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Antioxidant enzyme responses and physiological thresholds of metal stress in *Lemna minor* in dose-dependent phytoremediation of chromium and lead

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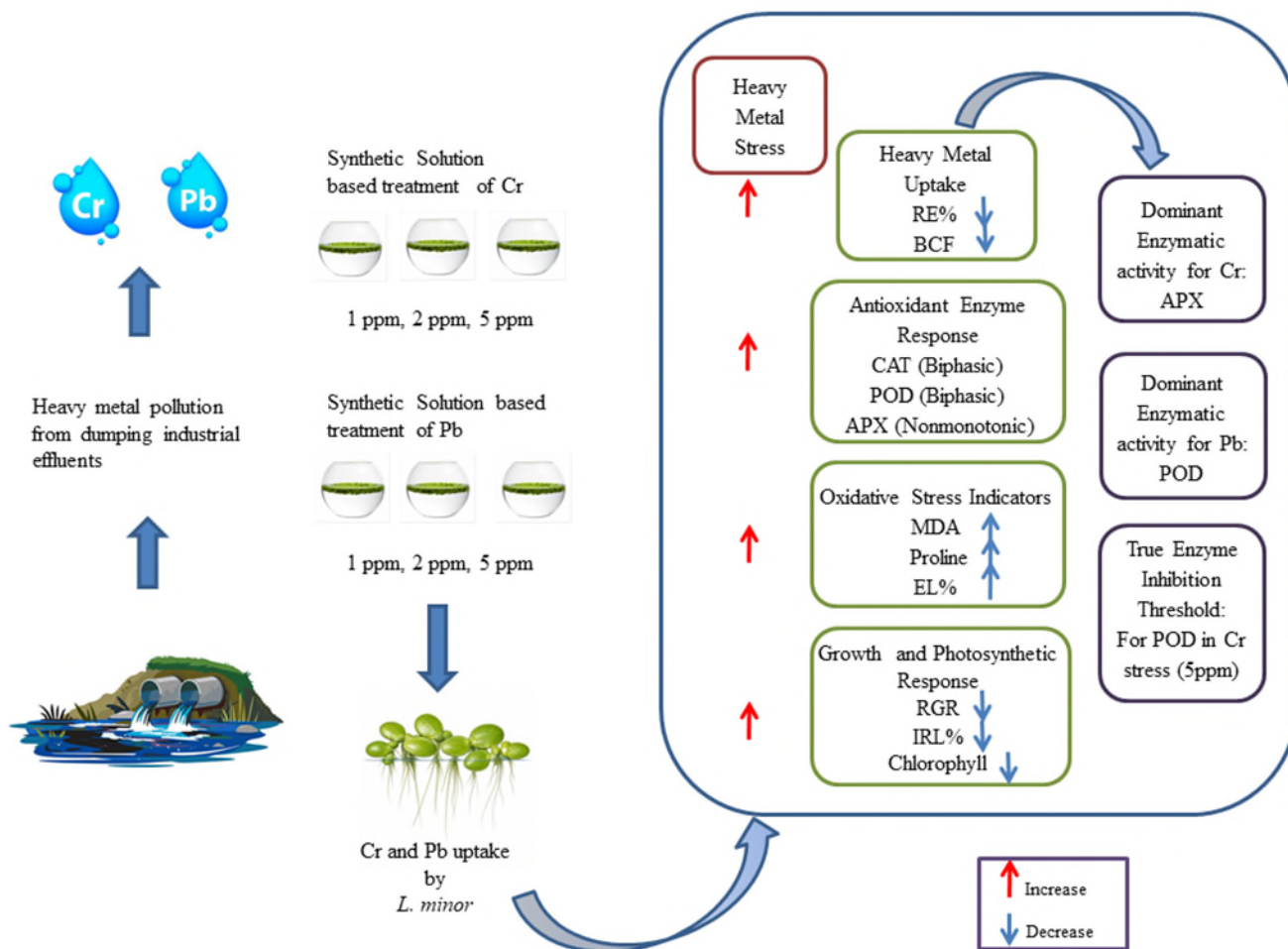
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Graphical Abstract

ABSTRACT

The presence of chromium (Cr) and lead (Pb) in aquatic environments is a major environmental concern because they are toxic and pose ecological risks. Despite the extensive research on the application of *Lemna minor* for phytoremediation, physiological mechanisms that link antioxidant defense and metal removal efficiency are not well understood. In this study, *L. minor* was exposed to different concentrations of Cr and Pb (1, 2 and 5 mg L⁻¹) in a dose-dependent manner and evaluated for its phytoremediation efficacy, physiological response, antioxidant defense, oxidative stress biomarkers and statistical modeling to elucidate the defense mechanisms over a period of 7 days. Removal efficiency (RE) and bioconcentration factor (BCF) were used to assess the phytoremediation performance, and the relative growth rate, root growth inhibition, chlorophyll, catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), malondialdehyde, electrolyte leakage, proline, and Enzyme Inhibition Index (EII) were used to evaluate the physiological and biochemical responses. One-way ANOVA, Tukey's HSD test, Pearson correlation and multiple linear regression were used to analyze the data. Removal of chromium was high and constant at 96.32–98.89%, while that of lead was reduced from 92.10% to 86.10% as the concentration increased. EII was considered an enzymatic indicator of Cr stress, whereas APX and POD were the most important regulators of chromium and lead removal by multiple regression, respectively. The results showed that coordinated antioxidant regulation is important for the efficiency of phytoremediation and showed the possibilities of using some parameters as mechanistic biomarkers for optimization of phytoremediation by *L. minor*.

Keywords: Chromium; Lead; *Lemna minor*; Phytoremediation; Antioxidant enzymes; Oxidative stress

Introduction

Heavy metal contamination in freshwater ecosystems is an emerging environmental problem (Chang et al., 2013), especially in developing countries where the metal load into natural water bodies is also rising due to rapid industrialization and insufficient wastewater treatment facilities. The leather tanning, electroplating, textile dyeing, and metal processing industries are significant sources of chromium (Cr) and lead (Pb), which remain in the water bodies and are harmful to human health and the environment (Aslanzadeh et al., 2024; Ahmed et al., 2025). In Bangladesh, industrial effluents have significantly polluted rivers such as the Buriganga, Dhaleshwari, and Turag rivers, and traditional metal removal methods can be expensive and cause secondary pollution (Ahmed et al., 2025; Das et al., 2025).

The mechanisms of action inducing toxicity for Cr(VI) and Pb are different. Cr(VI) is taken up into plant cells via sulfate transport systems and undergoes reduction in plant cells, generating ROS, which causes oxidative damage to cellular and photosynthetic components (Aslanzadeh et al., 2024; Gill & Tuteja, 2010). Arshad et al. (2024) and Kushwaha et al. (2024) reported that Pb²⁺ mainly acts as an ionic interferent in cellular metabolism, enzyme activity, chlorophyll biosynthesis and membrane function. Therefore, the physiological and biochemical responses to the presence of both metals in the plant might vary, even though they are both toxic to plants.

Phytoremediation is a sustainable means of removing dissolved metals from contaminated waters. *Lemna minor* is one of the most suited aquatic species, due to its ability to collect metals, easy harvesting, high biomass productivity, and rapid growth (Farid et al., 2022; Zhou et al., 2023). Exposure to metals, however, can cause progressive poisoning of plants and reduced metabolic activity, which may affect the ability of the plants to remediate.

Under metal stress conditions, antioxidant enzymes such as catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) are critical for maintaining redox homeostasis. Malondialdehyde (MDA), Electrolyte leakage (EL), Proline, Chlorophyll, and Growth responses are some complementary indicators of oxidative damage and physiological impairment (Gill & Tuteja, 2010; Hasanuzzaman et al., 2020). But most are usually used as stress indicators rather than as a measure of metal-removal efficiency.

Thus, an understanding of antioxidant response and phytoremediation efficiency is one of the main gaps in knowledge. The role of antioxidant enzymes as predictors of metal removal, as well as the enzyme response from induction to inhibition as metal concentration increases, and the specific enzyme response to Cr and Pb remain unknown. Therefore, the present study examined the dose-dependent response of *L. minor* to Cr(VI) and Pb by evaluating the efficiency of metal removal, bioconcentration factor, growth, chlorophyll concentration, CAT, POD, APX, MDA, EL and proline. To understand the correlation between the antioxidant response and the phytoremediation efficiency, Pearson correlation and multiple linear regression were used, and the enzyme inhibition index (EII) was used to determine the probable shift from enzyme induction to enzyme inhibition. This integrated approach is used to gain insight into metal-specific antioxidant regulation and to determine the potential enzymatic biomarker associated with the metal phytoremediation performance of *L. minor*.

Materials and methods

Sample collection

Approximately 500 g of healthy *L. minor* were collected from a pond adjacent to the Dhaleshwari River, Bangladesh (23°47'55.58"N, 90°15'28.08"E). The samples were cleaned, surface-sterilized, and acclimated for 7 days in 0.1× Hoagland's nutrient solution under controlled conditions following Farid et al. (2022) and Vámos et al. (2024) (**Fig. S1**). Uniform fronds were used for all experiments.

River water (RW) samples were collected from five sampling stations (S1–S5), spaced approximately 500 m apart within the BSCIC Tannery Industrial Zone of the Dhaleshwari River, using combined grab and composite sampling (Mercy et al., 2023). The samples were collected to determine the chromium and lead concentrations for establishing the synthetic treatment solutions used in the experiment. Samples were collected in acid-washed HDPE bottles, transported to the laboratory, and stored at 4 °C until analysis (Lipps et al., 2023; Zadeh, 2023). The *L. minor* and water samples were collected between mid-October and mid-November 2025. The geographical locations of the sample collection sites are presented in **Table 1** and **Fig. 1**.

Table 1 The coordinates of five sampling stations for the collected water samples

<i>Station No.</i>	<i>Station Code</i>	<i>Latitude</i>	<i>Longitude</i>
1	S1	23° 47' 19" N	90° 14' 30" E
2	S2	23° 47' 03"N	90° 14' 26"E
3	S3	23° 46' 47" N	90° 14' 23" E
4	S4	23° 46' 32"N	90° 14' 17"E
5	S5	23° 46' 15"N	90° 14' 17"E

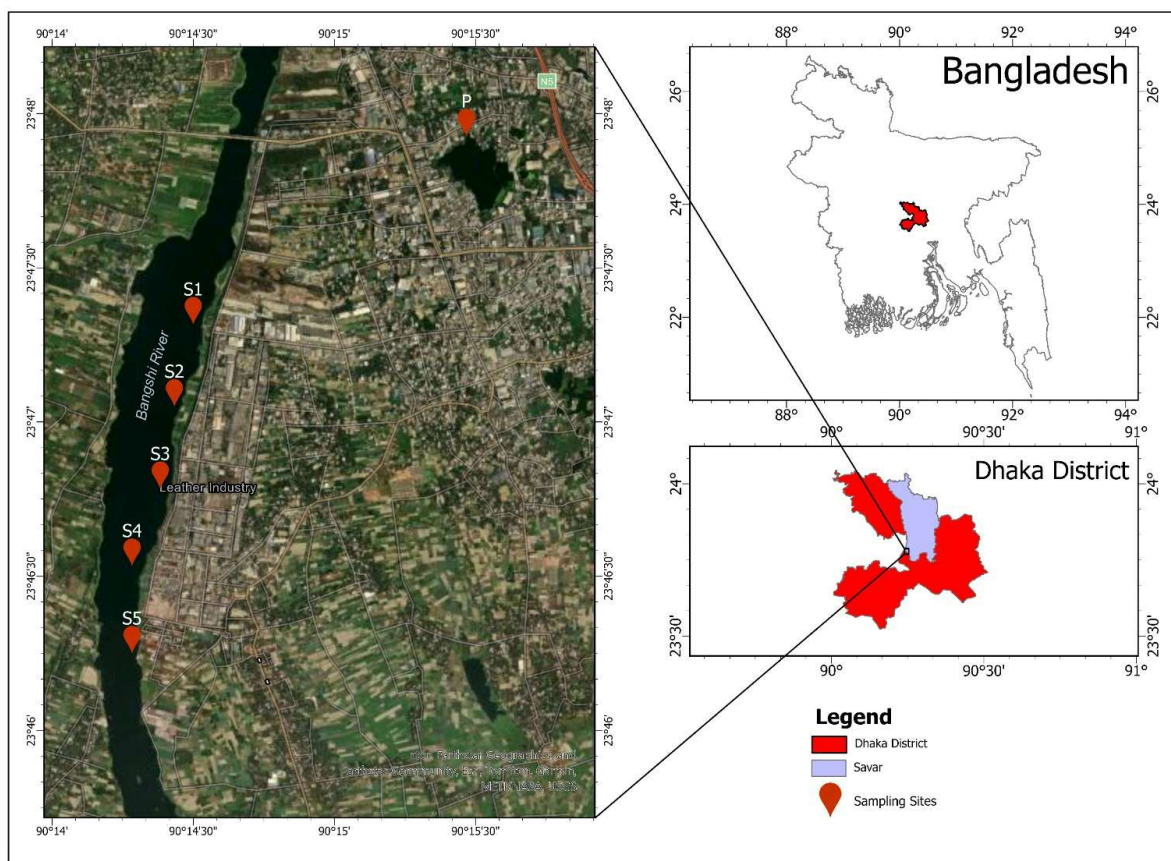


Fig. 1 Geographical location of sampling sites (water {S1, S2, S3, S4, S5}, *L. minor* {P})

Analysis of water samples and preparation of synthetic water solutions

The details of instruments, apparatus, chemicals, and reagents used are summarized in **Table S1**, which includes the instrument, apparatus, or chemical and its specification, purity/grade, and literature source. Chemical parameters (**Table S2**) such as pH, temperature, EC, TDS, salinity, DO, BOD and COD were measured, and Cr and Pb concentrations were measured in the initial stage using flame atomic absorption spectroscopy (AAS) (Lipps et al., 2023; Das et al., 2025). The concentrations of Cr and Pb in the river water (RW) were used to choose the environmentally relevant concentrations for the synthetic treatments.

Cr(VI) and Pb(II) stock solutions (1000 mg L^{-1}) were prepared according to a slightly modified procedure of Bayuo et al. (2020) using potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) and lead acetate [$\text{Pb}(\text{CH}_3\text{COO})_2$], respectively. Three concentrations: 1, 2, and 5 mg L^{-1} were prepared by serial dilutions in $0.1\times$ Hoagland's nutrient solution (Kassim et al., 2022). Before and after exposure, physicochemical properties were determined (**Table S3**) and target metal concentrations were confirmed using AAS (Niloy et al., 2025).

Experimental setup

Approximately 3 g (fresh weight) of acclimated *L. minor* fronds were exposed to 1, 2, and 5 mg L⁻¹ Cr(VI) or Pb(II) in 300 mL of 0.1× Hoagland's nutrient solution, with metal-free medium as the control (Boldrini et al., 2025). The treatment was carried out 3 times (n = 3), under controlled conditions (20 ± 2 °C, pH 7.0), for 7 days. After the exposure, water samples were taken for both physicochemical and metal analysis, and plant tissues were rinsed, blotted dry, and preserved at -80 °C for biochemical analysis (Monteiro et al., 2022; Zhou et al., 2022). The experimental design is shown in **Fig. 2**.

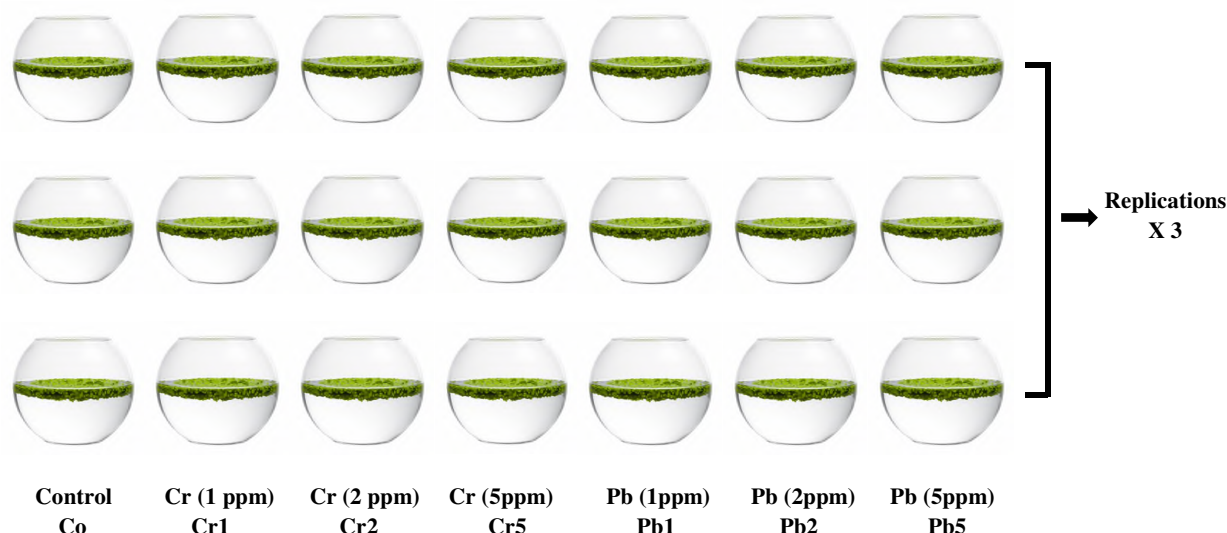


Fig. 2 Experimental setup of *L. minor* in treatment containers

Heavy metal analysis

L. minor fronds were washed with deionized water to remove the dirt, oven-dried for 24 h at 70 °C, and finely ground (Gan et al., 2016; Gebretekle et al., 2024). Dried tissues were digested in closed Teflon digestion tubes, using concentrated HNO₃ (65%) for 4 h at 120 °C (Mashhour & Haghaninia, 2026). The samples were digested in accordance with the above procedure and diluted accordingly and then the samples were analyzed for the concentration of Cr and Pb by flame AAS (model: PerkinElmer PinAAcle 900F). The concentration of metals was expressed in terms of mg kg⁻¹ dry weight (DW) of plant tissue (Das et al., 2025; Gebretekle et al., 2024).

The water samples were filtered using a 0.45 µm membrane filter, acidified to pH < 2 with trace-metal grade, ultra-pure nitric acid (69%) and analyzed (Lipps et al., 2023). Flame atomic absorption spectroscopy (AAS) was used to detect chromium and lead concentrations using matrix-matched calibration standards and wavelengths of 357.9 nm (Cr) and 283.3 nm (Pb).

Antioxidant enzyme activity assay

A common crude enzyme extract for CAT, POD, and APX assays was prepared following Zhao et al. (2022) with minor modifications. Fresh *L. minor* tissue (0.2 g) was homogenized in 5 mL of chilled 50 mM sodium phosphate buffer (pH 7.8) containing EDTA, PVP-40, and glycerol; 1.0 mM DTT was added for APX extraction (Kumar, 2022; Sun et al., 2022). The homogenate was centrifuged at $10,500 \times g$ for 20 min at 4 °C, and the supernatant was immediately used for enzyme assays (Ahmad et al., 2022; Hao et al., 2020; Hasanuzzaman et al., 2012).

Catalase (CAT) activity assay

Catalase (CAT) activity was determined by monitoring the decomposition of H_2O_2 at 240 nm following Pongprayoon et al. (2013); Aebi (1984); Beers and Sizer (1952), with minor modifications (Ahmad et al., 2022). Enzyme activity was calculated using the molar extinction coefficient of H_2O_2 ($\epsilon = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$) and expressed as $\text{U g}^{-1} \text{ FW}$, where one unit represents the decomposition of $1 \mu\text{mol } H_2O_2 \text{ min}^{-1}$. CAT activity was measured using **Equation (1)** as follows:

$$\text{CAT (U g}^{-1} \text{ FW)} = (\Delta A \text{ min}^{-1} \times V_t \times V_{\text{ext}} \times 1000) / (\epsilon \times l \times V_e \times \text{FW}) \quad (1)$$

Where $\Delta A \text{ min}^{-1}$ is the change in absorbance per minute; V_t , total reaction volume (3 mL); V_{ext} , extraction volume (5 mL); V_e , enzyme extract volume (0.2 mL); l , cuvette path length (1 cm); and FW is the tissue fresh weight (0.2 g) and 1000 is a conversion factor to normalize units from M to mM.

Peroxidase (POD) activity assay

Peroxidase (POD) activity was measured by monitoring guaiacol oxidation at 470 nm (Biniiaz et al., 2026; Kato & Shimizu, 1987). Activity was calculated using the extinction coefficient of tetraguaiacol ($\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$) and expressed as $\text{U g}^{-1} \text{ FW}$ where one unit represents the oxidation of $1 \mu\text{mol}$ guaiacol per minute (Ahmad et al., 2022; Kato & Shimizu, 1987). POD activity was measured using **Equation (2)** as follows:

$$\text{POD (U g}^{-1} \text{ FW)} = (\Delta A \text{ min}^{-1} \times V_t \times V_{\text{ext}}) / (\epsilon \times l \times V_e \times \text{FW}) \quad (2)$$

Where $\Delta A \text{ min}^{-1}$ is the change in absorbance per minute; V_t , total reaction volume (4 mL); V_{ext} , extraction volume (5 mL); V_e , enzyme extract volume added to the cuvette (1 mL); l , cuvette path length (1 cm) and FW, fresh weight (0.2 g).

Ascorbate peroxidase (APX) activity assay

Ascorbate peroxidase activity was determined according to a partially modified Nakano and Asada (1981) by measuring the H_2O_2 -dependent oxidation of ascorbate at 290 nm. Enzyme activity was calculated using the molar extinction coefficient of ascorbate ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). Activity was expressed as $\text{U g}^{-1} \text{ FW}$ (Nakano & Asada, 1981). APX activity was measured using **Equation (3)** as follows:

$$\text{APX (U g}^{-1} \text{ FW)} = (\Delta A \text{ min}^{-1} \times V_t \times V_{\text{ext}}) / (\epsilon \times l \times V_e \times \text{FW}) \quad (3)$$

Where: $\Delta A \text{ min}^{-1}$ is the change in absorbance per minute; V_t , total reaction volume (3 mL); V_{ext} , extraction volume (5 mL); V_e , enzyme extract volume added to the cuvette (0.3 mL); l , cuvette path length (1 cm) and FW, fresh weight (0.2 g).

Oxidative stress response assay

Electrolyte leakage estimation

Electrolyte leakage (EL) was determined following slightly modified Hasanuzzaman et al. (2026). Fresh tissue (0.2 g) was incubated in 10 mL deionized water for 2 h, after which initial conductivity (EC_1) was measured. Samples were then autoclaved (121 °C, 20 min), cooled, and final conductivity (EC_2) was recorded. Background conductivity of deionized water (EC_0) was measured separately. Electrolyte leakage was calculated using **Equation (4)** as follows:

$$EL (\%) = [(EC_1 - EC_0) / (EC_2 - EC_0)] \times 100 \quad (4)$$

The higher the EL% is, the greater the permeability of the membrane and damage to the cells. This is due to oxidative stress caused by metals (Hasanuzzaman et al., 2026).

Chlorophyll content estimation

Chlorophyll *a*, chlorophyll *b*, and total chlorophyll were determined spectrophotometrically following slightly modified Arnon (1949). Fresh tissue (0.2 g) was extracted with 80% acetone, and absorbance was measured at 663 and 645 nm. Pigment concentrations were calculated using **Equations (5–7)** as follows:

$$\text{Chlorophyll } a (\text{mg g}^{-1} \text{ FW}) = 12.7 \times A_{663} - 2.69 \times A_{645} \times \frac{V}{100 \times W} \quad (5)$$

$$\text{Chlorophyll } b (\text{mg g}^{-1} \text{ FW}) = 22.9 \times A_{645} - 4.68 \times A_{663} \times \frac{V}{100 \times W} \quad (6)$$

$$\text{Total chlorophyll (mg g}^{-1} \text{ FW)} = \text{Chlorophyll } a + \text{Chlorophyll } b \quad (7)$$

Where C is the chlorophyll concentration (mg L^{-1}), V is the extraction volume (10 mL), and W is the fresh weight of the sample (0.2 g). Final results are expressed as mg g^{-1} FW.

Estimation of lipid peroxidation (MDA)

Lipid peroxidation was quantified by measuring malondialdehyde (MDA) content using the thiobarbituric acid (TBA) method of Buege and Aust (1978), with minor modifications (Taguali et al., 2025). Fresh *L. minor* tissue (0.2 g) was homogenized in 5 mL of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at $4,000 \times g$ for 10 min. Two milliliters of the supernatant were mixed with an equal volume of 0.6% (w/v) TBA prepared in 10% TCA and heated at 100 °C for 15 min. After rapid cooling in an ice bath, the mixture was centrifuged at $10,000 \times g$ for 10

min, and absorbance was measured at 532 and 450 nm (Buege & Aust, 1978; Taguali et al., 2025). The concentration of MDA was calculated using **Equation (8)** as follows:

$$C (\mu\text{mol L}^{-1}) = (6.45 \times A_{532}) - (0.56 \times A_{450}) \quad (8)$$

Where 6.45 is derived from $1/\epsilon \times 1000$, $\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$ is the MDA–TBA adduct's molar extinction coefficient at 532 nm.

MDA content per unit fresh weight was then calculated using **Equation (9)** (Buege & Aust, 1978) as follows:

$$\text{MDA } (\mu\text{mol g}^{-1} \text{ FW}) = C / 155 \times (V_{\text{ext}} / \text{FW}) \times \text{DF} \quad (9)$$

Where C is the MDA concentration in the reaction mixture ($\mu\text{mol L}^{-1}$); V_{ext} is the extraction volume (5 mL); FW is the tissue fresh weight (0.2 g); and DF is the dilution factor (2), applied because the supernatant was combined in equal volumes with the TBA reagent before heating. Results were expressed as $\mu\text{mol g}^{-1} \text{ FW}$.

Proline content assay

Free proline content was determined using the acid-ninhydrin method (Yao et al., 2009) of Bates et al. (1973) with minor modifications (Oral et al., 2025). Fresh tissue was homogenized in 3% sulfosalicylic acid, centrifuged ($10,000 \times g$, 10 min), and the supernatant was reacted with acid-ninhydrin and glacial acetic acid at 100°C for 1 h. After toluene extraction, absorbance was measured at 520 nm, and proline concentration was calculated from an L-proline standard curve (Bates et al., 1973; Oral et al., 2025). The regression equation obtained from the standard curve was determined following **Equation (10)** as follows:

$$y = 0.1219x - 0.0126 \quad (10)$$

Where y is the absorbance measured at 520 nm (dimensionless); and x is the corresponding proline concentration in the reaction mixture ($\mu\text{g mL}^{-1}$). Rearranging to solve for concentration using **Equation (11)** as follows:

$$x (\mu\text{g mL}^{-1}) = (y + 0.0126) / 0.1219 \quad (11)$$

Final proline content per unit fresh weight was calculated following **Equation (12)** (Bates et al., 1973) as follows:

$$\text{Proline } (\mu\text{mol g}^{-1} \text{ FW}) = [x (\mu\text{g mL}^{-1}) \times V_{\text{ext}} (\text{mL})] / [115.5 \times \text{FW} (\text{g})] \quad (12)$$

Where V_{ext} is the total extraction volume (5 mL); FW is the tissue fresh weight (0.2 g); and 115.5 is the molecular weight of L-proline (g mol^{-1}), used to convert mass concentration to molar units. Results were expressed as $\mu\text{mol g}^{-1} \text{ FW}$.

Prior to metal exposure, baseline CAT, POD, APX, electrolyte leakage, total chlorophyll, proline, and MDA were determined, while background Cr and Pb concentrations were quantified in acid-digested oven-dried plant tissues using AAS (Gebretekla et al., 2024).

Calculation of BCF and RE %

The Bioconcentration Factor (BCF) was calculated as the ratio of metal concentration in plant tissue (mg kg⁻¹ DW) to the metal concentration in the culture solution (mg L⁻¹) at the corresponding time point using **Equation (13)** (Cross et al., 2023) as follows:

$$\text{BCF} = C_{\text{plant}} / C_{\text{solution}} \quad (13)$$

Where C_{plant} is the metal concentration in plant tissue (mg kg⁻¹ dry weight) and C_{solution} is the metal concentration in the treatment solution (mg L⁻¹) at the corresponding time point (Cross et al., 2023).

The Removal Efficiency (RE%) was calculated using **Equation (14)** (Arshad et al., 2024) as follows:

$$\text{RE\%} = [(C_0 - C_t) / C_0] \times 100 \quad (14)$$

Where C_0 is the initial metal concentration in solution (mg L⁻¹) and C_t is the metal concentration at time point t (Arshad et al., 2024). This parameter quantifies the percentage reduction in water metal concentration resulting from plant-mediated uptake and bioaccumulation over the experimental period.

Growth response assay

Relative growth rate (RGR) estimation

Relative growth rate was determined by dividing the growth rate in this study by that of the control. Natural logarithms were used to compensate for the exponential growth in time of biological growth, and aquatic macrophyte growth assessment was conducted according to the standard method using **Equation (15)** (Boldrini et al., 2025) as follows:

$$\text{RGR (g g}^{-1} \text{ day}^{-1}) = [\ln(W_2) - \ln(W_1)] / (t_2 - t_1) \quad (15)$$

Where: W_1 and W_2 represent the initial and final fresh weights, respectively; $(t_2 - t_1)$ is the duration of the exposure period in days. Results were expressed as g g⁻¹ day⁻¹ (Boldrini et al., 2025).

Inhibition of root length percentage (IRL%) calculation

Inhibition of root length percentage was calculated as the percentage reduction in root length in each treatment replicate relative to the mean root length of the control group using the approach of Chen et al. (2017) using **Equation (16)** as follows:

$$\text{IRL (\%)} = [(RL_{\text{control}} - RL_{\text{treatment}}) / RL_{\text{control}}] \times 100 \quad (16)$$

Where: RL_{control} is the mean root length of the control group (mm), $RL_{\text{treatment}}$ is the root length recorded for each individual treatment replicate (mm). Results were expressed as percentage inhibition relative to the control.

EII (Enzyme Inhibition Index) estimation

The Enzyme Inhibition Index (EII) was calculated according to Abdullah et al. (2020) to evaluate concentration-dependent changes in antioxidant enzyme activities relative to the control. Enzyme Inhibition Index (EII) was calculated separately for CAT, POD and APX at every treatment concentration following **Equation (17)** as follows:

$$\text{EII} = [(\text{Control activity} - \text{Treatment activity}) / \text{Control activity}] \times 100 \quad (17)$$

Positive values indicate enzyme inhibition relative to the control, whereas negative values indicate enzyme induction.

Statistical analysis

All the experiments were repeated three times ($n = 3$), and the data were reported as mean $\pm$ SD. The normality of the data was tested with Shapiro–Wilk and Anderson–Darling tests. One-way ANOVA and Tukey’s HSD test ($p < 0.05$) were used to assess differences between treatments. Pearson correlation analysis was employed to determine the relationship between metal concentration, physiological responses, activities of antioxidant enzymes and removal efficiency. Two sets of multiple linear regression (OLS) were conducted with the removal efficiency of Cr and Pb as dependent variables, and the determinants of CAT, POD and APX as independent variables. R^2 , adjusted R^2 , regression coefficients, t values, F and p values were used for evaluation of the model performance. The criterion of statistical significance was $\alpha = 0.05$. Data were analyzed and presented graphically in MS Excel 2010 and GraphPad Prism 10.3.

Quality control (QC) and quality assurance (QA)

The analytical accuracy and reliability were achieved by implementing the QA/QC procedures throughout the study. Aiming to minimize contamination, ultrapure (Millipore-Q) water and acid washed / ethanol rinsed glassware were used (Yurtsever & Cüvelek, 2024). Plant samples were processed immediately or stored at -80°C until analysis. All treatments were conducted in triplicate ($n = 3$), and results are reported as mean $\pm$ SD. Cr and Pb concentrations were determined by flame AAS using NIST-traceable standards ($R^2 \geq 0.995$). Analytical quality was assessed using reagent blanks, duplicate analyses, matrix-spike recoveries (90–110%), and certified reference materials where applicable. Method precision was maintained at $\text{RSD} < 10\%$, with procedural blanks and controls included in each analytical batch (Lipps et al., 2023; Parvin et al., 2022).

Results

Physicochemical changes in treatment media before and after the observation period

Table S2 presents the physicochemical characteristics (pH, EC, TDS, salinity, DO, BOD and COD) of the background pond water (Bg), control (Co), composite river water (Ri) and the six synthetic Cr and Pb treatments,

measured immediately after treatment set-up (0 h) and following seven days of exposure, following standard water and wastewater examination protocols (Lipps et al., 2023).

Bioconcentration factor and removal efficiency of *L. minor* under chromium and lead treatment

The efficiency of *L. minor* under (Cr and Pb) stress conditions is shown in **Fig. 3**.

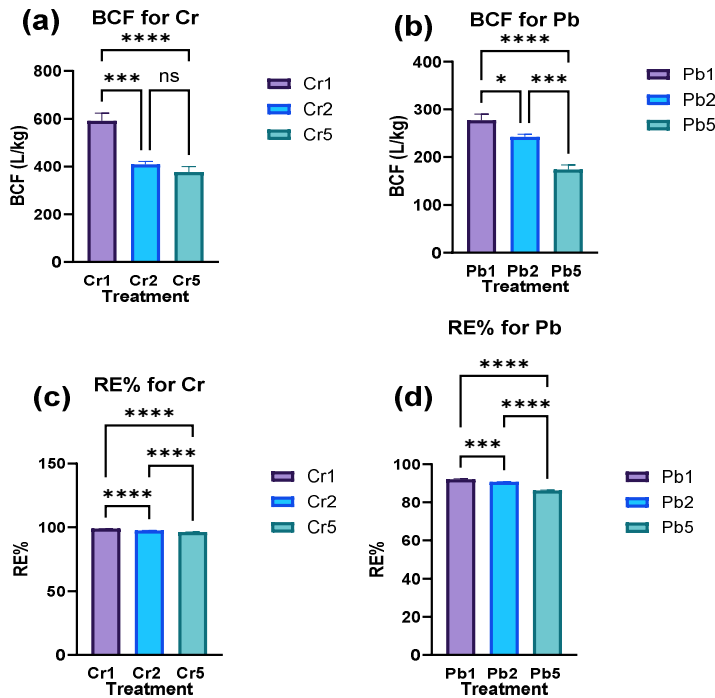


Fig. 3 Bioconcentration factor for (a) chromium and (b) lead across treatment concentrations. Removal efficiency for (c) chromium and (d) lead (Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Growth and photosynthetic responses under Cr and Pb stress

Changes in RGR, IRL%, and chlorophyll of *L. minor* under Cr and Pb exposure are shown in **Fig. 4 and Fig. 5**.

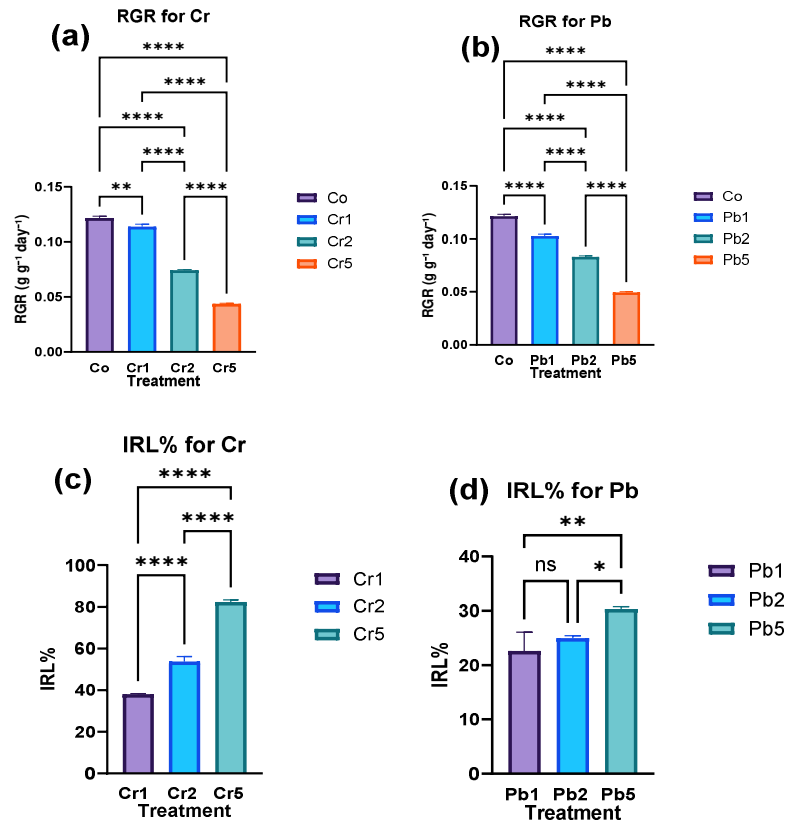


Fig. 4 Relative growth rate decline under (a) chromium and (b) lead with progressive suppression. Root length inhibition showing a severe response under (c) chromium and a mild response under (d) lead (Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

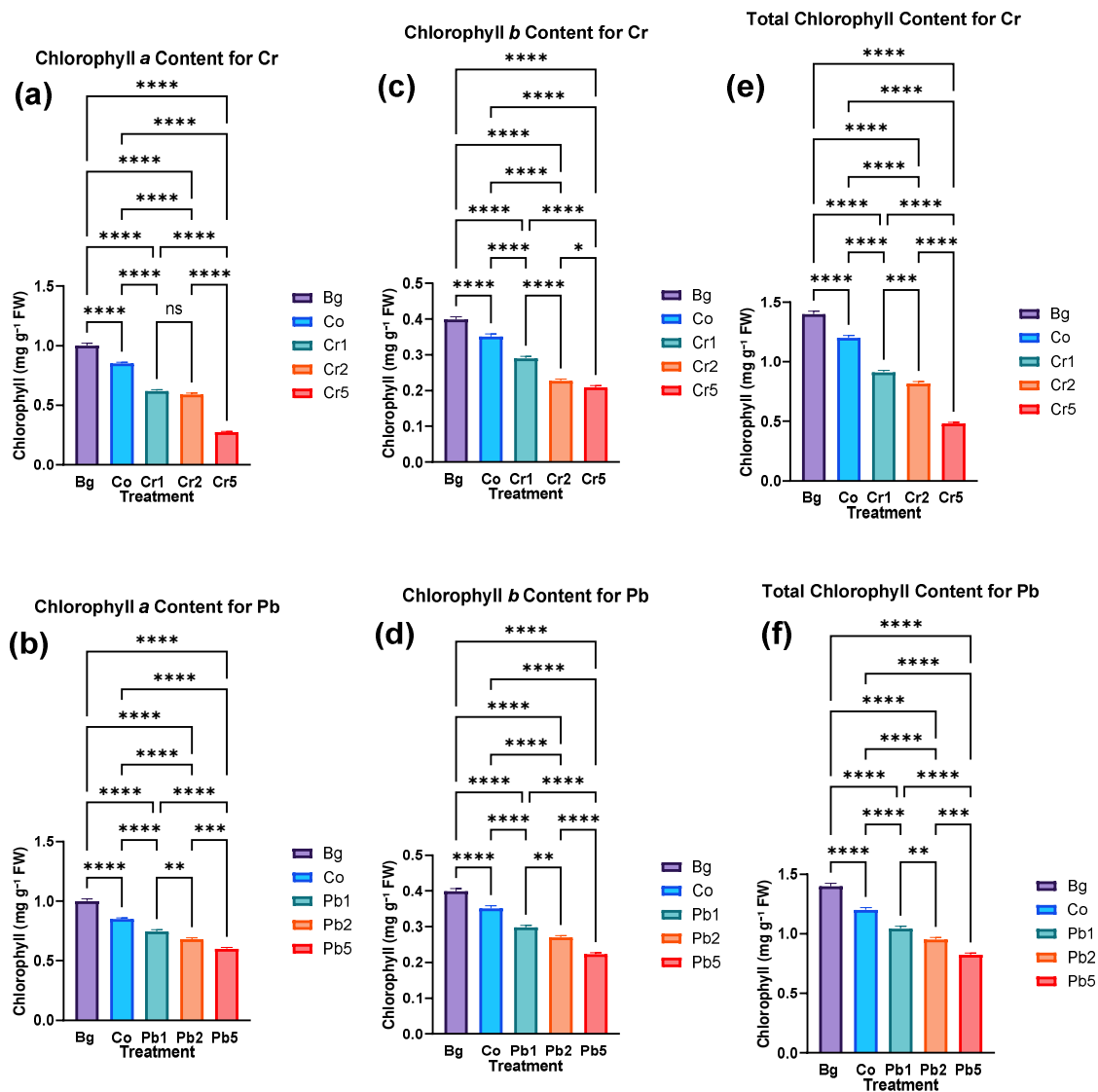


Fig. 5 Chlorophyll *a* content under (a) chromium and (b) lead; chlorophyll *b* content under (c) chromium and (d) lead; total chlorophyll content under (e) chromium and (f) lead (Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Oxidative stress responses under Cr and Pb stress

EL%, proline and MDA responses of *L. minor* under Cr and Pb exposure are shown in **Fig. 6**.

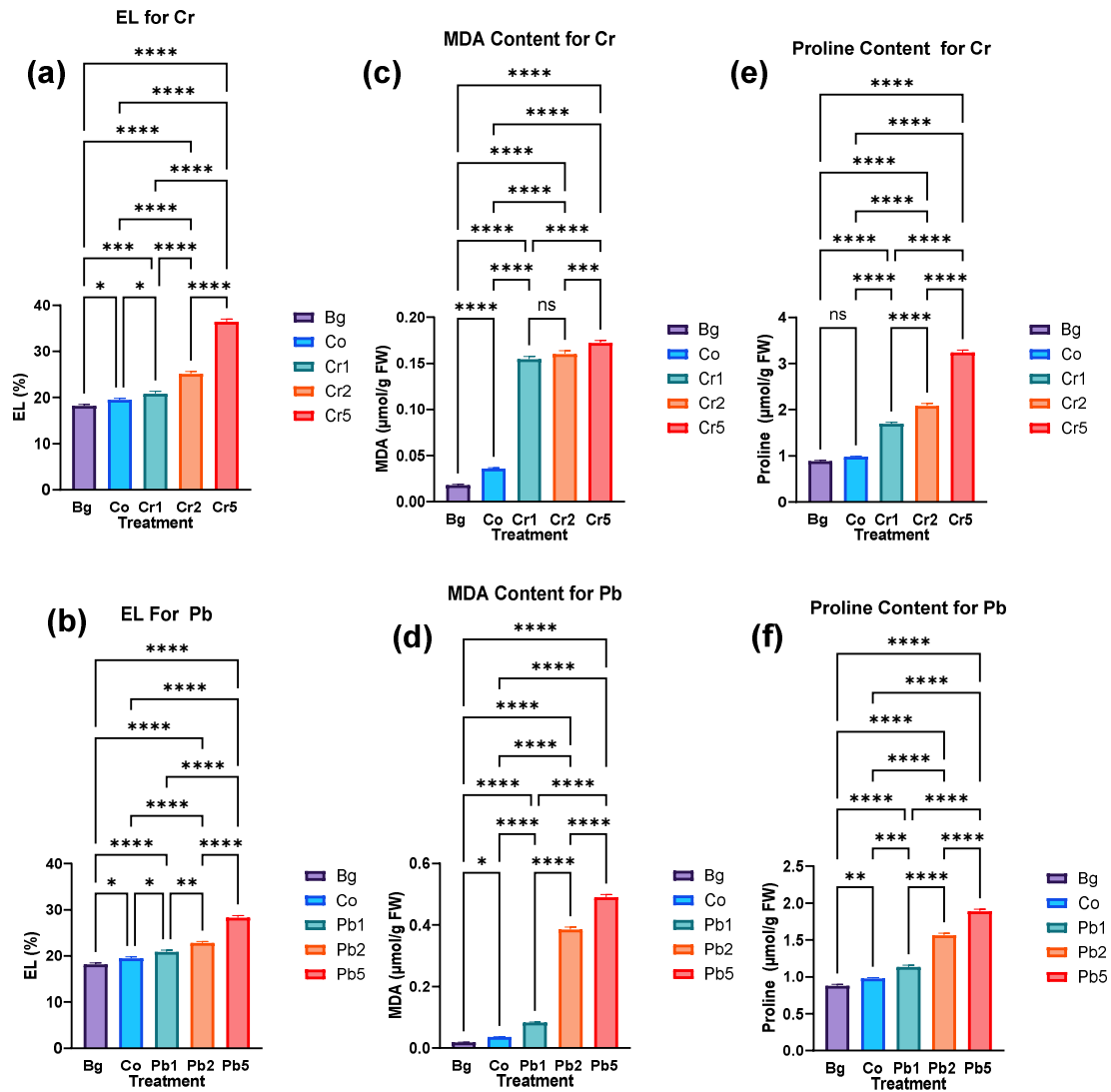


Fig. 6 Electrolyte leakage increasing progressively under (a) chromium and (b) lead; proline accumulation increasing under (c) chromium and (d) lead; malondialdehyde accumulation increasing under (e) chromium and (f) lead (Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Effect of chromium and lead on antioxidant enzymes

Dose-dependent antioxidant enzymatic activity and enzyme inhibition index (EII) under Cr and Pb stress are presented in **Fig. 7** and **Fig. 8**.

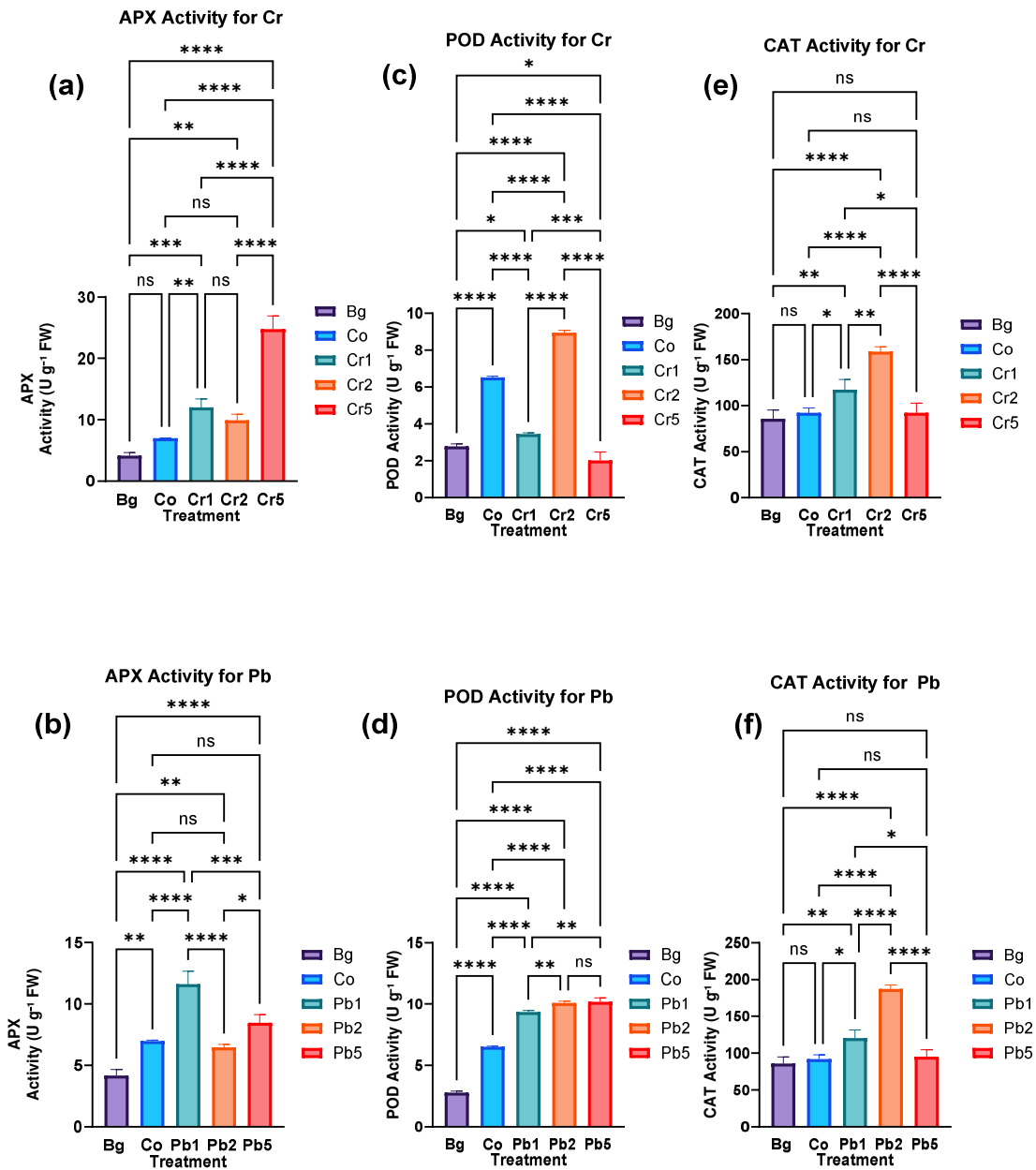


Fig. 7 Catalase activity showing biphasic peak-and-collapse under (a) chromium and (b) lead; peroxidase activity with biphasic response under (c) chromium and sustained induction under (d) lead; ascorbate peroxidase with pronounced induction under (e) chromium and modest response under (f) lead (Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

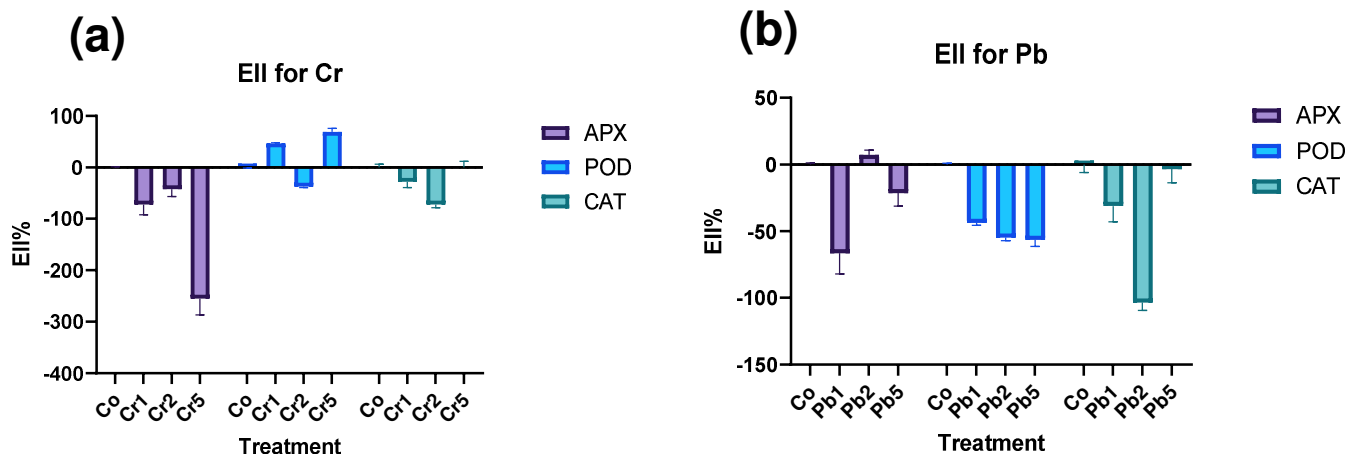


Fig. 8 Enzyme Inhibition Index (EII %) of CAT, POD, and APX across the chromium (a) and lead (b) concentration series. Positive values (above the zero line) indicate inhibition below control activity; negative values (below the zero line) indicate induction above control

The established antioxidant enzyme regulation strategies are metal- and concentration-specific. Under Cr stress, POD alone showed positive EII at Cr5, indicating true inhibition, whereas it remained induced under Pb stress. CAT remained near baseline under both metals. APX showed massive induction under Cr, minimal quantitative response under Pb.

Correlation between metal concentration and enzyme activity for chromium (Cr) and lead (Pb) stress

The pairwise Pearson correlation of metal concentration and enzymatic activity for chromium (Cr) and lead (Pb) stress is shown in **Fig. 9**.

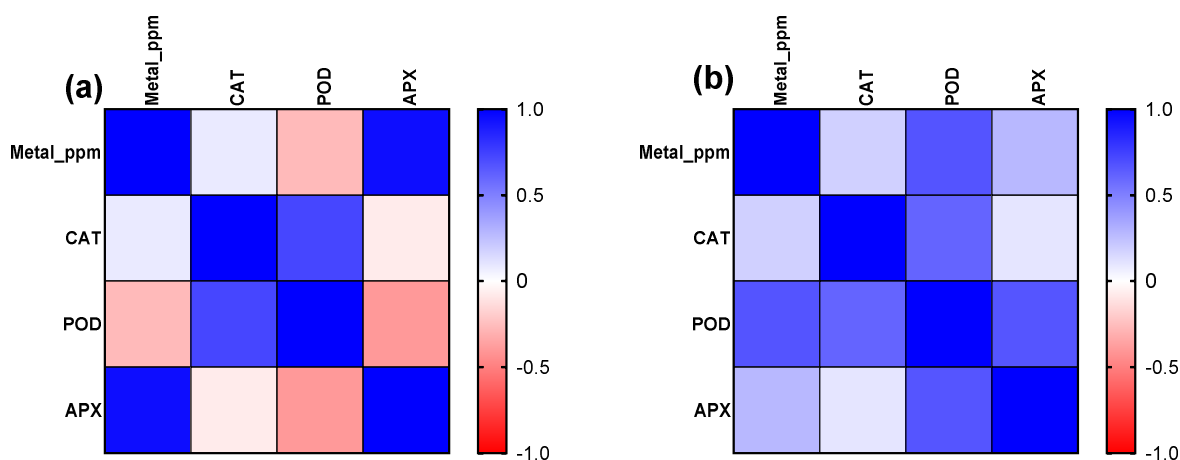


Fig. 9 (a) Metal-enzyme correlations under chromium showing APX dominance; (b) metal-enzyme correlations under lead showing POD dominance (Color intensity represents the strength and direction of the Pearson correlation coefficient (r), with blue indicating positive correlations and red indicating negative correlations)

Correlation of metal concentration and physiological parameters for chromium (Cr) and lead (Pb) stress

The pairwise Pearson correlation of metal concentration and physiological parameters for chromium (Cr) and lead (Pb) stress are shown in **Fig. 10**.

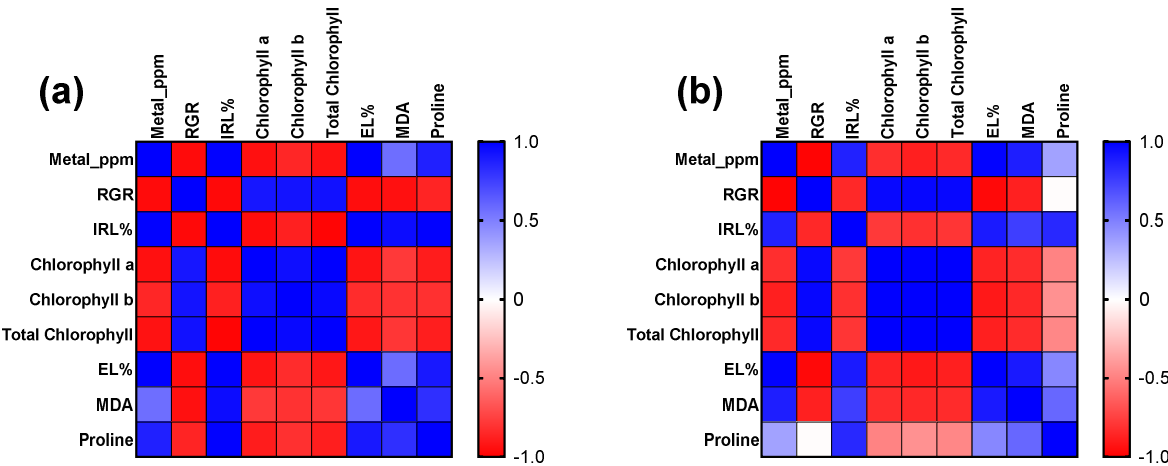


Fig. 10 (a) Metal-physiological correlations under chromium; (b) metal-physiological correlations under lead, distinguishing metal-specific stress response patterns (Color intensity represents the strength and direction of the Pearson correlation coefficient (r), with blue indicating positive correlations and red indicating negative correlations)

Correlation of enzyme activity and physiological parameters for chromium (Cr) and lead (Pb) stress

The pairwise Pearson correlation of enzymatic activity and physiological parameters for Chromium (Cr) and Lead (Pb) stress are shown in **Fig. 11**.

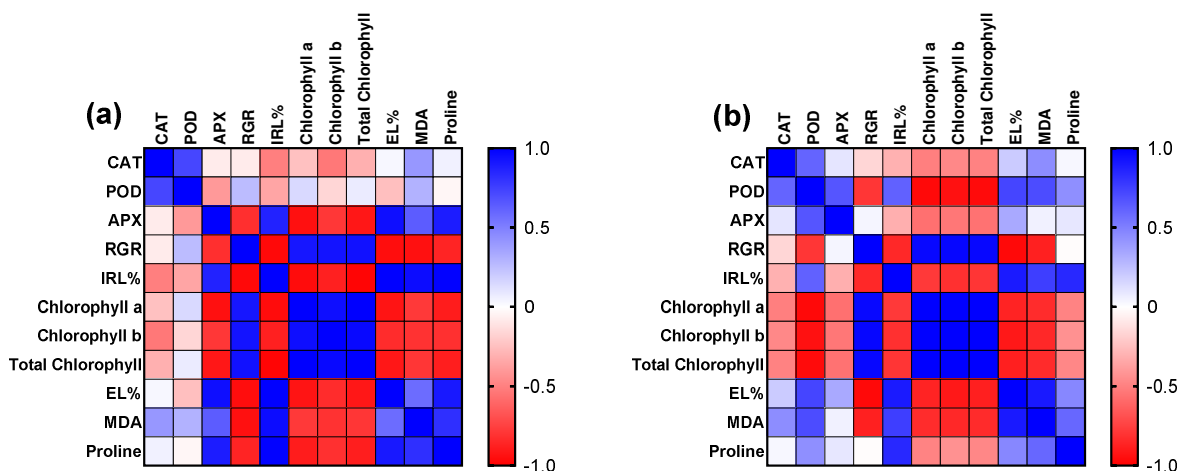


Fig. 11 (a) Enzyme-physiological coupling under chromium with APX showing strongest associations; (b) enzyme-physiological coupling under lead with POD showing strongest associations (Color intensity represents the strength and direction of the Pearson correlation coefficient (r), with blue indicating positive correlations and red indicating negative correlations)

Correlation between enzyme activity and phytoremediation efficiency for chromium (Cr) and lead (Pb) stress

The pairwise Pearson correlation of enzymatic activity and removal efficiency (RE%) are illustrated in the heatmap shown in **Fig. 12**.

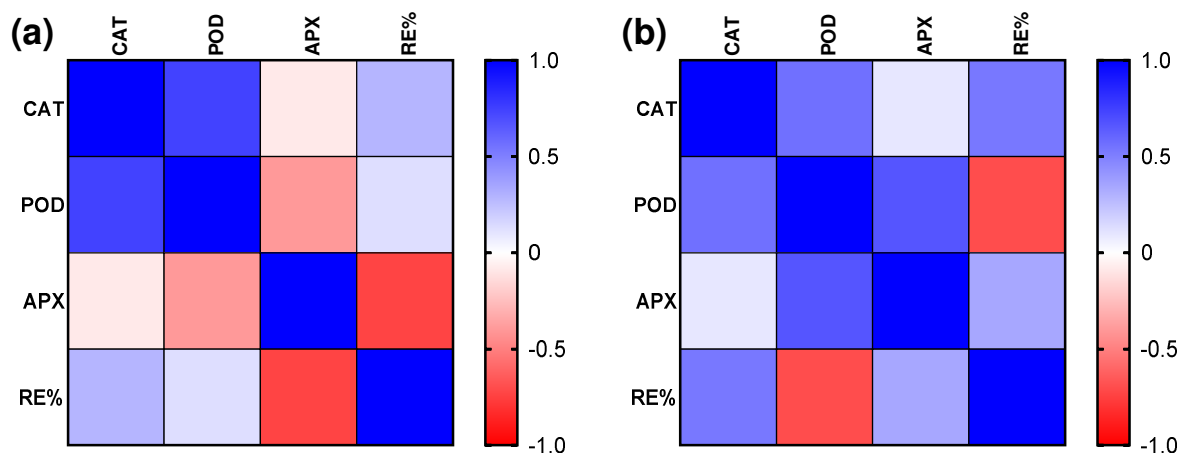


Fig. 12 (a) Enzyme-removal efficiency correlations under chromium showing APX negative association; (b) enzyme-removal efficiency correlations under lead showing POD negative association (Color intensity represents

the strength and direction of the Pearson correlation coefficient (r), with blue indicating positive correlations and red indicating negative correlations)

Multiple linear regression analysis

The predictive accuracy of the developed multiple linear regression models was evaluated by plotting the actual removal efficiencies against the model-predicted values for chromium and lead in **Fig. 13**.

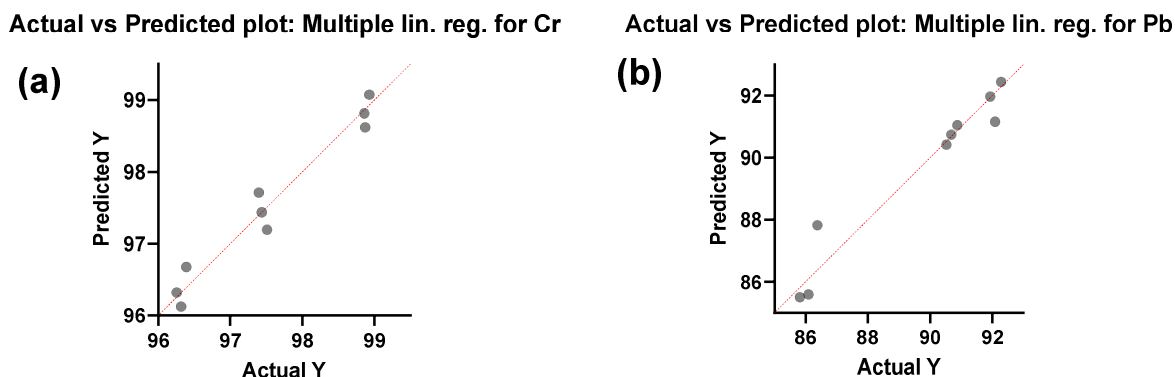


Fig. 13 Observed versus model-predicted (a) chromium removal efficiency (RE%) from the multiple linear regression of CAT, POD, and APX activity ($n = 9$; $R^2 = 0.959$) and (b) lead removal efficiency (RE%) from the multiple linear regression of CAT, POD, and APX activity ($n = 9$; $R^2 = 0.9432$)

Discussion

Physicochemical changes in treatment media before and after observation

Changes in treatment media before and after observation, through physicochemical means. The initial EC was significantly higher in synthetic metal treatments because of the inclusion of Cr and Pb salts, but dropped drastically after 7 days, especially in Cr₂ (59%) and Pb₂ (61%), which agrees with the removal of the metals from the aqueous phase by being taken up and adhered to by the plant (Farid et al., 2022). However, the TDS and COD levels rose due to the presence of dissolved organic compounds associated with membrane damage and tissue degradation caused by heavy metals in the water (Das et al., 2025). This resulted in the efficient removal of metal ions, but due to intracellular leakage of metal ions into the media, further oxidative stress was observed, resulting in an increase in electrolyte leakage and lipid peroxidation as observed (**Table S3**).

Metal removal behavior (RE and BCF)

The different Cr(VI) and Pb responses point to metal-specific uptake and sequestration in *L. minor*. The persistent removal of Cr(VI) could be attributed to its continued uptake and in vivo transformation, while the attenuation in the removal of Pb and its BCF could be linked to increased restrictions on the uptake and

sequestration of Pb^{2+} at higher exposure levels (Arshad et al., 2024; Kushwaha et al., 2024). Therefore, *L. minor* seems to be more stable in terms of Cr(VI) removal than in terms of accumulation of lead in the atmosphere under an increasing metal load (Fig. 3). These responses were similarly strengthened by the river-water treatment when exposed to a mixed-metal combination (Fig. S2c–d).

Growth and photosynthesis (RGR, IRL and chlorophyll)

Cr(VI) caused higher reductions in growth, root development and chlorophyll compared to the other metals, Pb, which is consistent with its higher ROS-generating potential (Gashi et al., 2024; Jorjani & Pehlivan Karakaş, 2024) (Figs. 4 and 5). The higher sensitivity of chlorophyll a indicates a preferential effect on photosynthetic function and pigment biosynthesis, while the relatively small pigment loss from Pb treatment is in line with its known ionic toxicity (Proctor et al., 2022). With increasing disruption of root growth and nutrient acquisition shown by the greater IRL under Cr, the reduced plant growth under Cr is mirrored by the corresponding reduction in root biomass and nutrient uptake. Within the range tested, overall, Cr caused greater physiological stress than Pb, and river-water exposure caused moderate growth impairment, but maintained photosynthetic activity (Table S4; Fig. S2b).

Oxidative stress (EL, MDA, and proline)

EL, MDA, and proline increased with increasing Cr(VI) and Pb exposure ($p < 0.05$), although their response patterns differed between metals (Fig. 6). Cr caused an initial decline in EL at Cr1 followed by a sharp increase at higher concentrations, whereas Pb induced a gradual increase. The subsequent increases in EL and MDA indicate progressive membrane disruption and lipid peroxidation under oxidative stress (Riyazuddin et al., 2022). Proline accumulation was greater under Cr than Pb, suggesting stronger osmotic and redox adjustment under Cr(VI) stress. Overall, these responses indicate increasing cellular stress with metal concentration and a greater oxidative burden under Cr than Pb (Ghosh et al., 2022; Kushwaha et al., 2024). River-water exposure also induced EL, MDA, and proline accumulation, indicating physiological stress under mixed-metal exposure (Table S4).

Antioxidant enzymes (CAT, POD, and APX)

CAT showed a biphasic response under both metals, with induction at intermediate concentrations followed by a decline at the highest dose. POD declined sharply at Cr5 but remained induced under Pb, indicating metal-specific antioxidant responses (Jorjani & Pehlivan Karakaş, 2024; Ziegler et al., 2025). APX showed the strongest response under Cr, increasing progressively to Cr5, whereas Pb induced a non-monotonic response with maximum activity at Pb1 (Fig. 7a–b). These patterns indicate greater reliance on APX under Cr(VI) and sustained POD activation under Pb, demonstrating metal-specific antioxidant regulation in *L. minor* (Naik et al., 2003). Comparable enzyme activities in river water further support antioxidant activation under mixed-metal exposure (Fig. S2a).

Enzyme Inhibition Index (EII)

EII revealed metal-specific patterns of antioxidant enzyme inhibition (Fig. 8). Under Cr(VI), POD showed positive EII at Cr1 and Cr5, indicating inhibition relative to the control, whereas the strongly negative EII of APX, particularly at Cr5, indicated marked induction. CAT remained near or below control levels. Under Pb, POD remained induced, whereas CAT and APX showed variable responses. These contrasting patterns indicate differential regulation of antioxidant enzymes under metal-induced oxidative stress, consistent with the distinct roles and stress-responsive regulation of CAT, POD, and APX in plants (Riyazuddin et al., 2022)

Pearson correlation

Pearson correlation analysis showed metal-specific relationships among metal concentration, physiological responses, antioxidant activity, and removal efficiency (Figs. 9–12; Tables S5–S12). Under Cr(VI), APX was strongly correlated with Cr concentration ($r = 0.947$, $p < 0.001$), while CAT and POD were not. Pb concentration was significantly correlated with POD ($r = 0.668$, $p = 0.009$), but not with CAT or APX. Cr was also strongly associated with EL, IRL, and proline, whereas Pb showed strong associations with EL, MDA, and IRL but not proline. APX and POD were negatively associated with Cr and Pb removal efficiency, respectively (Fig. 12), suggesting that increased enzyme activity primarily reflected stress intensity rather than improved removal (Akoglu, 2018; Schober et al., 2018). Overall, APX predominated under Cr(VI), whereas POD was more responsive under Pb.

Multiple regression

Multiple linear regression indicated metal-specific enzyme predictors of phytoremediation efficiency. Under Cr(VI), APX was the only reliable predictor, while CAT and POD showed high multicollinearity (VIF = 14.91 and 9.90); APX had acceptable collinearity (VIF = 3.66). Its negative association with Cr removal suggests that increased APX reflected greater oxidative stress rather than enhanced uptake (Hasanuzzaman et al., 2020). Under Pb, POD was the strongest enzymatic predictor, with low multicollinearity (VIF = 1.78–4.31), consistent with its role in Pb-induced oxidative and cell-wall responses (Hair et al., 2019; Kushwaha et al., 2024). Thus, enzyme-based prediction of phytoremediation efficiency should be metal-specific rather than universal.

Conclusions

The study supports a metal-specific role of antioxidant enzymes in phytoremediation. APX was the strongest enzymatic indicator under Cr(VI), whereas POD was more closely associated with Pb removal. Thus, no single enzyme appears suitable as a universal proxy for phytoremediation performance in *L. minor*, highlighting the need for contaminant-specific biomarkers. EII further showed that POD crossed a true inhibitory threshold only under Cr(VI) exposure, indicating its greater vulnerability under severe chromium stress.

Limitations of the Study

Limitations of the study were the relatively small sample size ($n = 9$ per metal), the short exposure duration (7 days) and the use of laboratory conditions, which might restrict extrapolation to natural aquatic environments.

Other antioxidant components (SOD, GR, non-enzymatic antioxidants) were not included in biochemical assessment and only limited measurements of CAT, POD, APX, MDA, proline, electrolyte leakage and chlorophyll were performed. Furthermore, the test concentrations of metals (1, 2 and 5 mg L⁻¹) were few, and toxicity thresholds could not be assessed at other concentrations.

Declaration

All authors have read, understood, and complied, as applicable, with the statement on "Ethical responsibilities of Authors" as found in the Instructions for Authors.

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Conflicts of interest/Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and materials

The manuscript has data included as supplementary material.

Authors' contributions

Zarin Raisa: Study concept, Development of methodology, Investigation, Sample collection, Conceptualization, Data calculation, Statistical elucidation, Graphs and Figures preparation, First draft preparation; Md. Arifur Rahman Bhuiyan: Study concept, Development of methodology, Conceptualization, Draft finalization, Review and editing, Overall supervision of the study; Mousona Islam: Data analysis, Laboratory facility support, Data calculation, Writing, Editing, Fateeha Noor: Draft review and editing, Proof read; Kishwar Jahan Sethi: Draft review and Editing; Mohammad Enayet Hossain: Laboratory facility support, Draft review; Sadika Mahbub: Draft review and editing, Proof read; Shanjida Akter Tanju: Draft review and editing; Maimuna Binte Hasan Raisa: Graphs and figures preparation, Draft review; Nabiha Tahsin: Draft review and editing.

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