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Assessment of Oxidative Stress in the Freshwater Fish (*Cyprinus carpio*) Exposed to Lead Dioxide

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ABSTRACT

To assess ecotoxicological risks, scientists use biomarkers to detect early signs of pollution. Various biochemical and cellular biomarkers, particularly those related to oxidative stress, have been studied in aquatic organisms, particularly fish, and are recommended for monitoring water quality. The objective of this study was to evaluate lead bioaccumulation and oxidative stress biomarkers in the organs of *C. carpio* exposed to different doses of lead dioxide (LD10, LD50, and LD90) in the laboratory. The analysis of animal tissue using atomic absorption spectroscopy showed significant lead accumulation in the gills, liver, and muscle of carp treated with LD10, LD50, and LD90. This study illustrated that fish could accumulate lead in their organs when exposed to high doses, regardless of whether the organs are in direct contact with water or not. The findings from biochemical biomarker assays indicate that freshwater animals undergo oxidative stress, which impacts enzymes and substrates responsible for their regulation. This biochemical response is evident in the different organs of both control and treated *C. carpio*, as evidenced by an increase in CAT and GST activity in the gills, liver, and muscles, with a higher increase observed in carp treated with lead dioxide in the following order: LD50 > LD10 > LD90. Moreover, a decrease was recorded in the glutathione (GSH) level in the organs of treated carp, followed by an increase in the MDA level in the organs (liver, gills, and muscle) of carp treated at different doses compared to controls. This response varies depending on the concentration of the pollutant studied and the organ.

INTRODUCTION

Heavy metals within aquatic ecosystems have the capability to endure in the environment and become integrated into food chains. Their entry into these aquatic systems primarily occurs through natural inputs and human activities **(Amira & Leghouchi, 2017)**. Aquatic environments are particularly sensitive to trace metal elements because of the simultaneous occurrence of bioaccumulation and biomagnification phenomena **(Ouro-Sama *et al*., 2014; El-Gaar *et al*., 2021; Ahmed *et al*., 2022; Salaah *et al*., 2022a)**.

Lead is a metal with a long history of use in various applications dating back to antiquity. Similar to many other metals, lead exhibits high reactivity in the environment **(Raj & Das, 2023)**. The toxic impact of lead (Pb) varies depending on factors such as the fish's life stage, water pH, hardness, and the presence of organic matter **(Dione *et al*., 2018)**. Lead is considered one of the most toxic metals for both humans and animals **(Naranjo *et al*., 2020; Salaah *et al*., 2022b)**.

The evaluation of eco-toxicological environmental risks involves the use of biomarkers intended to highlight an early stage of pollution **(Soltani, 2008; Salaah & El-Gaar, 2020)**. These biomarkers include those specific to oxidative stress, recommended for monitoring the quality of the aquatic environment **(Van der Oost *et al*., 2003; Khalil *et al*., 2017)**.

The species *Cyprinus. carpio* is commonly employed as a bioindicator since cyprinids represent the most significant group of teleost fishes cultured globally for commercial use. Moreover, they are highly sensitive organisms that are easy to manage, making them ideal for such purposes **(Casas-Hinojosa *et al*., 2023)**.

C. carpio is a large fish, growing up to 30cm in length. It has a "stout" body with a dark olive color, a yellowish belly, two barbels on the upper lip, and a large dorsal fin **(Kumaresan *et al*., 2018)**. Carp inhabit streams, lakes, ponds, and rivers, thriving in brackish and even slightly saline waters **(Stuart *et al*., 2021)**.

This study was designed to assess the bioaccumulation of lead and the biomarkers of oxidative stress in the organs of *C. carpio* when exposed to varying doses of lead dioxide. The following analyses were conducted:

- Lead quantification in various organs, including the gills, liver, and muscle.
- Assessment of oxidative stress biomarkers that involves measuring the activity of antioxidant enzymes such as catalase (CAT), glutathione (GSH), and glutathione S-transferase (GST), as well as assessing lipid peroxidation through malondialdehyde (MDA) levels in different tissues.

MATERIALS AND METHODS

1. Species studied

This study was conducted on common carp, which were captured from Oubeira Lake (EL-Kala, Algeria) (Fig. 1) using traps, then transported in containers to the laboratory. The carp were initially placed in large aquariums, and then identified using identification keys **(FAO, 2020)**. The samples had an average weight of 308.08 ±68.8g and length of 30.27 ±4.77cm.

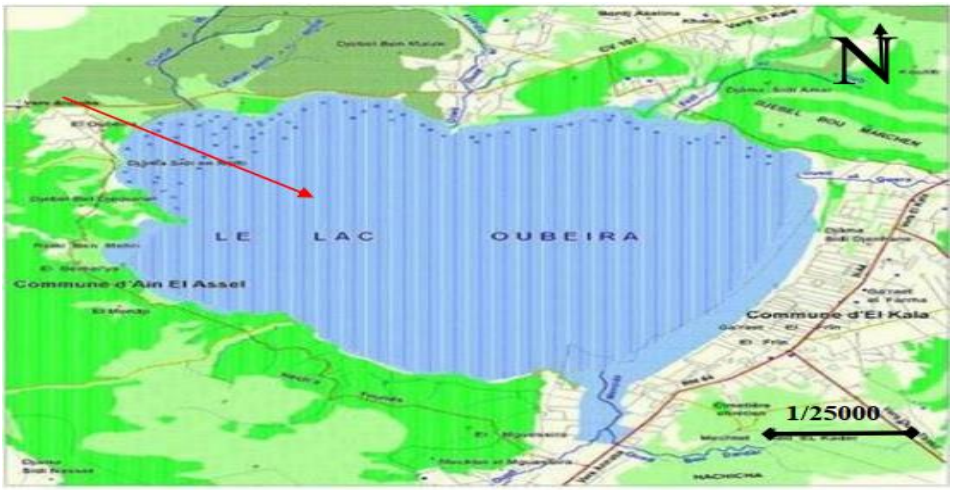

Fig. 1. Location of the study area **(Google Earth, 2020)**

2. Experiment principle

All fish were kept in the laboratory for at least nine days before being used. The first 48 hours serve as an installation period, followed by a seven-day acclimation period, totaling nine days, in water similar to the test water. Fish were maintained under the following conditions: photoperiod and temperature appropriate for the species, oxygen concentration at least 60% of air saturation value, and feeding three times a week until 24-48 hours before exposure. During the acclimation period, mortality was monitored according to the following criteria: if mortality exceeded 10% of the population within seven days, the entire batch was discarded; if mortality was between 5 and 10%, acclimation was extended for an additional seven days, and if mortality exceeded 5% during this second seven-day period, the batch was rejected; if mortality was less than 5% in seven days, the batch was accepted. After acclimatization **(OCDE, 2019)**, fish were divided into four groups; one served as control and the others as treated groups.

The fish were exposed to lead dioxide (PbO_2) for 96 hours under static, semi-static, or flow-through conditions. Fish were allotted five (5) fish per group based on the doses of lead dioxide (Sigma-Aldrich); they were exposed to 0, 2, 10, and 20mg/ L. During this period, any deaths and noticeable changes in appearance or behavior were recorded when possible, and the concentrations that result in 50% mortality of the fish (LD50) were determined (Table 1).

Table 1. Different concentrations used in bioassays

PbO_2	**LD [10]**	**LD [50]**	**LD [90]**	
Control group				**Srivastava & Srivastav, 2019**
Group A	2 mg/L			
Group B		10 mg/L		
Group C			20 mg/L	

3. Metal quantification in carp organs

Previously frozen carp organs were thawed and subsequently subjected to 48 hours of drying in an oven at 105°C to ensure thorough desiccation. The dried material was then pulverized and sifted through a 2mm mesh. The resulting powder underwent non-wet digestion using aqua regia (HCl/HNO_3, 3V/1V) **(Dauvillier, 1998)**. The equipment utilized in our investigation was the AA-6200 model (SHIMADZU-CORPORTION).

4. Extraction of enzymes

The measurements related to stress biomarker monitoring require that the collected organs, namely the liver, gills, and muscle, undergo prior homogenization followed by subcellular fractionation. These procedures were carried out in an appropriate buffer with the following composition: phosphate buffer at 100mM (8.44ml), glycerol at 20% (1.56ml), and phenylmethanesulfonylfluoride (PMSF) at 0.2mM (2µl).

The collected organs were placed in tubes to which a defined volume of this buffer was added (500µl per 100mg of tissue). Tissue homogenization was performed directly in the tube containing the organ to minimize material loss during successive transfers. The homogenizer's rotation speed was low enough to prevent sample overheating but sufficiently high to minimize homogenization time and avoid sample degradation.

Once the tissues were adequately homogenized in the buffer, centrifugation was carried out (at 10000 r/15 min, 4°C) to separate the different cellular fractions **(Barillet *et al*, 2006)**.

5. Biomarkers and enzyme

5.1. Catalase activity

Catalases, found in various tissues, are tetrameric enzymes containing heme groups. They help protect cells from oxidative stress by catalyzing the conversion of harmful hydrogen peroxide (H_2O_2) into water and oxygen **(Regoli & Principato, 1995)**. Catalase activity was measured using a spectrophotometer at a wavelength of 240nm by monitoring the change in optical density resulting from the dismutation of hydrogen peroxide, which is reacted in phosphate buffer (100mM; pH 7.5).

5.2. Glutathione (GSH)

Glutathione, also known as γ-L-cysteinylglycine, is a tripeptide and the primary antioxidant in cells due to its free SH group. To measure blood glutathione (GSH) levels, a modified version of Ellman's colorimetric method (1959) was employed using 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB) as the acidic reagent. This method is based on the oxidation of GSH by DTNB, resulting in the release of TNB **(Ellman, 1959)**.

5.3. Glutathione-S-transferase (GST) activity

Glutathione S-transferases (GSTs) are metabolic enzymes that facilitate the elimination of various substrates with electrophilic groups by conjugating them to glutathione, which has a nucleophilic –SH group **(Habig *et al.*, 1974)**.

5.4. Malondialdehyde (MDA)

Malondialdehyde (MDA) was quantified using the method outlined by **Draper and Hadley (1990)**. This technique involves a colorimetric measurement of the reaction between thiobarbituric acid (TBA) and malondialdehyde, which is produced from the lipid peroxidation of polyunsaturated fatty acids in cell membranes. The reaction forms a pink complex that was measured at a wavelength of 532nm.6.

6. Statistical analyses

6.1. Student's t-test

The Student's t-test was used to compare means pairwise between control carp and those treated at different doses. This test was conducted to compare lead concentrations and the measured biomarkers.

6.2. One-way analysis of variance

One-way analysis of variance (ANOVA) was used to examine differences between values recorded in the organs of carp treated with different lead doses.

RESULTS AND DISCUSSION

1. Lead concentration

The lead concentration recorded in different organs of the studied carp ranged from 0.128 to 5.334µg/ g (Fig. 2). Statistical analysis indicated a significant increase in lead levels in treated carp compared to controls, with the highest concentrations found in the gills and muscles of the treated carp. Additionally, comparing treated carp across different doses revealed significant differences, showing a decrease in lead concentrations in carp treated at LD90. ANOVA also showed a highly significant difference ($P< 0.001$) in lead concentrations among the various organs of the carp.

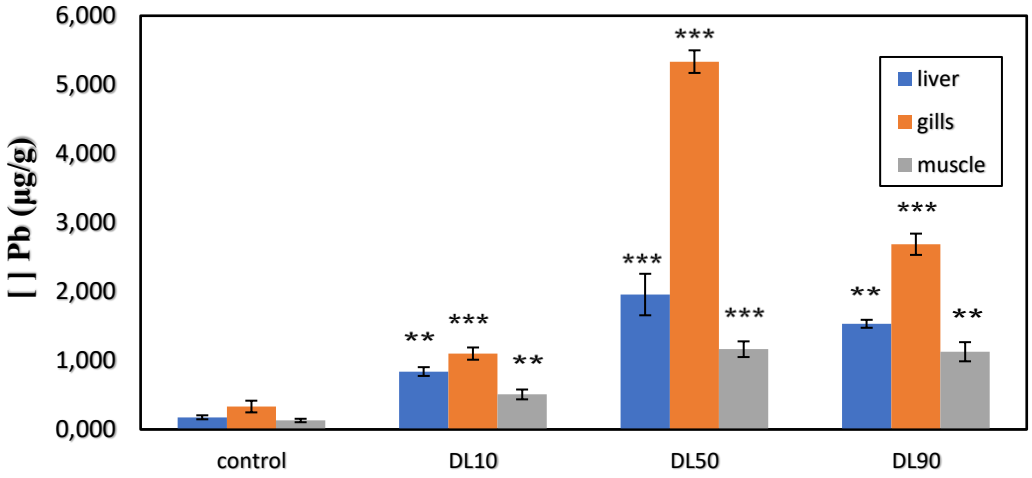

Fig. 2. Lead concentration (µg/g) in the organs of common carp exposed to lead dioxide for 96 h. (mean ± SD; n = 5; ** Significantly different at $P < 0.01$, ***: Significantly different at $P< 0.001$ with student "t" test).

2. Catalase activity (CAT)

The Student's t-test comparing control and treated carp demonstrated a highly significant increase ($P< 0.001$) in catalase activity in the gills, except at the LD90 dose, as well as in the liver and muscles. Additionally, this test showed significant differences between carp treated with different doses. ANOVA indicated a highly significant increase ($P< 0.001$) in catalase activity in the gills and muscles, and a significant difference ($P < 0.05$) in the liver (Fig. 3).

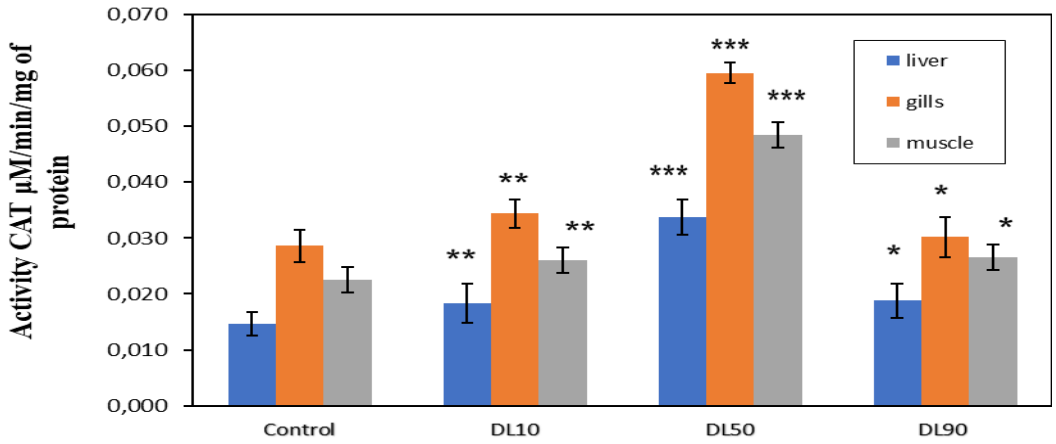

Fig. 3. Catalase activity (µM/min/mg of protein) in the three organs (liver, gills, muscle) of common carp exposed to lead dioxide for 96h. (mean ± SD; n = 5; * Significantly different at $P< 0.05$, ** Significantly different at $P<0.01$, ***: Significantly different at $P < 0.001$ with student "t" test).

3. Glutathione (GSH) level

Statistical analysis with the Student's t-test revealed a significant decrease ($P< 0.01$) in GSH levels in the organs of treated carp compared to controls. Additionally, this test showed significant differences between carp treated with different doses. ANOVA indicated a highly significant difference ($P< 0.001$) in GSH levels across the organs of the carp (Fig. 4).

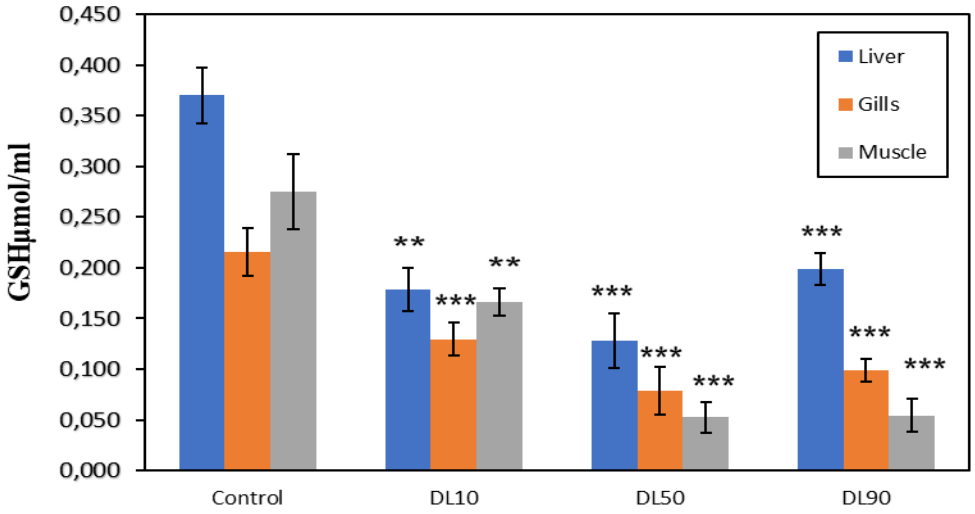

Fig. 4. Glutathione Level (µM/min/mg of protein) in the three organs (liver, gills, muscle) of common carp exposed to lead dioxide for 96h (mean ± SD; n = 5; ** Significantly different at $P<0.01$, ***: Significantly different at $P< 0.001$ with student "t" test).

3. Glutathione-S-transferase (GST) activity

Comparison of recorded GST values using the Student's t-test revealed a significant increase in the liver and gills of carp treated with LD10 and LD50, while no significant difference in the muscle. When comparing carp treated with different doses, there was a decrease in GST activity in the gills and muscles of those treated with LD90. ANOVA showed a significant difference in GST levels across the organs of the carp (Fig. 5).

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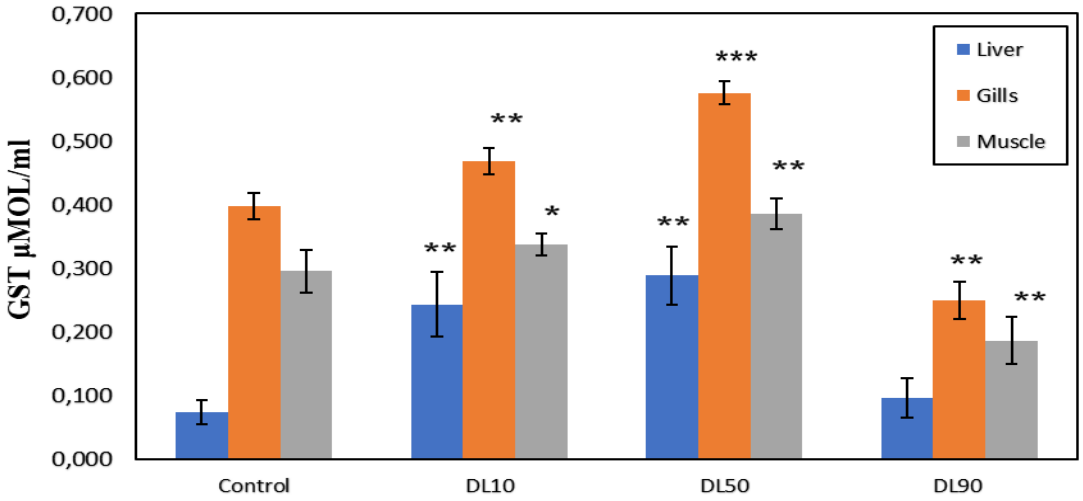

Fig. 5. Glutathione-S-transferase activity (µM/min/mg of protein) in the three organs (liver, gills, muscle) of common carp exposed to lead dioxide for 96h (mean ± SD; n = 5;* Significantly different at $P< 0.05$, ** Significantly different at $P<0.01$, ***: Significantly different at $P< 0.001$ with student "t" test)

4. Malondialdehyde (MDA) level

Statistical comparison using the Student's t-test between control and treated carp showed a highly significant increase ($P< 0.01$) in MDA levels in the organs of treated carp, except in the liver where no significant difference was observed at LD90. When comparing MDA levels between treated carp, a significant increase was noted only at LD50 (Fig. 6).

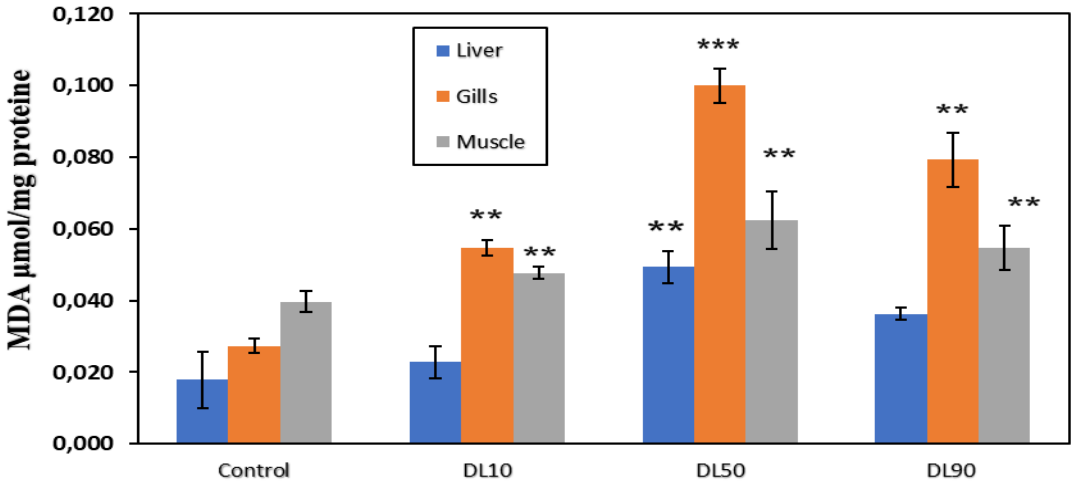

Fig. 6. Malondialdehyde level (µM/min/mg of protein) in the three organs (liver, gills, and muscle) of common carp exposed to lead dioxide for 96 h (mean ± SD; n = 5** Significantly different at $P< 0.01$, ***: Significantly different at $P< 0.001$ with student "t" test).

Heavy metals pose a significant threat to aquatic species due to their persistence in the environment, tendency to bioaccumulate, and above all, their toxicity **(Ouali *et al*., 2018)**. Their targets in these species primarily include the kidney, liver, gonads, muscle, gills, and brain. Consequently, heavy metals can affect not only productivity and reproductive capacities but also can lead to species mortality. The level of bioaccumulation is particularly high in carnivorous fish species **(Kouamenan *et al*., 2020)**.

Analysis of our samples using SAA revealed lead concentrations ranging from 0.128 to 5.334µg/ g in the different organs of the studied carp. Lead concentrations in the carp organs were as follows: gills > liver > muscle. It is noteworthy that the concentrations of lead in the same organ, subjected to different doses of lead dioxide, follow this order: LD50 > LD90 > LD10 > controls.

The high concentration of lead in the gills can be explained by the fact that gills serve as respiratory organs in fish, through which metal ions are absorbed **(Yulong, 2023)**. The gills are in direct contact with water and have a thin epithelium; thus, metals can easily penetrate through their cells **(Mélila *et al*., 2012)**.

The high lead concentration in the liver is likely due to bioaccumulation, as the liver is the center of metabolism and can therefore concentrate heavy metals. This increase can be attributed to the accumulation of Pb in organs as indicated by our results. Indeed, the accumulation of this metal catalyzes ROS capable of damaging biomolecules such as proteins, lipids **(Mélila *et al*., 2012; Salaah *et al*., 2022a)**, and DNA **(Crowther, 2023)**.

Reactive oxygen species (ROS) are generated as a result of oxidative stress triggered by harmful substances, such as lead, which provoke responses in aquatic organisms **(Soltani *et al*., 2012; Salaah *et al*., 2022b)**. When confronted with oxidative stress from heavy metals, organisms must uphold a finely tuned antioxidant system to safeguard delicate cellular elements **(Dai *et al*., 2018)**. The examination of the findings revealed a notable rise in catalase activity in both treated carp and the examined organs in comparison to the control group. Such elevation in catalase activity is frequently observed in the presence of pollutants, such as lead (Pb) **(Sellami *et al*., 2012; Salaah *et al*., 2022b; Soliman *et al*., 2023)**. They observed an augmentation in catalase (CAT) activity subsequent to carp exposure to lead. These findings indicate that this compound induces oxidative stress, disrupting the redox equilibrium in treated animals to favor reactive oxygen species (ROS) production **(Karadag *et al*., 2014)**. We can explain these results by the fact that heavy metals exposure in fish generates reactive oxygen species (ROS), which induces oxidative stress **(Shaukat *et al*., 2018; Lee *et al*., 2019)**.

The biochemical response in terms of CAT in carp, following contamination by PbO_2, varies depending on the time and dose of this pollutant, with a maximum recorded with the highest tested dose **(Sellami *et al*., 2012; Rehman *et al*., 2021)**.

The results of glutathione levels measured in the organs showed a significant decrease in treated carp compared to controls.

Tissue depletion of GSH is one of the main factors facilitating lipid peroxidation **(Zaidi & Soltani, 2011; Bouzenda *et al*., 2021)**. This enzyme possesses carboxylic acid groups, an amino group, a sulfhydryl group, and two peptide bonds as sites for metal reactions. Pb+2 exclusively binds to the sulfhydryl group, reducing GSH levels and potentially interfering with the antioxidant activity of GSH **(Shahsavani *et al*., 2012)**.

GST has been identified as a bioindicator for evaluating the environmental consequences of various organic or inorganic pollutants **(Mebarki *et al*., 2015)**. This enzyme plays a role in detoxifying xenobiotics and eliminating their metabolites **(Jing *et al*., 2017; Naz *et al*., 2019)**.

Assessment of GST activity in carp organs showed an increase in GST levels across all treated fish, with particularly high values observed in those exposed to the DL50 dose. Previous studies indicate that GST levels elevate after a 96-hour (4-day) exposure. This study assesses the effects of two pollutants, 2,4-D and azinphosmethyl, on marker enzyme activity in the liver of the fish species *Cyprinus carpio* (L.) **(Özcan Oruç & Üner, 2002)**. Another study on the effects of heavy metals in freshwater fish revealed a significant increase in glutathione-S-transferase activity in *Oreochromis niloticus* after exposure **(Rehman *et al*., 2021)**. Our findings further indicate that GST activity is most pronounced in the gills compared to the liver and muscle. These results can be ascribed to lead concentration in freshwater fish being directly related to GST activity, with GST acting as a protective enzyme that helps mitigate the toxic effects of lead **(Shaukat *et al*., 2018)**.

MDA serves as one of the frequently utilized markers for lipid peroxidation **(Jing *et al*., 2017)**. Statistical analysis of the MDA levels in the organs of carp treated with PbO_2 showed an increase in levels in all treated carp compared to controls, with a predominance of MDA levels in carp treated with DL50. This increase is likely due to lipid peroxidation caused by the presence of lead **(Khebbeb *et al*., 2010; Salaah *et al*., 2022b)**. Regarding the organs, it is the gills that contain the highest levels of MDA .

MDA has been extensively utilized as the predominant biomarker for evaluating lipid peroxidation in both biological and medical fields **(Boussoufa *et al*., 2021)**. It has been noted that there is a distinct rise in MDA levels subsequent to the incubation of certain unsaturated fatty acids with lead. Increased levels of thiobarbituric acid-reactive

substances (TBARS) have been observed in specific tissues of mammals following lead poisoning, potentially attributed to the generation of ROS without commensurate increases in antioxidant defenses **(Amira *et al*., 2018)**. Evidence also suggests that pollutant-induced lipid peroxidation occurs in various fish species **(Shahsavani *et al*., 2012)**.

Given that fish tissues contain high concentrations of polyunsaturated lipids and fatty acids, they are particularly susceptible to lipid peroxidation, a susceptibility that is further heightened by exposure to heavy metals **(Sifi & Soltani, 2019)**. In line with these findings, the current study reveals a significant elevation in MDA concentration in the liver, muscles, and gills of common carp exposed to lead **(Shahsavani *et al*., 2012)**.

CONCLUSION

This study aimed to investigate the bioaccumulation of lead in the organs of common carp, focusing primarily on antioxidant enzymes and oxidative stress biomarkers (CAT, GSH, GST, MDA). Chemical analysis of the organs using SAA revealed higher concentrations of lead in the gills compared to other organs. Furthermore, the highest concentrations of lead were observed in carp treated with DL50 for 96h. The evaluation of oxidative stress biomarkers indicated an increase in CAT activity, GST activity, and MDA levels, alongside a decrease in GSH levels in the liver, gill, and muscle tissues of *Cyprinus carpio*. This response varied depending on the pollutant concentration and the organ.

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