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# Ecotoxicological impact of copper on the growth rate, pigment and biochemical components in *Lemna minor*

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## Abstract

The study investigated the effects of copper (Cu) stress on pigment levels (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid), total soluble protein, carbohydrate, and proline levels in *Lemna minor L.* under controlled laboratory conditions. Cu treatments were applied at concentrations of 0, 1, 3, 5, and 7 mg /L over a duration of 96 hours. The findings of the study indicated that Cu influenced the growth rate, pigmentation, and biochemical state of *L. minor*. *Lemna minor* exhibited a reduction in growth rate, pigment levels, total soluble protein, and carbohydrate content, alongside an increase in proline concentration following 96 hours of Cu exposure. Increased copper concentrations (3 mg/l, 5 mg/l, and 7 mg/l) lead to reductions in chlorophyll a, total chlorophyll, and carotenoid levels, with chlorophyll b exhibiting the most significant decrease at 7 mg/l. The growth rate declines at concentrations of 5 mg/l and 3 mg/l. The biochemical contents of protein and carbohydrate reach a maximum decrease at a Cu concentration of 7 mg/L, while proline content exhibits an increase with rising Cu concentration. This study's findings indicate that *Lemna minor* is suitable for the phytoremediation of water bodies contaminated with low levels of Cu.

***Keywords**:* Copper, *Lemna minor*, Growth rate, Pigment content, Biochemical components, Phytoremediation.

### Intrduction

Numerous environmental issues are linked to the dispersion and subsequent accumulation of excessive metal trace elements (MTE) in the environment. MTE encompasses toxic metalloids and heavy metals, with copper (Cu) classified within this group (Sall et al. 2020). Furthermore, copper (Cu) poses a significant concern due to its ongoing release into the environment stemming from anthropogenic activities (Panfili et al. 2019). Plants require trace amounts of cationic Cu for metabolic functions, as it is integral to processes that facilitate normal growth and development (Matayoshi et al. 2020). This element is essential for electron transport in respiration and photosynthesis, and it also functions in signaling for protein transcription and iron mobilization (Panfili et al. 2017; Yruela et al. 1996). In contaminated environments, copper can induce negative impacts on plants, including nutritional disorders and alterations in physiological, morphological, and biochemical processes (Adrees et al. 2015). Furthermore, Cu can impede plant growth at minimal concentrations (Adrees et al. 2015). Copper (Cu) can influence photosystem II (PS II), reduce the levels of photosynthetic pigments, and damage thylakoid membranes (Cambrollé et al. 2013; Tanyolaç et al. 2007). Furthermore, copper (Cu) can impede the capacity of plants to assimilate carbon dioxide (CO2), correlating with a reduction in stomatal conductance (Rouphael et al. 2008). These effects may result in stunted growth, reduced biomass, and mortality in plants (Tanyolaç et al. 2007). Copper can induce oxidative stress via the Haber-Weiss and Fenton reactions, resulting in the excessive production of reactive oxygen species (ROS) (Matayoshi et al. 2020). This overproduction can be detrimental to plants, as ROS have the capacity to degrade membranes, lipids, proteins, and nucleic acids (Panfili et al. 2019; Tedeschini et al. 2015). Plants have enzymes and antioxidant molecules that can mitigate the harmful effects of reactive oxygen species (ROS), provided that the cellular capacity to manage oxidative damage is not surpassed (Matayoshi et al. 2020; Sherwood, 2020). *Lemna minor L*., commonly known as duckweed, is garnering attention as a free-floating wild aquatic species. Duckweed is prevalent across multiple continents, noted for its rapid growth and adaptability to adverse environmental conditions, including a broad range of temperature and pH levels. Additionally, it can be cultivated easily under controlled conditions (Del Buono et al. 2020; Regni et al. 2021). This plant exhibits antioxidant properties, leading to its examination in numerous studies (Del Buono et al. 2020). This study examines the importance of *L. minor* in phytoremediation efforts. The capacity for stress tolerance and the accumulation of heavy metals, as well as the uptake and detoxification of organic pollutants, is assessed using copper as a marker for anthropogenic contaminants in constructed wetlands situated between agricultural fields and surface waters. Thus, given the importance of $Cu^{+2,}$ the current study was undertaken to explore the responsiveness and feasibility of $Cu^{+2}$ phytoextraction by *L.minor* (duckweed). In addition, biochemical approaches were used to identify the mechanisms of HMs tolerance induced by $Cu^{+2}$ in *L.minor* by evaluating the roles of key components such as (i) relative growth rate, (ii) pigment content, (iii) protein content, (iv) carbohydrate content, and (v) proline content.

## Materials and methods

### Plant collection site

Fresh duckweed species of *Lemna* (*Lemna minor*) plants were collected from Prayagraj U.P. surface water pond at the Roxburgh Botanical Garden Department of Botany University of Allahabad,Prayagraj,U.P.

### Source and mode of copper treatment

This study utilized copper for heavy metal treatment, administered as $CuCl_2$ (MW = 99.00 g/mol; purity = 99%; Sigma Aldrich). To determine the appropriate dose of Cu, experiments were conducted using various concentrations: 0, 1, 3, 5, and 7 mg/l in the culture media (Hutner's media). These metal doses were administered to the plant samples of *Lemna minor* after 7 days of culturing. The plants were treated with the specified concentrations and observed for 4 days (96 hours) under laboratory conditions. The plants were harvested on the fourth day to assess fresh weight, relative growth rate, photosynthetic pigments, and biochemical changes. The concentrations of Cu employed in this study are relevant to environmental contexts and comparable to those found in aquatic environments.

### Determination of relative growth rate

The relative growth rate (RGR) serves as a useful measure for analyzing growth dynamics of *Lemna minor* over a specified time frame under butachlor stress, quantifying the increase of a quantity per unit of time relative to its initial value (Hoffmann and Poorter, 2002).

### Estimation of photosynthetic pigments

The concentrations of chlorophyll a (chl a), chlorophyll b (chl b), total chlorophyll, and carotenoids were assessed using the colorimetric method and the experimental equation outlined by Lichtenthaler (1987).

### Determination of total soluble protein content

The protein content was determined utilizing Bradford's (1976) method. Proteins were extracted by homogenizing 100 mg of leaves at 4 °C in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and 2% (w/v) polyvinylpyrrolidone (PVP). The protein content was determined by centrifuging the homogenates at 10,000 g for 10 minutes and analyzing the supernatants. To conclude, combine 0.1 ml of the sample solution with 2.5 ml of Coomassie brilliant blue G-250 reagent in a test tube. Allow the mixture to stand at room temperature for two minutes before measuring the absorbance at 595 nm. The total soluble protein content was assessed using bovine serum albumin as a reference protein solution.

### Determination of carbohydrate content

The carbohydrate content of each plant sample was measured according to the method developed by Loewus (1952). A 100 mg plant sample underwent hydrolysis for three hours in a hot water bath with the addition of five milliliters of 2.5N HCl. Upon reaching room temperature, the solution was neutralized with $Na_2CO_3$ until effervescence ceased. The solution was diluted with 100 ml of distilled water and subsequently centrifuged. Heat 4 milliliters of Anthrone reagent and 1 milliliter of supernatant in a boiling water bath for eight minutes. Following cooling, the absorbance of the solution was assessed at 630 nm.

### Estimation of proline content

After crushing 100 mg of fresh leaves in 3% aqueous sulfosalicylic acid and centrifuging at 10,000 g, acid ninhydrin and 3% glacial acetic acid were incorporated. Four milliliters of toluene were extracted by heating samples in a water bath at 95 °C for one hour, followed by cooling and vortexing for 15 seconds with a test tube mixer. The toluene layer was aspirated, and absorbance at 520 nm was measured with toluene serving as a blank (Bates et al. 1973).

## Result and Discussion

### Growth behavior

The observed results indicate that varying copper (Cu) concentrations, ranging from 0 mg/l to 7 mg/l, lead to a declining trend in *Lemna minor's* growth rate; however, the effects are not significantly pronounced (Fig1). At copper (Cu) concentrations of 3, 5, and 7 mg/l, reductions of 6.8% ($P < 0.001$), 14% ($P < 0.001$), and 9.3% ($P < 0.001$) were observed in *Lemna minor*, respectively as shown in figure 2 A.

### Pigment analysis

The influence of varying copper concentration doses, from 0 mg/l to 7 mg/l, on chl a content in *Lemna minor* demonstrates a notable inhibitory effect at Cu concentrations of 3, 5, and 7 mg/l. *Lemna minor* exhibits reductions of 53.22% ($P <0.001$), 73.16% ($P <0.001$), and 81.3% ($P <0.001$). The concentration of copper (Cu) at 7 mg/L results in a significant reduction of 60% in chlorophyll b content ($P < 0.001$) in *Lemna minor*. The total chlorophyll (chl a + chl b) content demonstrates a reduction of 46.2%, 65.1%, and 78.18% at concentrations of 3, 5, and 7 mg/L in *Lemna*, respectively. The effect on carotenoid content at varying Cu concentrations shows a significant reduction at 3, 5, and 7 mg/L. In *Lemna minor*, reductions of 43% ($P < 0.001$), 65% ($P < 0.001$), and 67% ($P < 0.001$) were observed, respectively (fig 2 B).

### Biochemical components

The protein content of *Lemna minor* decreased by 6% ($P <0.001$), 15.37% ($P <0.001$), 17% ($P <0.001$), and 18.29% ($P <0.001$) at copper concentrations of 1, 3, 5, and 7 mg/L, respectively, according to the results shown in Figure 2 C. As illustrated in figure 2 E, the carbohydrate content of the duckweed species *Lemna minor* decreased at varying copper concentrations after 96 hours of treatment. The carbohydrate content increased initially at 1 mg/L copper (Cu) concentration by 17% in *Lemna minor*, and then decreased at copper concentrations of 3,5 and 7 mg/L by 4.3% ($P <0.001$), 15% ($P <0.001$), and 56% ($P <0.001$) in *Lemna minor*, respectively, over control values. Figure 2 D displays the findings of the proline content and copper concentration in the duckweed species *Lemna minor* following 96 hours of treatment. A little increase in proline content has been noted as the concentration of copper (Cu) in *Lemna* has increased from 0 to 7 mg/L. After 96 hours of treatment, it was discovered that the concentration of copper (Cu) in *Lemna minor* increased by 37% (P <0.001), 45% (P <0.001), 56% (P <0.001), and 67% (P <0.001), respectively, at concentrations of 1, 3, 5, and 7 mg/L.

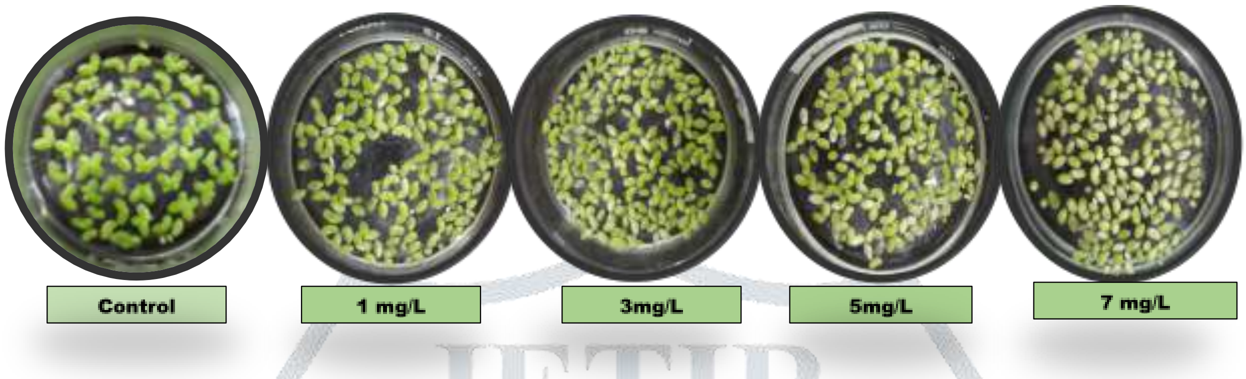


**Fig 1** Effect of different copper concentration on *Lemna minor* after 96 h of incubation period under laboratory condition at 0, 3, 5, 7mg/L concentrations of copper.

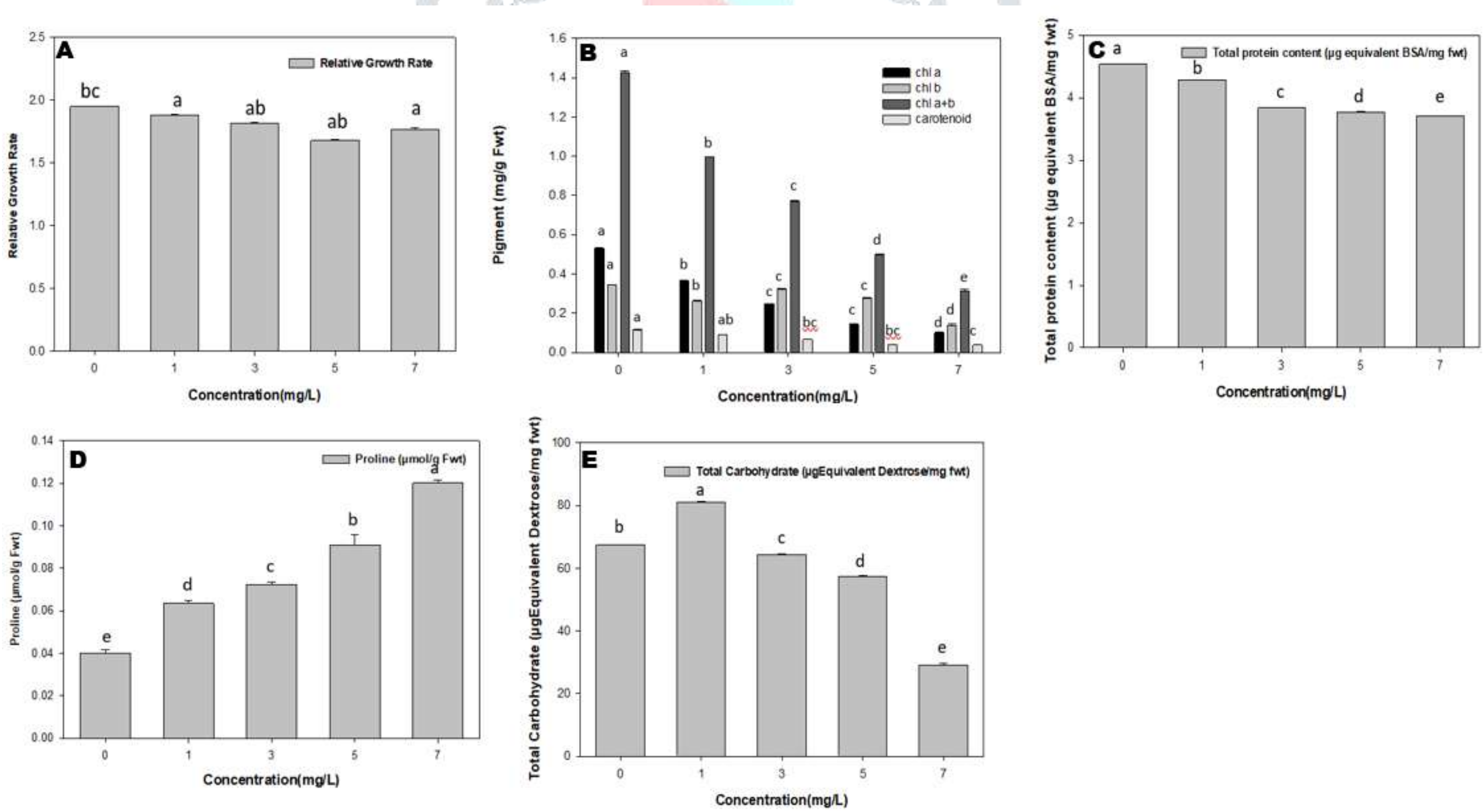


**Fig 2** Effects of increasing Copper concentrations (0–7 mg/L) on Relative growth rate(A), Pigment content (B) (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids) and biochemical parameters (Protein(C), Proline(D) and Carbohydrate(E)) in *Lemna minor* (A, B, C, D, E). Data are means ± SE. Different letters above bars indicate significant differences among treatments ($p < 0.05$).

**Table 1.** Effects of increasing copper concentration on growth, pigment content, biochemical parameters in *Lemna minor*.

| Concentration (mg/L) | Relative Growth Rate ±SE | Chl a ±SE (mg/g FW) | Chl b ±SE (mg/g FW) | Chl (a+b) ±SE (mg/g FW) | Carotenoid ±SE (mg/g FW) | Total Protein ±SE (µg BSA eq./mg FW) | Total Carbohydrate ±SE (µg Dextrose eq./mg FW) | Proline ±SE (µmol/g FW) |
|---|---|---|---|---|---|---|---|---|
| 0 | 1.948 ± 0.003 | 0.529 ±0.004 | 0.343 ±0.000 | 1.427 ±0.009 | 0.113 ± 0.002 | 4.536 ± 0.005 | 67.375 ± 0.144 | 0.040 ± 0.001 |
| 1 | 1.877 ± 0.006 | 0.363 ±0.002 | 0.260 ±0.007 | 0.995 ±0.002 | 0.088 ± 0.000 | 4.281 ± 0.002 | 81.000 ± 0.191 | 0.064 ± 0.001 |
| 3 | 1.815 ± 0.006 | 0.248 ±0.001 | 0.321 ±0.004 | 0.768 ±0.004 | 0.065 ± 0.000 | 3.838 ± 0.001 | 64.417 ± 0.220 | 0.072 ± 0.001 |
| 5 | 1.677 ± 0.006 | 0.142 ±0.002 | 0.277 ±0.002 | 0.498 ±0.005 | 0.040 ± 0.000 | 3.773 ± 0.003 | 57.333 ± 0.182 | 0.091 ± 0.005 |
| 7 | 1.766 ± 0.011 | 0.099 ±0.004 | 0.138 ±0.006 | 0.311 ±0.007 | 0.037 ± 0.000 | 3.706 ± 0.006 | 29.083 ± 0.481 | 0.120 ± 0.001 |

## Statistical analysis

The statistical analysis was performed using SPSS 16 and Sigma Plot 10

## Discussion

Copper exposure markedly reduces the content of photosynthetic pigments in *Lemna minor*. The reductions in chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids at concentrations of 3, 5, and 7 mg/L Cu align with established effects of copper toxicity on the photosynthetic apparatus in aquatic macrophytes (Singh et al. 2022; Miller, 2023), table 1. The interference of copper with chlorophyll synthesis and its role in enhancing pigment degradation via the generation of reactive oxygen species (ROS) is well established in the literature. Heavy metals such as copper catalyze the production of reactive oxygen species (ROS), which compromise chloroplast membrane integrity and interfere with pigment biosynthesis pathways, resulting in pigment loss and diminished photosynthetic efficiency. The observed effects correspond with the significant inhibitory influence on chlorophyll a, a crucial pigment for photosynthesis, underscoring the heightened sensitivity of *L. minor* to copper concentrations as low as 3 mg/L (Frankart et al. 2002; Miller, 2023; Upadhyay & Panda, 2009). Chlorophyll b, an essential accessory pigment for light harvesting, experiences degradation in the presence of elevated copper levels, as demonstrated by a 60% reduction at 7 mg/L Cu. This aligns with research indicating the susceptibility of chlorophyll b due to its proximity to the chloroplast membrane and its differential degradation under metal stress. The observed reduction in total chlorophyll content (46.2%, 65.1%, and 78.18% with increasing Cu doses) supports earlier research on *Lemna* species and other aquatic plants subjected to copper exposure (Kupper et al. 2009; Giannakoula et al. 2021). Carotenoids, which are photoprotective pigments that play a role in antioxidative defense, exhibit significant depletion (43-67% reductions), indicating an overwhelmed antioxidative capacity in *L. minor* under copper stress. The loss of pigment results in heightened vulnerability to photooxidative damage, which exacerbates growth inhibition and chlorosis (Li et al. 2023; Gulmez et al. 2023). Copper (Cu) exposure at concentrations ranging from 3 to 7 mg/L results in a significant yet moderate decrease in the growth of *Lemna minor*, with reductions of 6.8%, 14%, and 9.3%

observed at 3 mg/L, 5 mg/L, and 7 mg/L, respectively (P <0.001). This suggests that elevated copper levels adversely affect this species without inducing catastrophic stress. The observed decline is statistically significant, although it is less severe than the substantial growth inhibition documented in other studies involving much higher concentrations of copper or combined metals (Kanoun-Boulé et al. 2009). The observed percentage declines at 3–7 mg/L align with the range documented for *Lemna minor* in various studies, suggesting that growth inhibition and the onset of oxidative stress typically occur with elevated copper levels, though total growth cessation is not always evident unless concentrations are extreme (Parlak et al., 2016). Elevated copper concentrations have been demonstrated to inhibit protein biosynthesis in *Lemna minor* and related species, primarily as a result of direct metal toxicity and oxidative damage to cellular structures. The current data indicate reductions that are consistent with prior studies, which demonstrated that copper stress significantly decreases protein concentration in *Lemna minor* and similar duckweed species, suggesting impairment of metabolic enzymes and a disruption of normal physiological processes. This effect is attributed to the inhibition of enzyme activities by copper and its interference with nitrogen metabolism (Kanoun-Boulé et al. 2009; Hou et al. 2007). The observed increase in carbohydrate content at low copper exposure (1 mg/L) indicates a short-term, stress-induced metabolic adjustment, likely resulting from enhanced photosynthetic activity or the mobilization of stored carbohydrates as a protective mechanism. As copper concentration increases, the observed declines at elevated doses suggest a disruption of photosynthesis and a reduced capacity for carbohydrate synthesis in plants under significant metal stress. Similar trends have been observed, indicating that low metal doses can temporarily stimulate specific metabolic pathways, whereas excessive copper levels impair normal carbon assimilation and storage by damaging chloroplasts and inhibiting essential Calvin cycle enzymes (Hou et al. 2007; Kellaf et al. 2010). Proline serves as a recognized stress indicator in plants, accumulating as a defensive response to copper toxicity. The significant increase in *Lemna minor* under elevated copper concentrations likely indicates an adaptive response to metal-induced oxidative stress, facilitating osmotic balance, reactive oxygen species scavenging, and the protection of proteins and cellular structures. Recent studies indicate that elevated proline levels are commonly observed in response to heavy metal exposure, consistent with the current data. Increased proline accumulation correlates with improved tolerance to oxidative and osmotic stress induced by metallic ions (Panda, 2008).

## Conclusion

The current work shows that copper (Cu) stress has a significant impact on the physiological and biochemical characteristics of *Lemna minor* L. under controlled laboratory circumstances. Exposure to increasing Cu concentrations for 96 hours resulted in significant reductions in growth rate, pigment content, total soluble protein, and carbohydrate levels, with the most substantial decreases occurring at 7 mg/L. Among the pigments, chlorophyll b demonstrated the highest sensitivity, showing the greatest reduction at elevated Cu levels, while chlorophyll a, total chlorophyll, and carotenoids all decreased significantly, particularly at 3-7 mg/L. Growth performance was significantly compromised at 3 mg/L and 5 mg/L, suggesting the early manifestation of stress effects. In contrast, proline content rose with Cu concentration, indicating its significance in stress adaption and osmoprotection during heavy metal exposure. These reactions combined indicate that increased Cu concentrations cause oxidative and metabolic stress in *L. minor*, lowering photosynthetic efficiency and growth potential. However, the species' capacity to collect and respond to metal stress, as well as its tolerance at lower Cu levels, emphasize its potential usefulness in phytoremediation of aquatic settings contaminated with low to moderate Cu concentrations.

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