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Toxicity of Cobalt and Lead on *Tubifex tubifex*: Study on Lethal Effect and Changes in Antioxidants (Glutathione and Superoxide Dismutase)

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Abstract

The study aimed to assess the lethal and sublethal toxicity effects of heavy metals, Cobalt (Co) and Lead (Pb), on Aquatic oligochaete Tubifex tubifex, which is an essential environmental indicator. Experiments indicated that the median lethal concentration (LC50) was 998.2 mg/L for Co and 207.5 mg/L for Pb after 48 h of exposure, reproducing the higher toxicity of Pb compared to Co. The study also detected the effect of exposure to sublethal concentrations (680 mg/L for Co and 95 mg/L for Pb) over 7 and 14 days. The results showed a significant reduction in the activity of antioxidants. For Cobalt, the activity of superoxide dismutase (SOD) decreased from 98 U/mg on day 7 to 73 U/mg on day 14, while the concentration of glutathione (GSH) decreased from 20 µg/mL to 13 µg/mL. Upon exposure to lead, SOD activity decreased from 95 U/mg on day 7 to 68 U/mg on day 14, and GSH concentration declined from 18 µg/ml to 8 µg/ml. These results highlight the crucial role of antioxidants in counteracting oxidative stress induced by heavy metals, making them real biomarkers for monitoring environmental pollution. The study highlights the serious need to develop sustainable environmental strategies to reduce the effect of these pollutants on aquatic ecosystems.

Keywords:

Tubifex tubifex, sublethal toxicity, lethal toxicity, antioxidants, SOD, GSH.

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Introduction

Heavy metals are natural compounds in the environment produced by humans and their chemical composition is affected by their activities (Nasrallehzadeh Saravi et al., 2023). They are present in the Earth's crust and are released into the surface in several ways, including the breakdown of the rocks that contain them and their transformation into soil and then to the surface of the water (Khatiri et al., 2019). Also, the activity of volcanoes can release metals to the surface of the water and soil (Al-Amin et al., 2021). There are some non-essential metals such as lead that do not have a positive effect on living things because they do not have a nutritional or biochemical function for organisms, which regulate their internal concentrations of these elements through resistance mechanisms such as binding and detoxification through proteins with large molecular weights, including metalthionin (Roy et al., 2023), non-metalthionin (Tschuschke et al., 2002), (Didden & Römbke, 2001) and glutathione (Lucan-Bouché et al., 1999), or by isolating minerals inside the tissues of the organism as granules in the form of insoluble precipitated minerals (Klerks & Bartholomew, 1991), or by accumulating in the tail and causing deposition in it as in the annelids (Kokhia et al., 2022) including the Tubifex tubifex (Chapman & Wang, 2000). In addition, some organisms regulate the concentration of the metals in the body by sending it to the outer shell as in oysters (Almeida et al., 1998). The crayfish can excrete minerals through the gills into the water column (Allinson et al., 2000). The effect of the heavy metal depends on the amount of its absorption from the body, which is determined by its concentration and the length of the exposure period, which are directly proportional to the effect (Rahman et al., 2024), and also depends on the bioavailability of the metals (Jawad Hassan et al., 2020) The risks of metal toxicity occur at high biological readiness (Al-Jashaami et al., 2024).

Antioxidants are the first line of defense for organisms against the toxic effects of heavy metals, which lead to excessive production of reactive oxygen species (ROS), causing oxidative stress and disruption of cellular processes (Asghari, 2019). Organisms regulate this stress through a defense system that includes enzymatic and non-enzymatic antioxidants, which play a fundamental role in neutralizing ROS and reducing cellular damage resulting from heavy metal poisoning (Raza et al., 2021). Enzymatic antioxidants include the enzyme superoxide dismutase (SOD), which converts superoxide ions (O_2^-) to hydrogen peroxide (H_2O_2), reducing their toxicity within the cell. This role is complemented by the enzyme catalase (CAT), which converts H_2O_2 to water and oxygen, limiting its toxic effect. Glutathione peroxidase (GPx) plays a main role in eliminating lipid peroxides and decreasing damage to cell membranes when exposed to heavy metals (AbdElgawad et al., 2020). At the non-enzymatic level, glutathione (GSH) is one of the record important antioxidant compounds that help remove toxins resulting from heavy metals (Falih, 2024). Glutathione acts as a free radical receptor and binds to heavy metal ions, forming non-toxic compounds that are easily excreted from the cell. Studies indicate that high levels of GSH in cells are associated with increased resistance to oxidative stress caused by metal pollution (Kevans & Rasmussen, 2000). In addition, antioxidant compounds such as vitamins (C and E) play an important role in inhibiting oxidation processes, as they protect proteins and lipids from oxidative damage resulting from continuous exposure to heavy metals (Ziwei & Han, 2023). Research shows that some plants and aquatic organisms have adaptive mechanisms that enhance the activity of these antibiotics when faced with environments contaminated with heavy metals (Ali et al., 2019). Recent studies show that chronic exposure to heavy metals, such as lead and cobalt, leads to the weakening of these defense systems, which increases the accumulation of cellular damage and dysfunction of biological processes. Therefore, analyzing the levels of antioxidants and their changes due to exposure to environmental pollutants is an important biomarker for assessing the impact of oxidative stress caused by metal pollution in aquatic environments (Jan et al., 2015).

Aquatic Oligochaete have been widely distributed in Iraqi water bodies, especially the Tubificidae and Naidide families (Obaid, 2022), This group of organisms has some harms, as they contribute to the transfer of pollutants through the food chain from bacteria to fish, as they feed on sediment, algae, and bacteria. Aquatic Oligochaete have a long history in toxicity testing, as they were among the first organisms used in many studies due to their importance to the aquatic environment, the wide spread of their species, the ease of culturing, preserving, and handling them, and their ability to withstand the physical and chemical properties of bottom sediments, such as the size of the sediment particles and its content of organic matter. Some species include sediment-ingesters, which feed by placing their head in the sediment and their tail in the water column, thus they will be exposed directly to the pollutants in the water column then by ingesting the polluted sediments (Chapman, 2001).

Many species are Commonly used in acute and chronic toxicity and bioaccumulation tests include *T. tubifex*, as well as *Lumbricules varigates*, *Limnodrilus hoffmestri*, and *Branchiura swerbyi*, which have been used in many studies. Species belonging to the Tubificidae family represent good test organisms for selecting the environmental toxic effects of chemical compounds in sediments since sublethal effects disturb not only individuals but also the population level and thus the composition of the benthic community (Crayton et al., 2020).

Material and Method

1. Sampling

T. tubifex samples were collected from the Diyala River, Baqubah city center, from the river edge at a depth of 0.5 m and isolated from the clay using sieves measuring (0.5 mm) and then placed in plastic containers with a quantity of river water. In the laboratory, the samples were placed in a basin containing bottom sediments and dechlorinated water for acclimatization to laboratory conditions for a week, and two days before the test they were isolated from the sediment and placed in water to get rid of nutrients (starvation) (Frank & Robertson, 1979). Worms were placed on glass slides and a drop of ethyl alcohol (30%) was added to each one for anesthesia, then treated with a drop of lactophenol solution. The slide cover was then placed on the sample and the sample was left for several hours before examination. Diagnosis was made using the key (Brinkhurst & Jamieson, 1971).

2. Lethal Toxicity Tests

These tests were conducted to determine the 48hr LC50 using 3 replicates for each concentration of heavy metals used with one replicate for the control. Each replicate contained 10 fully mature, active worms placed in 250ml plastic containers containing 200ml of the experimental solution. The number of dead individuals was counted every 24 hours of exposure and throughout of the experiment, with dead individuals removed during that period. Complete decomposition of the worm body was considered the endpoint for each of the lead experiments, while the cessation of the dorsal blood vessel was the endpoint for the cobalt, the experiment was repeated when the mortality rate in the control was greater than 10% (Chapman, 2018). The toxicity of each metal in each treatment was estimated by calculating the median lethal concentration (LC50) using Polo plus probit and LOgit analysis.

3. Sub-Lethal Toxicity Tests

These tests were conducted for 7 and 14 days using active adult worms with three replicates in each treatment, 10 worms per treatment were exposed to mg of Cobalt, and mg. Of Lead; At the end of the test period, the

concentration of AchEase and Glutathione was measured at the end of the experimental period, the concentration of SOD and glutathione were measured.

3.1. Estimation of GH

and prepare the worm extract, we relied on what was mentioned in (Sutariya et al., 2012). The worms were washed, and cut, 20 g were weighed, 10 ml of hydrochloric acid was added to them, and they were stored in a glass vial and placed in an aluminum thermal vessel at 50 °C for 5 hours to complete the decomposition process. Using a pipette, 100 microliters were drawn and pipetted into the evaporator to remove moisture with nitrogen gas. After drying, it was dissolved by adding 100 microliters of acetonitrile exposed to ultrasonic waves for one minute, and transferred to a thermal vessel at 50 °C. Heat 50 for 30 minutes to complete the decomposition and inject 100 microliters of it into the HPLC column, noting that the mobile phase is a flow of an equal mixture 50/50 (volume/volume) of water and acetonitrile at pH = 7 at 1 ml/minute and the column is 25 cm x 4.6 mm and the detector = 465 nanometers.

3.2. Estimation of SOD Activity

According to (Beauchamp & Fridovich, 1971) estimated by collect the worms, were washed, homogenized with a buffer solution consisting of 50 mM phosphate buffer and 0.1 mM EDTA, and homogenized with tissue at a ratio of 10:1 (tissue weight/buffer volume) by centrifugation at 12,000×g for 20 min at 4°C. Then the upper fluid portion containing proteins was isolated from the SOD inclusion and 50 ml of it was mixed with the reaction solution consisting of (50 mM phosphate buffer (pH 7.8): 2.8 m + 0.1 mM EDTA: 0.1 mL + 13 mM methionine: 0.3 mL + 75 µM NBT: + 0.1 mL 2 µM riboflavin: 0.1 mL) to measure SOD activity by the Nitroblue Tetrazolium (NBT) method. The mixture was placed under white light for 10-15 minutes, after which the absorbance was measured at 560nm. To measure the activity, the following formula was used: Inhibition (%) = [(Absorbance of Control−Absorbance of Sample)/Absorbance of Control] ×100

Result and Discussion

Lethal Toxicity Tests

After conducting a series of preliminary tests to determine the median lethal concentration (LC_{50}) of Co and Pb on *T.tubifix*. The results (Figure 1) showed that LC_{50} value was 998.2 mg/l for Cobalt, and 207.5 mg/l for Lead (Figure 2) measured after 48 hours of exposure.

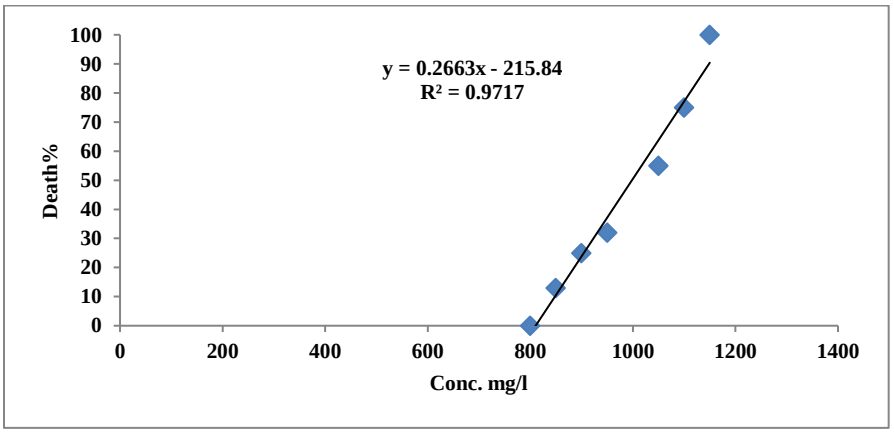

Figure 1. The percentage of mortality of *T. tubifex* after 48 hours of exposure to different concentrations of Cobalt, showing the straight-line equation and correlation value

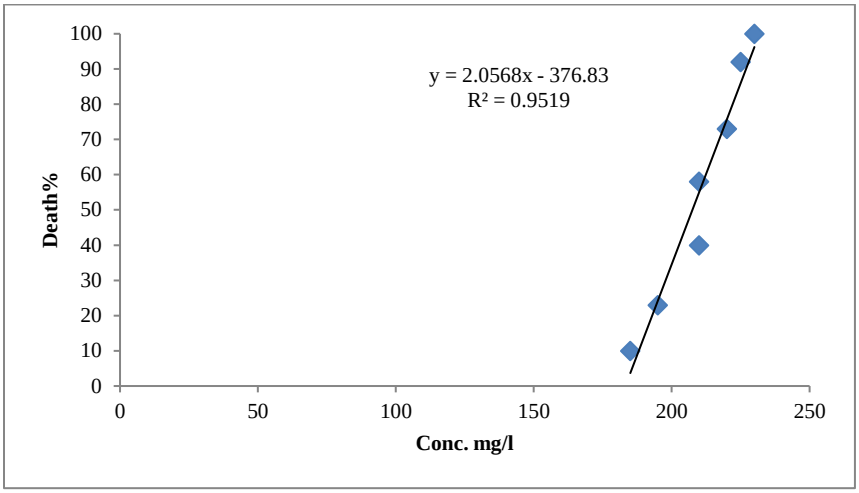

Figure 2. The percentage of mortality of *T. tubifex* after 48 hours of exposure to different concentrations of Lead showing the straight line equation and correlation value

The results of the current study showed that the resistance of individuals of *T. tubifex* to cobalt is higher than lead, the acute toxicity of metals varies according to the species of aquatic organisms and even between similar species, and this depends on the amount absorbed by the body, and this result may be due to the low level of absorption of cobalt, as (Mar et al., 1998) indicated that cobalt is slow in its toxic effect and that it works to reduce the toxicity of other metals, as it has an antagonist effect with other metals. The resistance shown by individuals of the species *T. tubifex* to increasing concentrations of metals may be due to their ability to remove toxicity in response to increased pollution. This ability depends on the history of exposure, as (Klerks & Bartholomew, 1991) found that *Limnodrilus hoffmesteri* taken from polluted areas showed high resistance to a mixture of cadmium, nickel, and cobalt. This resistance is not due to the low level of their accumulation in the body, but rather to the high level of the metal associated with the protein Metaltheionien (MT). The current study noted that the death of the worms begins with the degradation of the posterior segments, due to the accumulation of metals in the caudal region, which causes cell death (Lucan-Bouché et al., 1999), also expose to heavy metals, causes rupture in the body wall and breakdown of both the lining of the digestive tract and the amoeboid cells (Chapman & Brinkhurst, 1984). One of the most important reasons for the breakdown of the last rings of the body is that the presence of pollutants negatively affects the transmission of nerve impulses and consequently the contraction and relaxation of muscles, which slows down the blood flow to the last rings of the body and gradually leads to the permeation of death into those segments (Jawir, 1977). The deposition of metals on the body also directly affects worms because they reduce the efficiency of gas exchange through the skin (Stephen Whitley, 1968), also essential and non-essential metals, when found in high concentrations, bind to the basic components of the cell, causing the destruction of the cell membrane, changing the specificity of enzymes, disrupting cellular functions, destroying the structure of DNA, and thus cell death (Bruns et al., 2000).

Sub-Lethal Toxicity Tests

The variation in glutathione concentration and SOD activity was measured after exposing fully active mature *T.tubifix* to sublethal concentrations: 680mg/l of cobalt and 95mg/l of lead for 7 and 14 days. The results (Table 1) showed a decrease in glutathione levels after 7 days of exposure to sublethal concentrations of cobalt and reached 20 micrograms/ml, reflecting an initial response to oxidative stress. It decreased further with continued exposure for 14 days, indicating that continued oxidative stress led to the depletion of glutathione stores. Cobalt works to produce reactive oxygen species (ROS), which leads to the depletion of glutathione as part of the

cells' response to get rid of oxidative stress. This is consistent with previous studies that showed that heavy metals reduce GSH levels due to their use in removing ROS (Valko et al., 2006; Liu et al., 2009). This decrease was greater when exposed to sublethal concentrations of lead, where it reached 18 micrograms/ml and then decreased to 8 micrograms/ml (Table 2). Lead disrupts the oxidative balance more than cobalt, as it increases the production of ROS and inhibits the enzymes responsible for recycling GSH, such as glutathione reductase. The sharp decrease in GSH after 14 days One day it reflects the great oxidative stress and damage to the defense systems (Patrick, 2006; Gurer & Ercal, 2000). Also, long-term exposure to lead negatively affected the activity of SOD by disrupting the enzymatic reactions through its association with the sulfhydryl groups (SH) in the active sites of the enzyme, and this was confirmed by many studies on the ability of lead to inhibit the antagonistic enzymes (Matović et al., 2015; Elssaidi et al., 2020).

Table 1. Effect of exposure to sublethal concentration of cobalt (mg/l) on GSH (µg/mL) levels and SOD activity (U/mg) at 7 and 14 days exposure

Antioxidant	control	7 day	14 day	Sig.
SOD	90±8.6	98±3.2	73±1.04	0.00
GSH	23±.0.41	20±1.8	13±0.51	0.00

Table 2. Effect of exposure to sublethal concentration of Lead (mg/l) on GSH (µg/mL) levels and SOD activity (U/mg) at 7 and 14 days of exposure

Antioxidant	control	7 day	14 day	Sig.
SOD	90±8.6	95±2.8	68±3.05	0.00
GSH	23±3.01	18±1.54	8±0.31	0.00

Conclusion

The results of the study indicate the negative effect of both lead and cobalt on *T. tubifex*. The sublethal tests showed a significant decrease in the levels of GSH and the activity of the SOD after the organisms were exposed to sublethal concentrations of cobalt (680 mg/L) and lead (95 mg/L) for 7 and 14 days. This decrease shows the harmful effect of these metals on the antioxidant system, which may result in oxidative stress that disrupts the vital functions of the organism, it also points to the essential role played by antioxidants in protecting against heavy metal toxicity, and the importance of using these biochemical indicators in assessing environmental risks resulting from heavy metal pollution.

Heavy metals, such as lead and cobalt, have damaging effects on organisms in the aquatic environment, which may disturb the ecological balance. Therefore, it is necessary to adopt effective and sustainable environmental clarifications to protect aquatic systems and confirm their long-term stability, which include strategies and procedures aimed at reducing the accumulation of heavy metals in the environment, including reducing the discharge of heavy metals from factories and agricultural lands into water, and using advanced methods to cleanse water and soil from these metals, and monitoring pollution levels frequently to take preventive measures in a timely manner and repair damaged ecosystems by planting metal-absorbing plants or using microorganisms to treat soil and water.

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