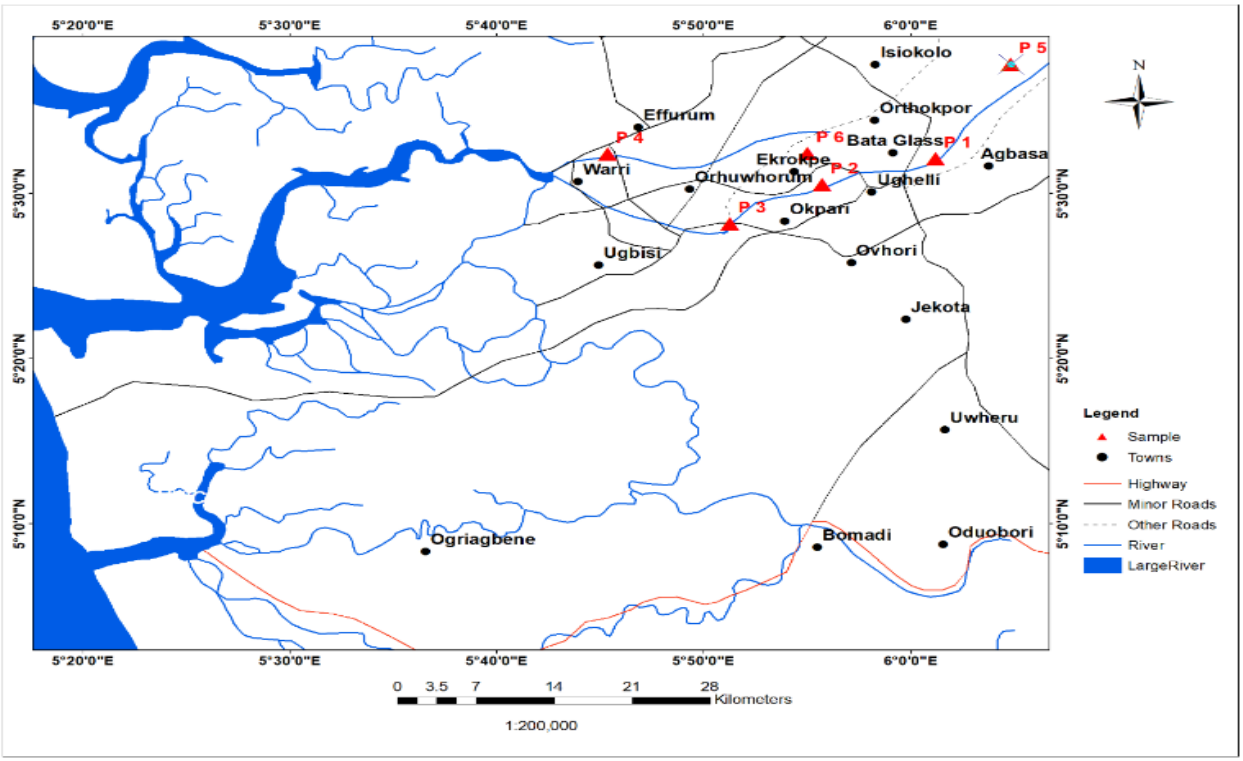

Fig. 1. Map showing sample sites (Ogana *et al*., 2023)

Where P1= up-stream, P2 = effluent discharge point, P3 = down-stream, P4 =Ekpan pond

Experimental Design

The fish samples were harvested from Banegbe River at three different points,

1 At the point of discharge of the thermal power plant effluent into the river,

2 Up-stream from the place of discharge of the power station effluent

3 Downstream from the place of discharge of power station effluent

The fish samples were grouped as follows:

Group 1 = Control 1;

Group 2 = Control 2

Group 3 = Upstream 1;

Group 4 = Upstream 2

Group 5 = Upstream 3;

Group 6 = Upstream 4

Group 7 = Downstream 1;

Group 8 = Downstream 2

Group 9 = Downstream 3;

Group 10 = Downstream 4

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Group 11 = Middle-stream 1 Group 12 = Middle-stream 2

Group 13 = Middle-stream 3 Group 14 = Middle-stream 4

Collection of fish samples

A total of 56 African knifefish (*Papyrocraruns afer*) were collected by professional local fishermen using a fishing net in the river. The fish samples were of similar weight and size. Live specimens of *P. afer* were transferred into water buckets and brought to the laboratory for further analysis. Fish blood samples were collected for the determination of biochemical parameters.

Test groups: Forty-eight of these fish samples were collected from the Banegbe River, Ughelli.

B. Control 1: Four fish of the same species were collected from the Orogodo River, Agbor.

C. Control 2: Four were bought from a pond at Ekpan, Warri, all in Delta State, Nigeria.

METHODS

Determination of Malondialdehyde (MDA) Concentration

The concentration of malondialdehyde (MDA) was measured following the procedure outlined by Farombi *et al.,* (2002). A 20-fold dilution of the liver homogenate was prepared, and 0.4 ml of each diluted sample was combined with 1.6 ml of 0.15 M Tris KCL buffer. Subsequently, 0.5 ml of 20% TCA was added, followed by 0.5 ml of 52 mM TBA, and the mixture was incubated in a water bath for 45 minutes at 80°C. After the incubation period, the mixture was cooled and centrifuged for 10 minutes at 3000 rpm. The absorbance of the resulting clear supernatant was assessed against distilled water as a reference blank at a wavelength of 532 nm. The MDA concentration in each sample was calculated by dividing the absorbance by the molar extinction coefficient.

Assay of Catalase Activity

The catalase activity of the serum was determined according to the method of Sinha (1972). The standard curve of H_2O_2 was prepared. The sample was diluted at a ratio of 1:50. To this, 4 ml of 0.2M hydrogen peroxide was added to 5 ml of 0.1M phosphate buffer at pH 7.0, followed by 1.0 ml of the appropriately diluted sample preparation, which was mixed gently with the H2O2/buffer solution at room temperature. A portion of 1.0 ml from the mixture was extracted and introduced to 2 ml of dichromate/acetic acid reagent at one-minute intervals, with a steady absorbance reading recorded at 570 nm. The monomolecular velocity constant K for the catalase-catalyzed decomposition of hydrogen peroxide was calculated using the equation for a first-order reaction.

$$K = \frac{1}{\Delta t} \; x \; In\left(\frac{S_0}{S}\right)$$

Where S_0 is the initial H_2O_2 concentration and S is the concentration of H_2O_2 at a particular time interval given as t (minutes). The value of K is plotted against t, and the velocity constant of catalase K (0) at 0 minutes is determined by extrapolation (that is, the intercept on the vertical axis). The catalase contents of the sample were expressed in terms of catalase fähigkeit or' Kat. Kat. F = K (0/mg protein per ml.)

Assay of Superoxide Dismutase (SOD) Activity

SOD activity was assayed using the method of Fridovich (1989). The sample underwent a dilution of 20 times, after which 0.2 ml was combined with 2.5 ml of a freshly prepared adrenaline solution, followed by a rapid inversion mixing in the cuvette. Consequently, the reference cuvette included 2.5 ml of buffer, 0.3 ml of adrenaline, and 0.2 ml of the sample. The change in absorbance was measured at 480 nm for 150 seconds, with readings taken every 30 seconds.

The results were calculated as follows

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$$\text{Increase in absorbance per minute} = \frac{A^3 - A_0}{2.5}$$

Where A_0 = absorbance after 30 seconds

A_3 =absorbance after 150 seconds

%inhibition = (increase in absorbance for substrate) x 100/ (increase in absorbance for blank)

Assay of Glutathione Peroxidase (GPX) Activity

Glutathione peroxidase assay was evaluated by the methods of Paglia and Valentine (1967), Kraus and Ganther (1980). A vial of reagent was mixed with 6.5 mL of buffer to create the reagent mixture. This process was carried out for the entire sample size. Ten milliliters of normal saline was utilized to dilute 10µl of Cumene Hydroperoxide, which was shaken thoroughly to ensure proper mixing. A total of 200 mL of distilled water was employed to reconstitute the vial of Diluting Agent. A volume of twenty microliters of the sample was placed into a test tube, designating it as sample (S), followed by the addition of 100µl of the reagent mixture and 40µl of Cumene Hydroperoxide, ensuring that the entire reaction medium was well-mixed. In a separate test tube marked as reagent blank (RB), 1000µl of the reagent mixture was pipetted. The initial absorbance readings for sample (S) and the reagent blank (B) were taken after one minute, and the timer was started simultaneously. These readings were taken again after one minute and then after two minutes. The absorbance of the reagent blank was deducted from that of the sample. The concentration of Glutathione peroxidase was determined using this formula.

U/l of Haemolysate = 8412 x Change in Absorbance (ΔA) 340nm/minute.

Assay of glutathione reductase (GR) Activity

GR activity was quantified following the method described by Kenji (1999). Fish liver samples were analyzed using the post-mitochondrial fraction (supernatant). An assay solution was prepared containing 50 mM phosphate buffer (pH 7.6), 1 mM EDTA, 0.1 mM NADPH, 1 mM GSSG, and 0.1% bovine serum albumin (BSA). This mixture was allowed to pre-incubate for 5 minutes at room temperature. The reduction in absorbance caused by 0.1 ml of cytosol was measured at 340 nm using a Beckman spectrophotometer (DU 640), compared to a blank that included all assay mixture components minus GSSG. The enzyme's activity was determined by applying the molar extinction coefficient of NADPH (£340 = 6.22 x 10^3 M-1 cm-1), with the results presented as nmol NADPH oxidized per minute per mg of protein.

Determination of metallothionein

Metallothionein was conducted following the procedure outlined by Linde et al. (2008). The liver tissues were homogenized in a buffer composed of 0.5 M sucrose and 20 mM Tris-HCl buffer at pH 8.6, which included 0.01% β-mercaptoethanol. The homogenate was distributed into 3 mL aliquots. These homogenates underwent centrifugation at 30,000 x g for 20 minutes to obtain a supernatant that contained metallothionein. Subsequently, to each 1 mL of the supernatant obtained, 1.05 mL of cold absolute ethanol (at -20 °C) and 80 µL of chloroform were introduced. The samples were then centrifuged at a low temperature (0-4 °C) at 6000 x g for 10 minutes. Finally, three volumes of cold ethanol were added to the resulting supernatant, which was then stored at -20 °C for one hour. The metallothionein was isolated and measured by centrifuging the supernatant at 6000 x g for 10 minutes, after which the resulting pellets were washed with a homogenization buffer consisting of ethanol and chloroform. These pellets were subsequently dried under a stream of nitrogen gas and then resuspended in 300 µL of 5 mM Tris-HCl and 1 mM EDTA at pH 7. The pellets were further resuspended in 4.2 mL of 0.43 mM 5,5-dithiobis (nitrobenzoic acid) in a 0.2 M phosphate buffer at pH 8 and allowed to incubate at room temperature for 30 minutes. The concentration of reduced sulfhydryl groups was then assessed by measuring the absorbance at 412 nm using a spectrophotometer. To accurately quantify metallothionein in the samples, a standard curve was generated using GSH as a reference. The quantity of metallothionein in the samples was estimated with the GSH standard, based on the assumption that 1 mole of MT contains 20 moles of cysteine.

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Determination of fat content by the Soxhlet extraction method

A clean 250 ml flask was placed in an oven at 105-110°C for approximately 30 minutes to dry. An accurate weight of 2g of the fish muscle sample was placed into labeled thimbles. The corresponding labeled cooled boiling flasks were also weighed. About 300 mL of petroleum ether (with a boiling point of 40-60°C) was added to the boiling flasks. A small amount of cotton wool was used to lightly plug the extraction thimble. The Soxhlet extractor apparatus was set up and allowed to reflux for around six hours. The thimble was carefully removed, and the petroleum ether in the upper container of the setup was collected and poured into a flask for reuse. Once the flask was nearly empty of petroleum ether, it was taken out and dried in the oven at 105-110°C for one hour. The flask was then moved from the oven to a desiccator to cool down before being weighed.

CALCULATION

$$\% \text{ fat} = \frac{\text{Weight of fat} \times 100}{\text{Weight of sample}}$$

Statistical Analysis

The data were expressed as mean ± SD, and a test of statistical significance was carried out using one-way analysis of variance (ANOVA) and post hoc tests to determine if a significant difference exists between the mean of the test and control groups. The data obtained were analyzed using Statistical Product and Service Solutions (SPSS), version 18. $P < 0.05$ was considered significant.

RESULTS

Malondialdehyde (MDA), catalase, superoxide dismutase, glutathione-peroxidase, and glutathione reductase activities of fish samples

MDA level, catalase, superoxide dismutase, glutathione-peroxidase, and glutathione-reductase activity of the fish presented as mean ± SD are presented in Table 25. The level of MDA, a natural by-product of lipid peroxidation, in the serum of group 1 (Orogodo River fish) was significantly lower when compared with different locations at Banegbe River and group 2 (Ekpan pond fish). There was a significant ($p<0.05$) increase in MDA level in the order of**:** middle-stream (group 11- group 14) >down-stream (group 7 to group 10) >up-stream (group 3 to group 6) was observed in different locations at the Banegbe River.

The catalase activity of group 1 (control I) significantly decreased ($p<0.05$) when compared with different locations at Banegbe River and group II (control II, Ekpan pond). The activity of the enzyme significantly increased ($p<0.05$) in the order: middle-stream (group 11- group 14) > down-stream (group 3 to group 6) > up-stream (group 7 to group 10) at the different locations of the Banegbe River.

The activity of SOD significantly decreased ($p<0.05$) in control 1 when compared with different locations at Banegbe River and second control group 2. The activities of SOD were contaminants dependent too. The level of PAHs, PCBs, and heavy metals in fish from the pond was higher when compared to fish from the Orogodo River. The SOD value significantly increased ($p<0.05$) at different location at Banegbe in the following order; down-stream (group 7 to group 10) > middle-stream (group11- group14) >up-stream (group 3 to group 6).

The activities of glutathione peroxidase and glutathione reductase of group 1 (control) significantly decreased ($p<0.05$) when compared with different locations at Banegbe River and the second control (group 2). These enzyme activities increased significantly ($p<0.05$) in the following order: effluent discharge point (group 11-group 14) > upstream (group 3 to group 6) > downstream (group 7 to group 10). The results also show variation in the activities of the enzymes at the same point of collection at the Banegbe River.

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Table 1: Pro- and anti-oxidant parameters.

Treatment Group	MDA Conc (mg/dl)	Catalase Activity (IU/L)	SOD Activity (IU/L)	GPx Activity (IU/L)	GR Activity (IU/L)
Group 1	1.24±0.95	2.35±0.28	54.63±8.83	22.10±1.22	20.27±1.00
Group 2	2.82±0.99	2.82±0.56	67.01±14.01	23.36±2.77	24.71±2.15
Group 3	1.57±0.55	2.51±0.32	56.09±5.50	23.80±1.73	24.67±1.90
Group 4	1.59±0.60	2.48±0.33	58.54±3.46	24.02±3.09	22.63±0.81
Group 5	1.78±0.16	2.26±0.19	66.14±1.59	24.64±2.71	22.19±1.52
Group 6	1.81±0.45	2.38±0.35	61.28±6.25	27.31±1.62	21.87±1.39
Group 7	1.80±0.60	3.50±0.23	61.19±3.67	26.55±2.00	22.32±1.44
Group 8	1.83±0.30	2.91±0.72	65.01±5.27	27.77±1.06	22.62±0.58
Group 9	1.95±0.37	2.83±0.66	70.84±1.30	27.58±0.74	22.42±0.44
Group 10	2.46±0.35	3.13±0.70	74.14±10.32	27.43±2.74	23.71±0.84
Group 11	2.04±0.22	2.77±0.74	67.18±6.33	28.21±1.40	22.61±0.53
Group 12	2.11±0.50	2.91±0.23	69.73±10.72	23.92±2.16	24.71±0.70
Group 13	2.59±0.29	2.50±0.33	65.47±3.57	28.72±4.35	25.52±1.30
Group 14	3.20±0.75	2.37±1.03	61.26±14.57	25.10±7. 31	26.64±0.85

Results are expressed in Means ± SD (n = 3)

Group 1 = Control 1; Group 2 = Control 2

Group 3 = Upstream 1; Group 4 = Upstream 2

Group 5 = Upstream 3; Group 6 = Upstream 4

Group 7 = Downstream 1; Group 8 = Downstream 2

Group 9 = Downstream 3; Group 10 = Downstream 4

Group 11 = middle-stream1; Group 12 = Middle-stream 2

Group 13 = Middle-stream 3:' Group 14 = Middle-stream4

Metallothionein and Fat contents of fish samples

Table 2 below shows the metallothionein and fat contents of fish samples. From the results, it was shown that the metallothionein activity of the fish in group 1 significantly ($p<0.05$) increased when compared with different locations at Banegbe River and control 2. There was a significant increase ($p<0.05$) in the level of metallothionein across the groups was observed from up-stream (group 3 to group 6) < down-stream (group 7 to group 10) < middle-stream (group 11- group 14). The value of metallothionein significantly varied within the same point of collection at the Banegbe River.

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Significant decrease ($p<0.05$) in the fat content of fish from Banegbe River when compared to group 1 (control 1) was observed. Generally, there was a significant decrease in the fat content of the fish from middle-stream (group 11- group 14) and down-stream (group 7- group 10) when compared with up-stream (group 3 to group 6) of the Banegbe River.

Table 2: Metallothionein and fat contents of the fish.

Groups	Metallothionein	Fat contents
Group 1	2.53±0.58	14.30±3.56
Group 2	3.49±0.30	9.20±0.91
Group 3	2.54±0.21	13.20±2.04
Group 4	2.73±0.43	11.73±6.60
Group 5	2.73±0.44	11.75±5.30
Group 6	2.57±0.41	14.39±3.34
Group 7	2.73±0.30	11.75±5.57
Group 8	3.06±0.38	8.67±0.76
Group 9	2.73±0.60	11.46±3.83
Group 10	2.91±0.27	8.84±4.55
Group 11	2.87±0.40	9.73±1.27
Group 12	2.98±0.21	9.32±3.38
Group 13	3.43±0.51	7.570±3.05
Group 14	3.25±0.32	7.77±6.11

Results are expressed in Means ± SD (n = 3)

DISCUSSION

The connection between environmental pollution and the stress response in fish suggests that exposure to certain polluted conditions can lead to the emergence of infectious diseases in the host. The harmful effects of numerous contaminants on aquatic life occur through oxidative damage resulting from the generation of reactive oxygen species (ROS). Normally, ROS are eliminated from the cell by the antioxidant defense mechanisms. However, when the production of ROS exceeds normal levels, the balance between its creation and elimination is disrupted, leading to oxidative stress (Li *et al*., 2010). The antioxidant defense system comprises enzymes such as glutathione peroxidase, catalase, superoxide dismutase, glutathione reductase, and glutathione-S-transferase (Menezes *et al*. 2011). As per the Environmental Risk Assessment (ERA), the elements of the antioxidant defense are categorized into biotransformation phase II components (such as GST and reduced/oxidized glutathione) and oxidative stress indicators (SOD, CAT, GSH-Pxand GR) (Van der Oost *et al.*, 2003)

Antioxidant enzyme activities of fish were determined to establish the possible environmental impact of the toxic effect of effluent from industries within the vicinity on the Banegbe River. According to the authors, antioxidant enzyme levels depend on age, nutrition, and spawning of the fish samples. Our current findings suggest that the changes observed in the liver antioxidant enzymes of the fish indicate the accumulation of persistent organic

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pollutants (PAHs, PCBs, and heavy metals) within the fish. These contaminants impact various organs, especially the gills, which come into direct contact with environmental pollutants. Nevertheless, the majority of these pollutants are absorbed into the bloodstream and transported to the liver, the primary organ responsible for detoxifying xenobiotic substances. The liver has shown to be more resilient to oxidative stress compared to other tissues, exhibiting the highest activities of SOD and CAT (Atli *et al*., 2006)

SOD functions as a scavenger of oxygen radicals within tissues, transforming the superoxide anion radical into H2O and H2O2 (Nordberg and Arner, 2001). The increase in SOD levels observed at the middle-stream and downstream locations may be attributed to the pollutant inflow from industrial discharges. In agreement with our findings, Farombi *et al.* (2007) reported that SOD activity was heightened in the liver tissues of fish from the Ogun River, Nigeria, adjacent to industrial operations. Khalil *et al*. (2017) reported increased level of SOD in O *niloticus* from Bosetta Branch of River Nile. Also, Lushchak (2011) and Kanak *et al*. (2014) noted that higher SOD activity in the presence of heavy metals might result from the fish adapting to the pollutants or from brief exposures that are very short in duration. Nevertheless, other research has shown that the presence of pollutants can inhibit SOD activity (Ameur *et al*., 2012). Sedeño-Diaz and Lopez-Lopez *et al*. (2012) discovered that SOD activity may be either increased or decreased based on the level of pollutants and the time of year. Prolonged exposure to low concentrations of pollutants can also lead to a reduction in SOD activity (Pandey *et al*., 2005)

Catalase (CAT) is an enzyme found in the majority of cells that facilitates the conversion of hydrogen peroxide into water and oxygen. Many researchers regard CAT as a crucial and sensitive indicator of oxidative stress, often surpassing superoxide dismutase (SOD) in demonstrating the biological impacts on the redox state of marine organisms (Regoli *et al*., 2002a).

Our present result showed a significant ($p<0.05$) increase in catalase activity in the fish sample from middle-stream and down-stream of Banegbe, with the range of 2.37±1.03 to 2.91±0.23 and 2.83±0.66 to 3.50±0.233.13±0.70, respectively, when compared to up-stream and the control (Orogodo river fish), with 2.26±0.19 to 2.51±0.32 and 2.35±0.28, respectively. The elevation in the CAT of these fish from middle-stream and down-stream is the mobbing effect of hydrogen peroxide produced by SOD. Also, fish from the Ekpan pond water had a significant ($p<0.05$) increase too, with the value of 2.82±0.56, which indicates a high presence of pollutants in the pond water.

The increase in CAT activity may be related to coping with the increased oxidative stress caused by pollutant exposures, while the decrease may be related to possible direct binding of pollutant (metal ions to -SH groups) on the enzyme molecule. Khalil *et al*. (2017) reported an increased level of CAT in O *niloticus* from the Bosetta Branch of the River Nile. Also, Sedeño-Diaz and Lopez-Lopez (2012) discovered increased CAT activity associated with higher concentrations of pollutants, particularly hydrocarbons and pesticides. Atli *et al.* (2006) showed notable changes in CAT activity both in vivo and in vitro across various tissues of O. *niloticus* following acute and chronic exposure to metals. A study involving the freshwater fish *Channa punctatus* also confirmed the increased CAT activity in the presence of herbicides and pesticides (Nwani *et al*., 2010).

SOD activity was significant ($p<0.05$) higher in the fish from middle-stream of Banegbe River. While the CAT was higher in the fish from downstream of the river. Although CAT degrades the hydrogen peroxide produced by the dismutation of superoxide ion by SOD during oxidative stress. Hence an increase in SOD activity should result in increased CAT activity. However, this is not always observed in practice and appears to be species-dependent (Lushchak, 2011). Higher CAT activity was also recorded in different fish species after Cu and Cd exposures (Sanchez *et al.*, 2005).

Vlahogianni *et al*., (2007) found an increase in the activity of SOD and CAT in *Mytilus galloprovincialis* from a polluted coastal area of the Saronikos Gulf in Greece, compared to a non-polluted site. Similarly, Batista *et al*., (2002) reported elevated levels of both enzymes at a contaminated spot along the Una River in Astyanax bimaculatus. The increase in CAT activity observed at the polluted locations of the Banegbe River indicates that there is an overproduction of H_2O_2 by SOD, which permeates into the cells and leads to oxidative damage

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Glutathione peroxidase (GPX) activity also increased significantly ($p<0.05$) in the fish sample from middle-stream and down-stream of Banegbe, with the range of 23.92±2.16 to 28.72±4.35 and 61.19±3.67 to 70.84±1.30, respectively, when compared to up-stream and the control (Orogodo river fish), with 56.09±5.50 to 66.14±1.59 and 54.63±8.83. The elevation of these enzymes could be attributed to oxidative stress from xenobiotics. Glutathione peroxidase is a well-known enzyme that acts as an antioxidant in response to oxidative stress, requiring glutathione as a cofactor. It facilitates the oxidation of GSH to GSSG while utilizing H2O2. This enzyme has selenium atoms in its active sites and transfers reducing equivalents from glutathione to H2O2, resulting in the production of water and GSSG. GPX serves as an antiperoxidative enzyme found within both the cellular and mitochondrial matrices. An increase in glutathione peroxidase levels was observed by Sarkar *et al*., (2017) in the liver of zebrafish subjected to arsenic oxide exposure.

While numerous compounds have antioxidant properties, the resilience is due to the functional overlap among various antioxidant enzymes and the relationship between their functions. This implies that if the cellular antioxidant defense operates as a physiological system, then alterations in the function of a specific antioxidant component should be accompanied by corresponding changes in the activities of others; thus, the level of Glutathione reductase (GR) was also increased. GR is crucial for cellular protection against reactive oxygen species. It keeps glutathione in its reduced form, which is essential for GSH-Ph activity; thus, GR plays a key role in regulating the redox balance within the cell (Djordjevic *et al*., 2010). Our present result showed a significant ($p<0.05$) increase in Glutathione reductase activity in the fish sample from middle-stream and down-stream of Banegbe, with the range of 22.61±0.53 to 26.64±0.85 and 22.32±1.44 to 23.71±0.84, respectively, when compared to up-stream and the control (Orogodo river fish), with 21.87±1.39 to 24.67±1.90 and 20.27±1.00, respectively. Similar to this is the finding of Saglam *et al.*, 2014, that reported a significant increase in glutathione reductase activity of Oreochromus niloticus exposed to metals in different hardness. Matos *et al.*, (2007) reported elevation of glutathione reductase of Nile tilapia (Oreochromis niloticus) exposed to carbaryl. And Li *et al.*, (2011) also reported increased activity of glutathione reductase. The GR activity within the groups of upstream, middle-stream, and downstream was varied, just like other enzymes reported in this work.

Lipid peroxidation (LPO) has been identified as a significant factor leading to the loss of cellular function under conditions of oxidative stress, commonly indicated by TBARS in fish (Oakes and Van der Kraak, 2003). Given that the typical outcome of ROS-induced damage involves the peroxidation of unsaturated fatty acids, our findings demonstrated an increase in LPO in fish from the middle-stream and downstream areas, in comparison to the upstream and Orogodo river, where LPO levels were notably lower.

Pollutants can produce ROS capable of damaging tissues such as DNA, proteins, and lipids (Pandey *et al.*, 2003). Similar to this result is the increase in lipid peroxidation of Mytilus galloprovincialis at the polluted site of Venice Lagoon when compared to less polluted sites Pampanin *et al*., (2005). Romeo *et al*., (2000) reported that cadmium increases the formation of lipid peroxidation in the kidney of Sea bass fish. Sarkar *et al.*, (1998) reported an increase in lipid peroxidation and alteration in antioxidants in cadmium-induced oxidative stress in rats. Vlahogianni *et al.*, (2007) reported an elevation in lipid peroxidation of *Mytilus galloprovincialis* in the polluted coastal area of Saronikos Gulf of Greece when compared to the non-polluted area, which is also in the same our results. Farombi *et al*., (2007) indicated significant elevation of lipid peroxidation and decreased antioxidant levels in all the organs of African catfish from Ogun River. The elevation in antioxidant enzymes and lipid peroxidation is evidence of the presence of some heavy metals above the permissive limit in water at the same sampled locations of Banegbe river water and fish (Ogana *et al.*, 2023).

There was a significant ($p<0.05$) reduction in the fat content of the fish from middle-stream and downstream of the Banegbe River when compared to upstream and control (orogodo river fish). The decreased level of the fat content could be related to meeting the additional energy required under stress caused by pollutants. Romanic *et al.*, (2014) have observed a positive correlation between the lipid content of fish muscles and the concentration of organochlorine pesticide Jagatheeswari, (2005) found that lipid level in liver and intestine of fish *Cyprinus carpio*, were seem to be decreased with increased concentrations of phosalone in 2, 4 and 6 days. Metallothionein protects against environmental stress caused by various contaminants, especially metals (Lukkari *et al*., 2004). MT has been proposed as an effective biochemical marker, with its assessment in fish body tissues serving as an indicator for identifying metal pollution in aquatic environments. Significant increases in MT levels have been observed in certain tissues (including gills, liver, kidney, intestine, and muscles) that play roles in metal

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absorption, storage, and elimination (Min *et al*., 2016). The elevation of metallothionein in Table 2 shows homeostasis of essential metals like Cu and Zn and detoxification of non-essential metals in the fish. Middle-stream and downstream of the Banegbe river have higher concentrations than upstream and the Orogodo river, as shown in Table 2. MT are normally present in tissues in trace amounts; exposure to metals induces their production (Rajeshkumar *et al*., 2018). Zhang and Wang, (2005) reported the Elevation of MT concentration in fish exposes to Zn. The activation of MT can also be triggered by various cytotoxic compounds, hormones, vitamins, antibiotics, and cytokines through intracellular signaling pathways, along with stresses such as inflammation, physical injury, high oxygen levels, and ultraviolet radiation (Bonneton *et al*., 1996)

In conclusion, the expression of metallothionein and elevation of the antioxidant enzymes in fish from the river is an indication of the presence of pollutants, especially heavy metals, in the Banegbe River, particularly at middle-stream and downstream, according to Ogana *et al*., (2023), which is likely a result of industrial effluent from within the middle-stream environment.

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