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METALS CONTENT AND ANTIOXIDANT ENZYME ACTIVITY IN NILE TILAPIA POPULATION OF RIVER JEHLUM

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ABSTRACT

Heavy metal contamination of the aquatic environment had recently become a major concern for aquatic organisms as well as public health. The present study was conducted to assess antioxidant enzymes catalase (CAT) and peroxidase (Px) activity in response to heavy metals from various organs of *Oreochromis niloticus*. Samples of fish and water were collected from different sites of River Jehlum. Fish were dissected to obtain different organs, extracted organs were homogenized in phosphate buffer for enzyme assay and antioxidant activity was measured. Some physico-chemical parameters, including Temp, DO and pH were measured on-site, while others (Mg, Ca and CO_2) were checked in the laboratory. Data was statistically analyzed with software STATISTIX by applying one-way ANOVA. After ANOVA, comparison of means was done through Tukey's test. The results indicated significant bioaccumulation of heavy metals in the liver, kidney, gills, heart and muscles. The trend of metals at all sites was recorded as Zn > As > Hg > Pb > Cr. Result indicated significant bioaccumulation of heavy metals in the liver, kidney, gills, muscles and heart of *O. niloticus*. The catalase activity level was maximum in the liver of fish, while peroxidase activity was higher in the kidney as compared to other organs. Antioxidant activity was measured in the following order: liver > kidney > gills > heart > muscles. This study is helpful in understanding the

effects of heavy metal accumulation in the aquatic ecosystem and identified potential solutions that could be helpful in reducing its negative impacts.

INTRODUCTION

Water is a crucial resource for humans and all living beings, but when it becomes scarce or polluted, it can lead to decrease in productivity and mortality of living species (Singh *et al*., 2020). Water pollution occurs when harmful substances contaminate water sources like rivers, lakes, wells and streams, posing a threat to humans and the environment (Kumar *et al*., 2020). The presence of heavy metals and persistent organic pollutants in river systems is leading to their contamination. Moreover, heavy metals are among the most harmful contaminants in aquatic habitats and can pose a threat to fish. Fish accumulate heavy metals, which then pass on to organisms that feed on them, making it a concern for human consumption as well (Ullah *et al*., 2017). The River Jehlum, which originates in the Southern Himalayas and Pir Panjal Range in Jammu and Kashmir, passes through several districts (Sarwar *et al*. 2007). Various water management infrastructures have been developed in the Jehlum basin to increase its capacity for the benefit of the region (Khan *et al*. 2004). The various environmental stressors present in water include hardness, temperature variations, dissolved oxygen, ammonia, pH and partial pressures (Nofal *et al*., 2019).

Fish, the top consumers in aquatic food webs, are highly susceptible to pollution due to their constant exposure and inability to escape harmful effects (Islam *et al*., 2023). They are ideal organisms for toxicology and toxic genomics studies due to their high sensitivity to environmental changes (Sula *et al*., 2020). Fish accumulate metals through various pathways, including food intake, suspended particulate matter and metal ion exchange (Jiang *et al*., 2019). Nile Tilapia, which belongs to the Oreochromis family, is an indigenous fish species known for its resilience and adaptability. It originates from the Nile River Basin in Africa (Ahmad *et al.* 2023). The Nile Tilapia (*Oreochromis niloticus*) is extensively farmed and nurtured in multiple areas worldwide, including Pakistan (Salayo *et al*., 2022). It is a nutritious source of protein and essential nutrients, providing a relatively inexpensive protein source for local consumption (Tom *et al*., 2021). However, the Nile Tilapia faces significant challenges in an environment contaminated with heavy metals, such as the pollution of the River Jehlum. Heavy metals can bio accumulates within the food chain, affecting the development, physiological processes, mortality and reproduction of *O. niloticus*. This raises concerns about the potential adverse effects on individual fish and the broader aquatic ecosystem (Temesgen *et al*., 2019).

Fish have a complex system of protection from damage caused by oxygen, similar to mammals (Zhao *et al*., 2012). They use different enzymes to keep their systems in balance. These include catalase, superoxide dismutase, peroxidase and glutathione-s-transferase. The essential enzymatic components in fish, including alkaline phosphatase, sodium-potassium adenosine tri phosphatase, calcium adenosine tri phosphatase and catalase (Bernet *et al*., 2001). This network of antioxidant defenses showcases the adaptability of fish in effectively managing oxidative stress and maintaining balance in diverse aquatic environments (Welker *et al*., 2013).

Catalase, an enzyme in fish, is a crucial part of their antioxidant defense system. It breaks down hydrogen peroxide into oxygen and water, neutralizing ROS produced during aerobic respiration. This enzymatic activity protects fish cells from oxidative stress. The presence of catalase in fish is vital for their overall well-being, maintaining the balance of ROS produced during metabolic processes (Foulkes, 2000). Its efficiency and high turnover capacity make it essential for rapid hydrogen peroxide decomposition, which is crucial for health and survival of fish, especially in aquatic environments with oxidative stressors (Loncar and Fraaije, 2015).

Peroxidase is an enzyme that contains heme and acts as a catalyst in various activities, such as the oxidation of multiple compounds. The peroxidase enzyme consists of several isoforms that carry out a range of metabolic processes in living beings, including cell adhesion, phagocytosis, immune cell function and antioxidant capabilities. It is an oxidoreductase that catalyzes various reactions, including the reduction of hydrogen peroxide and the oxidation of inorganic and organic compounds. When oxidative stress occurs, peroxidase acts as the first line of defense against reactive oxygen species. Heavy metal poisoning can cause fluctuations in peroxidase levels. During redox reactions, hydro peroxides are reduced to hydroxyl molecules and peroxidases are transformed into water. Therefore, the amount of peroxidase can be used to assess heavy metal toxicity in fish (Abbott *et al*., 2009).

The external substances act as triggers, causing fish to undergo oxidative stress by generating reactive oxygen species (Slaninova *et al*., 2009). Oxidative stress is a critical component of fish physiology, influencing the balance of ROS generation and antioxidant defenses. Fish cells also produce free radicals through mitochondrial respiration (Kumar *et al*., 2018). When ROS generation exceeds the antioxidant defenses, oxidative stress occurs, resulting in protein and lipid oxidation, gene expression modifications and alterations in cellular redox status. This can have a substantial impact on fish physiology, potentially compromising overall health and the well-being of aquatic ecosystems (Livingstone, 2003).

Mercury pollution in aquatic environments is a significant concern as it is first discharged as a toxic substance that accumulates in the liver of fish and circulates throughout their bodies. Symptoms of mercury poisoning in fish may include convulsions, gill separation, lack of coordination, liver inflammation and damage to blood. Lead is a metal that is commonly found in aquatic environments and it poses a threat to fish (Guerreiro and Fuentes, 2019). Lead has a stronger affinity for transport proteins such as calcium pumps and ion the function and integrity of cell membranes, which makes them more vulnerable to oxidative damage, resulting in decreased cellular function and greater toxicity (Pandey and Madhuri, 2014). Zinc accumulates in fish tissues through gills and the digestive tract with its deposition order typically being liver, kidney, intestine, gill and muscle causing stress leading to death (Garari *et al*., 2021). The toxicity of chromium can vary based on factors such as species, age and water conditions. It can affect DNA, enzymes and nuclear proteins. Arsenic a semi-metal poses significant risks to aquatic ecosystems due to natural and human-induced processes (Basu *et al*., 2014). Fish are exposed to low arsenic levels, leading to bioaccumulation in organs like the liver and kidney, causing hyperglycemia, enzymatic depletion and immune system dysfunction (Mishra *et al*., 2016).

METHODOLOGY

The study entitled “Spatial variation in heavy metal levels and antioxidant enzymes activities in *Oreochromis nilotics* from Jehlum River” was carried out in Aquaculture Biotechnology Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad. The main objective of this study was to analyze the spatial variations of heavy metal concentrations in *Oreochromis nilotics* and to evaluate variations in antioxidant enzyme activity across different locations of River Jehlum.

Fish Sampling

Nile Tilapia (*Oreochromis niloticus*) specimens were specifically chosen from the primary public fishing locations along the River Jehlum in Pakistan. These sampling sites include Jehlum Bridge, Mangla Dam, Pind Dadan Khan,Trimmu head works and Rasul Barrage.

Extraction of organs

Various organs of the fish, including the liver, kidney, gills, muscles and heart were dissected and stored in labeled polythene bags. Each organ sample was divided into two subsamples, one for catalase activity and other for peroxidase activity. Three replicates were prepared for each organ to analyze both antioxidant enzymes.

Water sampling

Water samples were collected from River Jehlum to evaluate a range of physicochemical parameters.

Determination of physicochemical parameters of water

Water samples were gathered from specific locations along River Jehlum to analyze various physicochemical parameters. The determination of these parameters in water samples proceeded as follows

Temperature

Temperature is a crucial water parameter for conducting any research involving fish. In this study the temperature of water samples was recorded using a Thermometer. The temperature

measurement was conducted by immersing the sensor of the meter directly into the water sample.

pH

Samples collected from different sites along the river were analyzed to assess the pH using a pH meter (HANNA-HI,98107).

Dissolved oxygen

The dissolved oxygen content of water samples collected from various sites was determined using the instrument HANNA-HI,9143. Dissolved oxygen levels were measured in parts per million (ppm).

Total hardness

A 50 ml water sample was obtained and a precise amount of buffer was added to maintain the pH. The mixture was titrated with an EDTA solution with a normality of 0.01. The titration concluded upon the appearance of a blue color, indicating the endpoint. The total hardness was subsequently calculated using the provided formula:

$$\textbf{Total hardness(mg/l)} = \frac{\textbf{Volume of EDTA used for titration x A x 1000}}{\textbf{Volume of the sample (mL)}}$$

Subsequently,

A = 0.1 ml of EDTA at the endpoint of the calcium ion indicator corresponds to an equivalent amount of calcium carbonate in milligrams.

Calcium

To adjust the pH of a 50 ml water sample to a range between 12-13, NaOH was added. Mercuric oxide was employed as the indicator. The titration was conducted using EDTA with a normality of 0.01 until a purple color appeared, indicating the endpoint. The formula for calculating calcium content is provided below:

$$\textbf{Calcium (mg/l)} = \frac{\textbf{Volume of EDTA used for titration x 400.8}}{\textbf{Volume of the sample(mL)}}$$

Magnesium

The magnesium content in the water sample was approximated using the calcium and total hardness values, applying the subsequent formula:

Carbon dioxide

The carbon dioxide content in water was determined via titration, employing Na_2CO_3 as the titrant. The formulation of sodium carbonate indicated the presence of carbon dioxide in the water samples. The reaction was completed at pH 8.30, with phenolphthalein serving as the indicator, transitioning from colorless to pink at the endpoint. The following formula was utilized to calculate the value of carbon dioxide:

$$\textbf{Carbon dioxide } (\textbf{mgL}^{-1}) = \frac{\textbf{Na}_2\textbf{CO}_3\textbf{ used } \times \textbf{1000}}{\textbf{Volume of sample (mL)}}$$

Measuring of catalase and peroxidase activity

To measure the activity of antioxidant enzymes

Peroxidase and catalase, liver, kidney, gills and muscles were extracted from the fish. Three replicates were created from each extracted organ for analysis.

Homogenization of fish organs

✧ One gram of sample was weighed from each extracted organ.

✧ Phosphate buffer with a pH of 7.0 was added to the weighed organ sample at a ratio of 1:4 (one part organ and four parts buffer solution).

✧ The sample and buffer mixture were homogenized using a pestle and mortar for fifteen minutes.

✧ The homogenized mixture was then subjected to filtration using muslin cloth.

✧ Whatman's filter paper number 1 was used to filter the liquid obtained from muslin cloth.

✧ The filtrate was centrifuged at 10,000 rpm for fifteen minutes to separate sediments and supernatants for the enzyme assay.

✧ All steps involving enzyme separation were conducted at a temperature of 4°C to preserve the integrity of the samples.

✧ The separated and supernatants were preserved in a refrigerator at 4°C until further analysis was conducted.

Analysis of catalase activity

Catalase activity was measured by using Bergmeyer improved method (1974) at 240nm. Activity of catalase, an antioxidant enzyme is its ability to decompose H_2O_2 into oxygen and water which was determined by using Chance and Mehlay method (1977).

Reagents and chemicals required for CAT activity

1. Volume of 50mm of phosphate buffer at pH 7.0.

2. Volume of 10mm of hydrogen peroxide.

Phosphate buffer's preparation

In a conical flask, 0.371g of NaH_2PO_4 and 0.270g of Na_2HPO_4 were taken and the volume was raised to approximately 200ml with distilled water. The pH of the buffer solution was maintained at 7.0.

Preparation of buffer solution for CAT activity

Hydrogen peroxide 0.44ml was introduced into a 50mM phosphate buffer to create a 10 ml solution of H_2O_2.

Procedure

✧ A blank solution of 3ml was pipetted into a cuvette and inserted into a spectrophotometer.

✧ At a specific wavelength of 240nm, the spectrophotometer's reading was adjusted to zero.

✧ In another cuvette, a sample solution of buffer substrate was placed into the spectrophotometer.

✧ The enzyme extract of 0.05ml was added to a second cuvette containing a substrate buffered solution. To initiate the reaction between the enzyme extract and the substrate buffered solution, the mixture was allowed to react for three minutes.

✧ After o one-minute interval, the absorbance was measured at 240nm using the spectrophotometer.

Calculations

To calculate catalase activity, the following formula was utilized:

$$\textbf{CAT activity (unit/ml)} = \frac{\Delta A/Min \times dilution \times 2ml}{0.04Mm\text{-}cm\text{-}1 \times 0.05mL}$$

Where,

ΔA	Absorbance at 240 nm;
Min	Time of Reaction;
2mM	Buffer and enzyme used;
$0.04Mm^{-1}$ cm^{-1}	H_2O_2 coefficient of absorption;
0.05ml	Enzyme Concentration;

Analysis of peroxidase activity

Peroxidase activity was assessed by its ability to convert H_2O_2 into non-toxic metabolites, employing a spectrophotometer set a wavelength of 470nm. The Chance and Mehaly method (1977) was utilized for this analysis

Chemicals and reagents requires for peroxidase assay

1. Guaiacol
2. Hydrogen peroxide
3. Phosphate buffer (0.2M) at pH 6.5

Phosphate buffer 0.2 M at 6.5 pH

Guaiacol (750ml) and phosphate buffer (47ml) were combined using a vortex agitator. Then 0.3ml of hydrogen peroxide was added to the solution. The resulting reaction mixture consists of:

Procedure

✧ A blank solution (3ml) was pipetted into a cuvette and inserted into the spectrophotometer.

✧ The spectrophotometer was adjusted to zero at a wavelength of 470nm.

✧ In another cuvette, buffer substrate solution was placed into the spectrophotometer and allowed to react for three minutes.

✧ Then, 0.06ml of enzyme extract was added to the buffer substrate solution and the cuvette was inserted back into the spectrophotometer.

✧ Absorbance was measured after a one minute at 470nm.

Calculation

The activity of peroxidase was determined using the following formula:

$$\textbf{Activity (UmL}^{-1}\textbf{)} = \frac{\Delta A/3}{26.60 \times \frac{60}{3000}}$$

Where:

A = Absorbance at 470nm

Detection of heavy metals by digestion method

To analyze the heavy metals content, weighed 1.0 gram of each organ was taken in open mouth conical flask. Concentrated Nitric acid (HNO_3) was measured by using glass cylinder (300 mL) and added in weighed sample. Then placed on hot plate when it reaches boiling point, Perchloric acid (10mL) was added and placed again on hot plate. The reacted mixture was heated until 1Ml of colorless solution was left behind. Then flask was removed and diluted with distilled water (100mL) following crystal clear appearance. Whatman's filter paper was used to remove all the particles from digested sample prior to minerals analysis (AOAC, 1990).

Determination of heavy metals

Selected metals such as zinc (Zn), lead (Pb), mercury (Hg), arsenic (As) and chromium (Cr) in processed samples were quantified using a Hitachi polarized Zeeman atomic absorption spectrometer (AAS) model 8200 from Japan, following the methodology outlined in AOAC (1990).

Statistical analysis

The data obtained from the observation were analyzed to determine the mean and standard deviation (MEAN±SD) using Microsoft Excel (2010). Analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) Post Hoc test were conducted on the data using Statistics' 8.1 software. Variations among different organs of fish, sampling sites and activity of antioxidant enzymes i.e. catalase and peroxidase were analyzed at the significance of $p<0.05$.

RESULTS

Comparison of metal content in different organs of *O. niloticus* at different sites of River Jehlum.

Comparison of metal content in different organs of *O. niloticus* taken from Pind Dadan Khan

In present study Hg, Pb, Zn, Cr and As were selected to check their concentration in different organs of *O. niloticus* in Pind Dadan Khan River Jehlum. The results showed that the elevated levels of selected metals at Pind Dadan Khan show following order Zn>Cr>Pb> As>Hg.

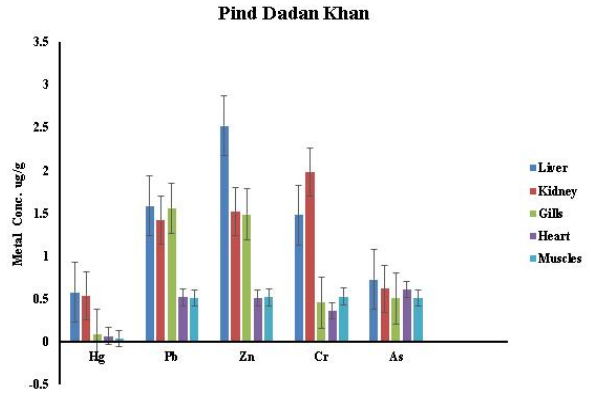

Figure 1: Graphical representation of metal content in different organs of *O. niloticus* taken from Pind Dadan Khan

Comparison of metal content in different organs of *O. niloticus* taken from Jehlum Bridge

In present study Hg, Pb, Zn, Cr and As were selected to check their concentration in different organs of *O. niloticus* in Jehlum Bridge River Jehlum. The results showed that the elevated levels of selected metals at Jehlum Bridge show following order Zn>Pb>As> Cr>Hg.

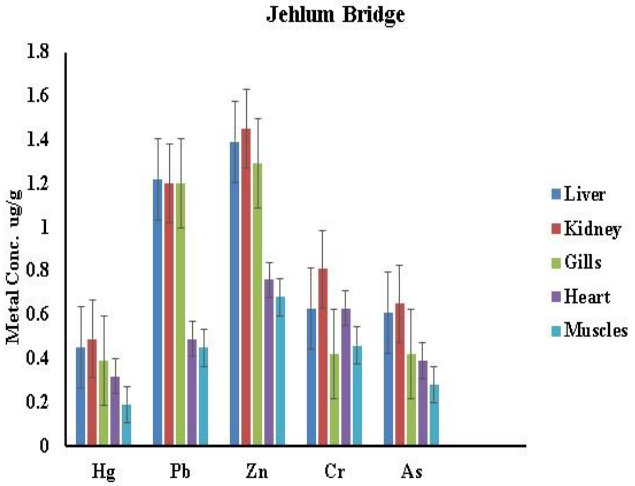

Figure 2: Graphical representation of metal content in different organs of *O. niloticus* taken from Jehlum Bridge

Comparison of metal content in different organs of *O. niloticus* taken from Rasul Barrage

In present study Hg, Pb, Zn, Cr and As were selected to check their concentration in different organs of *O. niloticus* in Rasul Barrage River Jehlum. The results showed that the elevated levels of selected metals at Rasul Barrage show following order Zn>As>Cr> Pb>Hg.

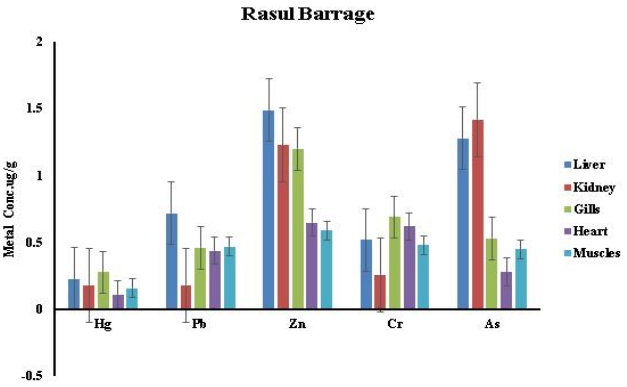

Figure 3: Graphical representation of metal content in different organs of *O. niloticus* taken from Rasul Barrage

Comparison of metal content in different organs of *O. niloticus* taken from Mangla Dam

In present study Hg, Pb, Zn, Cr and As were selected to check their concentration in different organs of *O. niloticus* in Mangla Dam River Jehlum. The results showed that the elevated levels of selected metals at Mangla Dam show following order Zn>As>Cr> Hg>Pb.

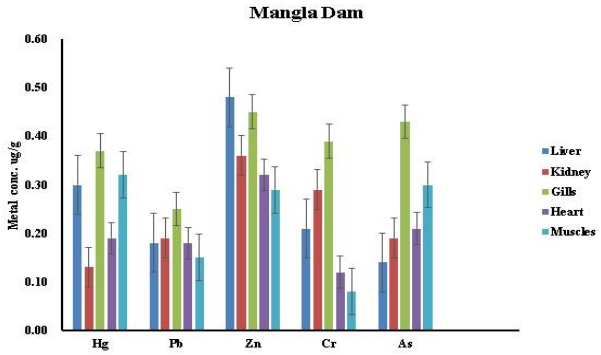

Figure 4: Graphical representation of metal content in different organs of *O. niloticus* taken from Mangla Dam

Comparison of metal content in different organs of *O. niloticus* taken from Trimmu Headworks

In present study Hg, Pb, Zn, Cr and As were selected to check their concentration in different organs of *O. niloticus* in Trimmu Headworks River Jehlum. The results showed that the elevated levels of selected metals at Trimmu Headworks show following order Zn>As>Cr> Pb>Hg.

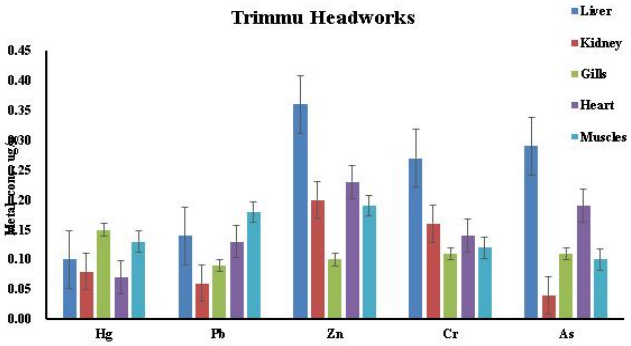

Figure 5: Graphical representation of metal content in different organs of *O. niloticus* taken from Trimmu Headworks

Comparison of bioaccumulation of different metals in different organs of *Oreochromis niloticus* sampled from different sites of River Jehlum

The research findings revealed a significant increase in the concentration of various metals (Zn, Cd, As, Pb, Cr) at Pind Dadan Khan compared to other sampling locations along the River Jehlum, including Rasool Barrage, Mangla Dam, Jehlum Bridge and Trimmu Headworks. This heightened concentration led to a greater bioaccumulation of these metals in the liver of *Oreochromis niloticus*, unlike other organs from fish sampled at different points along the river. The order of metal concentration across all sites was recorded as Zn > Cd > As > Pb > Cr. Furthermore, the hierarchy of metal bioaccumulation in different organs of *O. niloticus* was observed as Liver > Kidney > Gills > Muscles > Heart. In terms of site contamination, the order was Pind Dadan Khan > Rasool Barrage > Mangla Dam > Jehlum Bridge > Trimmu Headworks.

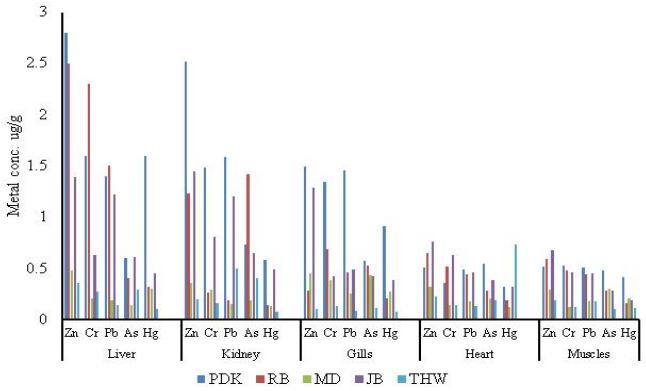

Figure 6: Graphical representation of bioaccumulation of different metals in different organs of *Oreochromis niloticus* sampled from different sites of River Jehlum

DISCUSSION

The current research was conducted to investigate the activity of antioxidant enzymes catalase and peroxidase in relation with metal bioaccumulation in different organs of *Oreochromis niloticus*, collected from five different locations of River Jehlum. Catalase activity was highest in liver (294.00±2.21) as compared to other organs of fish from the Pind Dadan Khan. The order of CAT activity in *O. niloticus* at different sites was recorded as liver > kidney > gills > muscles > heart. The liver of *O. niloticus* fish collected from Rasul Barrage exhibited the highest peroxidase activity (165.00±2) as compared to other organs of fish. The enzymatic activity in fish organs followed the order of liver > kidneys > gills > muscles > heart. The difference in enzymatic activity at every site is due to the presence of pollutants. These findings are correlated with the research conducted by Bibi *et al.* (2017) who, in their study investigated that the main source of water pollution that are industrial wastes and other anthropogenic activities. Compared to Jehlum Bridge, Rasool Barrage, Mangla Dam and Trimmu Headworks at Jehlum River, *O. niloticus* collected from Pind Dadan Khan Barrage had higher antioxidant enzyme activity in variety of organs. In comparison to the heart and muscles tissues of *O. niloticus*, the mean value of Zn was found to be greater in the liver, kidney and gills. These findings are related with a research by Ojeda *et al.* (2012) utilizing an atomic absorption spectrophotometer to examine the effects of metals As, Cu and Cr on *Parachanna obscura*, *Tilapia zilli* and *Heterobranchus bidoraslis* from Ogun River.

The concentration of Zn was found to be higher in the water sample from Pind Dadan Khan (1.23). The highest concentration of As (0.095) was found in water samples from Rasul Barrage. The average temperature at River Jehlum was 27°C, with the

highest recorded values at Pind Dadan Khan (25℃) and lowest at Mangla Dam (24℃). The increase in water temperature changes may be attributed to the addition of sewage and industrial waste. These results are correlated with the findings of Singh *et a*l. (2020) evaluating impact of anthropogenic activities on physico-chemical parameters of water and mineral uptake in Catla catla from river Ravi, Pakistan. The pH was almost neutral but variable at different points, with values ranging from 7.57- 8.54. Agricultural runoff, containing acidic fertilizers and pesticides and the decomposition of sewage and organic matter produce acids that lower water pH. Additionally, chemical reactions triggered by pollutants can generate acidic compounds, further reducing pH levels (Naeem *et al*., 2021).

Conclusion

On the basis of this study and previous studies, it is concluded that antioxidant enzymes are helpful in preventing the harmful effects of metals. Moreover, they are cautionary indicators for severe damage to organisms living in aquatic environment. The current study also concluded that water contamination at Pind Dadan Khan Barrage, Rasul Barrage, Mangla Dam, Jehlum Bridge and Trimmu Headworks, River Jehlum, met standards acceptable for human use and fish culture but presence of Zn, Hg, As, Pb and Cr in several organs of *O.niloticus* from riverine habitat called for routine inspection in order to stop the accumulation of various metals and it is crucial to conduct further studies on antioxidant enzyme systems in various aquatic animal models to gain a deeper understanding.

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