

# Phytoremediation Potential of *Azolla microphylla* and *Salvinia molesta* for Copper (Cu) Removal: Effects on Biomass, Antioxidant Activity, and DNA Integrity

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## ARTICLE INFO

### Article history

Received: May 6<sup>th</sup> 2026

Revised: May 19<sup>th</sup> 2026

Accepted: June 2<sup>nd</sup> 2026

### Keywords

*Azolla microphylla*

Copper (Cu)

DNA damage

*Salvinia molesta*

Superoxide dismutase (SOD)

## ABSTRACT

Industrial waste is one of the major sources of environmental pollution due to the presence of heavy metals that pose risks to ecosystems and human health. Copper (Cu) is an essential micronutrient; however, excessive concentrations in aquatic environments can become toxic. Aquatic plants *Azolla microphylla* and *Salvinia molesta* have been recognized for their potential as phytoremediation agents for heavy metal contamination. This study aimed to evaluate the effectiveness of both species in reducing Cu concentration, assessing biomass changes, determining metal accumulation in plant tissues, analyzing Superoxide Dismutase (SOD) enzyme activity, detecting DNA damage through comet assay, and evaluating changes in water quality after treatment. Phytoremediation was conducted using a static system with Cu concentrations of 0, 10, 25, 50, 75, and 100 ppm for 14 days. Data were analyzed using one-way ANOVA at a 95% confidence level. The results showed that *Azolla microphylla* reduced Cu concentration by up to 95.32%, whereas *Salvinia molesta* achieved a reduction of 72.42%. The dry biomass of both plants decreased with increasing Cu concentrations, indicating growth inhibition due to metal toxicity, while Cu accumulation in plant tissues increased, demonstrating the ability of both species to absorb and accumulate metals during the phytoremediation process. SOD enzyme activity did not show significant differences among treatments, suggesting that Cu exposure did not induce severe oxidative stress or that the plants had adapted through alternative antioxidant mechanisms. Comet assay results also revealed no detectable DNA damage in leaf tissues, indicating that cellular stability was maintained throughout the treatment period. In addition, dissolved oxygen (DO) levels increased after 14 days of treatment. These findings indicate that *Azolla microphylla* and *Salvinia molesta* have potential as effective phytoremediators for reducing Cu contamination in aquatic environments through metal uptake and accumulation mechanisms without causing significant cellular damage.

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How to Cite: Astuti, S.N.C.T. & Nugroho, A.P. (2026). Phytoremediation Potential of *Azolla microphylla* and *Salvinia molesta* for Copper (Cu) Removal: Effects on Biomass, Antioxidant Activity, and DNA Integrity. *Journal of Biotechnology and Natural Science*, 6(1), 16-27.

## 1. Introduction

Over the past two to three decades, environmental pollution has emerged as a global issue receiving considerable attention from academics, governments, non-governmental organizations, and the general public (Sembel, 2015). The escalation of industrial and non-industrial activities has led to the increasing release of hazardous substances into the environment, resulting in both direct and

indirect ecological damage. One of the most prevalent contaminants in aquatic ecosystems is heavy metals, including copper (Cu).

Copper is an essential micronutrient that plays a crucial role in plant metabolism. However, excessive accumulation of Cu is toxic and may cause membrane permeability disruption, inhibition of protein synthesis, impairment of enzymatic activity, lipid peroxidation, and oxidative stress, ultimately affecting the redox balance in plants (Chen et al., 2018; Mittler, 2002). A study by Astuti and Nugroho (2022) demonstrated that Cu exposure in *Vigna radiata* significantly inhibited seed germination through increased accumulation of reactive oxygen species (ROS), cellular structural damage, and reduced activity of metabolic enzymes. These findings underscore the high toxicity potential of Cu, even during the early stages of plant development.

Phytoremediation is a plant-based technology utilized to reduce or eliminate heavy metal contaminants from the environment (Talebi et al., 2019). This process operates through mechanisms such as biosorption and bioaccumulation, whereby plants particularly aquatic species are capable of absorbing dissolved and bioavailable metals from aquatic systems (Rahman & Hasegawa, 2011). At the molecular level, this capability is supported by gene regulation involved in metal transport, detoxification, and antioxidant responses. This is evidenced by the findings of Astuti et al. (2023), which showed that *Salvinia molesta* exhibits phytoremediation capacity for Cu through these mechanisms, thereby enhancing plant tolerance to metal stress and reinforcing its potential as a phytoremediator.

One group of plants with considerable potential for phytoremediation is floating aquatic ferns, characterized by rapid growth and high biomass production (Talebi et al., 2019; Soerjani et al., 1987). In addition to *Salvinia molesta*, which has been demonstrated to possess metal remediation capabilities, other species such as *Azolla microphylla* have also shown similar potential. Numerous studies have reported that the genus *Azolla* is effective in absorbing heavy metals such as Pb, Cd, and Ni (Asih & Rachmadiarti, 2019; Naghipour et al., 2018). However, information regarding the capacity of *Azolla microphylla* to absorb Cu remains limited, necessitating further investigation to evaluate its potential and to determine the optimal Cu concentrations for phytoremediation studies. Conversely, if these plants do not function as Cu hyperaccumulators, metal exposure may induce oxidative stress due to excessive accumulation of reactive oxygen species (ROS) and an imbalance between ROS generation and detoxification by the antioxidant system (Mittler, 2002). This condition can lead to cellular damage, including DNA damage. Therefore, analysis of superoxide dismutase (SOD) enzyme activity is essential to evaluate the antioxidant response of plants, while the comet assay serves as a sensitive method for detecting DNA damage caused by oxidative stress (Collins, 2014).

This study provides integrated evidence linking Cu removal efficiency with physiological and genotoxic responses, a combination that is still rarely reported, particularly in floating aquatic ferns. By integrating these aspects, this research offers a more comprehensive understanding of phytoremediation performance, not only in terms of pollutant reduction but also in evaluating the biological impacts on plant systems. Based on these considerations, this study aims to evaluate the potential of *Azolla microphylla* and *Salvinia molesta* in reducing Cu concentrations in contaminated aquatic environments, to analyze changes in plant biomass, to assess oxidative stress levels through SOD enzyme activity and comet assay, and to examine changes in water quality parameters (pH, temperature, and dissolved oxygen) following phytoremediation.

## 2. Methods

This study employed an experimental method using a Completely Randomized Design (CRD) consisting of six Cu concentration treatments (0, 10, 25, 50, 75, and 100 ppm) with three replicates for each plant species, namely *Azolla microphylla* and *Salvinia molesta*. The study included plant acclimatization, preliminary testing, and the main phytoremediation experiment using a static system for 14 days.

The main equipment used included an analytical balance (AND GR-200, accuracy  $\pm 0.01$  g) for weighing samples and chemical materials, a DO meter (Lutron DO-5510) for dissolved oxygen measurement, a digital pH meter (Hanna HI98107) for pH determination, a digital thermometer for temperature measurement, a drying oven (Memmert IN55) for dry biomass analysis, a centrifuge

(Hettich Zentrifugen 320R) for enzyme extract preparation, a UV-Vis spectrophotometer (Thermo Scientific GENESYS™ 10UV) for measuring Superoxide Dismutase (SOD) enzyme activity, as well as a fluorescence microscope and electrophoresis apparatus for *comet assay* analysis.

Research materials consisted of *Azolla microphylla* and *Salvinia molesta* plants, CuSO<sub>4</sub> crystals as the Cu source, extraction buffer, reducing buffer, pyrogallol, *normal melting agarose* (NMA), *low melting agarose* (LMA), lysis solution, alkaline buffer, neutralization buffer, and SYBR Safe stain. The Cu stock solution was prepared using distilled water to minimize the influence of dissolved ions on Cu solubility, speciation, and the reproducibility of phytoremediation treatments. The treatment medium used settled well water prior to experimentation.

Observed parameters included the effectiveness of Cu concentration reduction in water, changes in plant biomass based on total dry weight, Superoxide Dismutase (SOD) enzyme activity, DNA damage assessed using the *comet assay*, water quality parameters (temperature, pH, and dissolved oxygen/DO), and plant morphological changes. Morphological damage indicators included chlorosis (yellowing of leaves), necrosis (leaf tissue death), changes in leaf size or shape, and root or leaf abscission caused by Cu exposure.

The preliminary test was conducted using 25 g of plant material in 5 L of Cu solution with concentration variations ranging from 0.1 to 1000 ppm. Plants were exposed for 14 days under static phytoremediation conditions. Morphological observations were used to determine two safe concentrations that did not cause more than 50% damage, which served as the basis for selecting concentrations in the main experiment. In the main experiment, plants were exposed to six treatments: 0 ppm (control), 10 ppm, 25 ppm, 50 ppm, 75 ppm, and 100 ppm for 14 days, each with three replicates.

Superoxide dismutase (SOD) activity was analyzed for each treatment after 14 days and compared with the control using a modified method of Marklund and Marklund (1974). Approximately 0.2 g of *Azolla microphylla* and *Salvinia molesta* samples were frozen in liquid nitrogen, ground, and homogenized with 1.2 mL of extraction buffer (potassium phosphate buffer pH 7.8; 0.1 mM EDTA; 1% PVP). The homogenate was centrifuged at 15,000 rpm for 20 minutes at 4°C. The pellet was resuspended in 0.8 mL buffer and centrifuged again under the same conditions for 15 minutes. The resulting supernatants were combined as crude enzyme extracts. The reaction mixture consisted of 0.5 mL reducing buffer (Tris-HCl pH 8.2; 1 mM EDTA), 0.5 mL ddH<sub>2</sub>O, 200 µL supernatant, and 50 µL pyrogallol. Absorbance was measured at 325 nm for 3 minutes using a spectrophotometer, with pyrogallol solution as the blank.

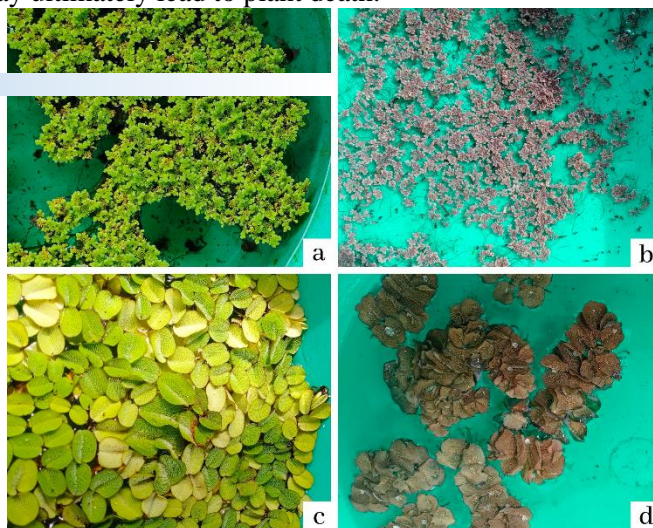
DNA damage was assessed using the comet assay after 14 days of exposure, with the control serving as a comparison, using cell suspensions derived from the SOD preparation supernatant. For base layer preparation, 1% normal melting agarose (NMA) in 1× PBS was heated until clear and maintained in a water bath. Microscope slides were dipped into the NMA until two-thirds of the surface was coated and then incubated in a humid chamber at 4°C overnight. Subsequently, 0.75% low melting agarose (LMA) in 1× PBS was heated to 60–70°C until clear, mixed with the cell suspension (90 µL LMA + 10 µL cells), and layered onto the NMA-coated slides. Slides were covered with coverslips and incubated in a humid chamber at 4°C for 4 hours. Coverslips were then removed, and slides were immersed in cold lysis solution (2.5 M NaCl; 100 mM EDTA; 10 mM Tris; 1% Triton X-100; 10% DMSO; pH 10–10.5) at 4°C overnight. Horizontal electrophoresis was performed in alkaline buffer (300 mM NaOH; 1 mM EDTA; pH 12.6) at 25 V and 300 mA for 30 minutes. Slides were subsequently rinsed with neutralization buffer (400 mM Tris; pH 7.5) and stored in a humid chamber prior to staining with SYBR Safe. Observations were conducted using a fluorescence microscope (10×100 magnification) equipped with a green filter and Cytovision 3.6 software, and analyzed using Comet Score Pro to determine tail intensity, tail moment, and tail length.

The observed parameters included the percentage reduction of Cu concentration, total plant dry weight, SOD activity, comet assay results, and water quality parameters (pH, temperature, and dissolved oxygen). Data were analyzed using one-way ANOVA at a 95% confidence level. When significant differences were detected, Duncan's Multiple Range Test (DMRT) was applied to compare differences among treatments.

### 3. Results and Discussion

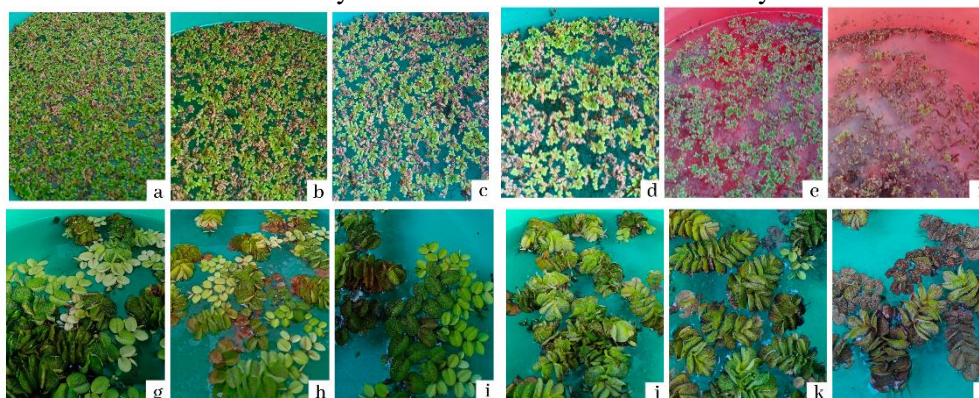
#### 3.1. Results of Preliminary and Main Experiments

Copper (Cu) heavy metal solutions imposed stress on *Azolla microphylla* and *Salvinia molesta*, resulting in toxic effects on both plant species. The preliminary experiment was conducted to determine concentrations that did not induce toxic effects in these species. The results after 14 days of exposure indicated that at a concentration of 1000 ppm, both *Azolla microphylla* and *Salvinia molesta* exhibited leaf discoloration, inhibited growth, thinning of leaves, structural damage, and shortened roots, ultimately indicating plant mortality due to Cu toxicity (**Figure 1**). These findings are consistent with the report of Mangkoediharjo and Samudro (2010), which states that heavy metals at certain concentrations may stimulate plant growth; however, at elevated concentrations, they inhibit growth and may ultimately lead to plant death.



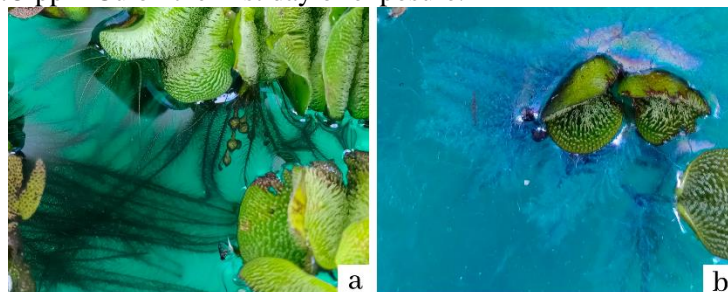
**Figure 1.** Effects of Cu exposure on (a) *Azolla microphylla* (control), (b) *Azolla microphylla* (1000 ppm), (c) *Salvinia molesta* (control), and (d) *Salvinia molesta* (1000 ppm) following 14 days of treatment in the preliminary experiment.

In the main experiment, after 14 days of exposure, both species demonstrated the ability to uptake Cu, with morphological conditions that still allowed relatively sustained growth (**Figure 2**). The selected concentrations were based on previous studies, in which *Azolla microphylla* and *Azolla filiculoides* exhibited optimal growth at Pb concentrations of 5–10 ppm, Cd at 5 ppm, and Ni at 10 ppm (Asih & Rachmadiarti, 2019; Naghipour et al., 2018). Although literature regarding Cu uptake by *Azolla microphylla* and *Salvinia molesta* remains limited, various species within the genera *Azolla* and *Salvinia* are known to effectively absorb and accumulate other heavy metals.



**Figure 2.** Effects of Cu on (a) *Azolla microphylla* (control), (b) *Azolla microphylla* (10 ppm), (c) *Azolla microphylla* (25 ppm), (d) *Azolla microphylla* (50 ppm), (e) *Azolla microphylla* (75 ppm), (f) *Azolla microphylla* (100 ppm), (g) *Salvinia molesta* (control), (h) *Salvinia molesta* (10 ppm), (i) *Salvinia molesta* (25 ppm), (j) *Salvinia molesta* (50 ppm), (k) *Salvinia molesta* (75 ppm), and (l) *Salvinia molesta* (100 ppm) in the main experiment after 14 days of exposure.

The process of metal uptake and accumulation in plants occurs through three continuous stages, namely metal absorption by the roots, translocation to other plant parts, and localization of metals within specific cells as a protective mechanism to prevent disruption of plant metabolism (Setyaningsih, 2007). In this study, Cu uptake by the roots of *Salvinia molesta* was clearly observed, as shown in **Figure 3**, which illustrates the morphological differences between control plants and plants exposed to 75 ppm Cu on the first day of exposure.



**Figure 3.** Effects of Cu on the roots of (a) *Salvinia molesta* (control) and (b) *Salvinia molesta* (75 ppm) in the main experiment on day 1.

Based on these observations, *Salvinia molesta* is presumed to possess the ability to absorb  $\text{Cu}^{2+}$  ions through direct contact with the aquatic medium. Plants require essential nutrients for metabolic processes such as photosynthesis and respiration; therefore,  $\text{Cu}^{2+}$  ions from  $\text{CuSO}_4$  solutions can be absorbed through the roots and subsequently stored in root tissues or translocated via the xylem to other parts of the plant (Prasad, 2003). The movement of ions from the solution to the root surface occurs through diffusion, defined as the movement of ions from a region of higher concentration to a region of lower concentration (Sutarman & Miftahurrokhmat, 2019). Upon reaching the root surface,  $\text{Cu}^{2+}$  ions enter the plant through ion exchange mechanisms, in which  $\text{H}^+$  ions on the root surface are replaced by metal ions. In addition, heavy metal uptake is also mediated by specific transporters in the root plasma membrane, such as the ZIP protein family involved in the uptake of  $\text{Zn}^{2+}$  and  $\text{Fe}^{2+}$ , and the NRAMP family, which is responsible for the transport of various divalent metal ions including Cu, Ni, Zn, and Mn (Ali et al., 2013).

The response of *Azolla microphylla* to Cu exposure differed from that of *Salvinia molesta*. In *Azolla microphylla*, root shortening and detachment were observed, while leaves exhibited chlorosis symptoms. These changes represent adaptive responses to environmental stress caused by heavy metal contamination (Asih & Rachmadiarti, 2019). According to Palar (2012), damage induced by heavy metal exposure commonly includes necrosis and chlorosis in both root and leaf tissues, as observed in *Azolla microphylla* in this study.

### 3.2. Reduction of Cu Heavy Metal Concentration in Water

The reduction of Cu concentration during phytoremediation (Table 1) showed that *Azolla microphylla* and *Salvinia molesta* were able to decrease Cu levels across all treatments. *Azolla microphylla* exhibited the highest reduction efficiency of 95.32% at 50 ppm, while *Salvinia molesta* achieved a maximum reduction of 72.42% at 10 ppm and 100 ppm. This variation in removal efficiency is consistent with the statement of Talebi et al. (2019), who reported that phytoremediation effectiveness is influenced by the level of metal contamination, plant growth capacity, biomass production, and the developmental stage of the plant.

**Table 1.** Cu reduction during 14 days of phytoremediation by *Azolla microphylla* and *Salvinia molesta*

| Treatment<br>Cu (ppm) | Percentage Reduction (%)  |                          |
|-----------------------|---------------------------|--------------------------|
|                       | <i>Azolla microphylla</i> | <i>Salvinia molesta</i>  |
| 0                     | 0.00±0.00 <sup>a</sup>    | 0.00±0.00 <sup>a</sup>   |
| 10                    | 92.83±5.61 <sup>cd</sup>  | 72.42±17.05 <sup>b</sup> |
| 25                    | 85.71±1.15 <sup>b</sup>   | 69.14±21.44 <sup>b</sup> |
| 50                    | 95.32±2.02 <sup>d</sup>   | 51.51±25.13 <sup>b</sup> |
| 75                    | 84.43±5.79 <sup>d</sup>   | 55.41±32.42 <sup>b</sup> |
| 100                   | 87.22±6.39 <sup>bc</sup>  | 72.42±9.72 <sup>b</sup>  |

Note : Values are presented as mean. Different letters within each column indicate significant differences according to Duncan's Multiple Range Test (DMRT) at a 95% confidence level.

Several studies have demonstrated the high potential of *Azolla* species as phytoremediators of heavy metals. *Azolla filiculoides* has been reported to remove Ni, Cd, and Pb by 76.82%, 92.84%, and 97.12%, respectively (Naghypour et al., 2018). *Azolla pinnata* is capable of absorbing 70–94% of Cd (Rai, 2008). Furthermore, Jafari et al. (2010) reported that *Azolla microphylla* exhibits removal efficiencies of 94% for Pb and 98% for Zn.

In the present study, the water used consisted of a  $\text{CuSO}_4$  solution; however, the reduction values refer to Cu ion concentrations measured using atomic absorption spectroscopy (AAS). Copper is an essential heavy metal required by organisms in trace amounts, becoming toxic when present in excess. In plants, Cu is absorbed in the form of  $\text{Cu}^{2+}$ , with an optimal sufficiency level of approximately 6 ppm (Lakitan, 2015). Excess concentrations may disrupt metabolic processes due to its toxic potential.

The reduction of Cu concentration in the aquatic medium is presumed to result from uptake by both plant species through phytoabsorption, a form of phytoextraction. This mechanism involves metal uptake via the roots followed by translocation and accumulation within plant tissues (Alkorta et al., 2004). After absorption as  $\text{Cu}^{2+}$  ions, the metal may form organic complexes such as Cu–EDTA and Cu–DTPA, and subsequently accumulate in roots, stems, and leaves (Rosmarkam & Yuwono, 2002). Copper exhibits a strong affinity for cell wall components and is capable of catalyzing the formation of hydroxyl radicals (OH); therefore, its excessive presence can interfere with normal physiological functions in plants.

### 3.3. Effect of Cu Exposure on Total Plant Dry Biomass

The results of total dry biomass measurements of *Azolla microphylla* and *Salvinia molesta* after 14 days of exposure (**Table 2**) indicate that both species exhibited a decrease in biomass with increasing Cu concentrations. The control treatment (0 ppm) produced the highest dry biomass in both plant species, indicating optimal photosynthetic conditions in the absence of metal stress. Increasing Cu concentrations led to a reduction in biomass due to metal accumulation within plant tissues, which inhibited growth and metabolic activity. This finding is consistent with Yruela (2005), who reported that plants exposed to excess Cu generally exhibit reduced biomass and chlorosis due to Cu toxicity at concentrations ranging from 20–30 ppm within plant tissues.

**Table 2.** Total dry biomass after 14 days of phytoremediation by *Azolla microphylla* and *Salvinia molesta*

| Treatment<br>Cu (ppm) | Total Plant Dry Biomass (% DM) |                         |
|-----------------------|--------------------------------|-------------------------|
|                       | <i>Azolla microphylla</i>      | <i>Salvinia molesta</i> |
| 0                     | 5.20±0.17 <sup>a</sup>         | 5.04±1.69 <sup>a</sup>  |
| 10                    | 4.04±1.26 <sup>bc</sup>        | 4.63±0.44 <sup>ab</sup> |
| 25                    | 4.19±0.52 <sup>b</sup>         | 4.32±0.35 <sup>bc</sup> |
| 50                    | 4.43±0.33 <sup>c</sup>         | 4.87±0.33 <sup>bc</sup> |
| 75                    | 3.97±0.19 <sup>bc</sup>        | 4.96±0.59 <sup>c</sup>  |
| 100                   | 3.33±0.15 <sup>d</sup>         | 4.61±0.40 <sup>c</sup>  |

Note : Values are presented as mean. Different letters within each column indicate significant differences according to Duncan's Multiple Range Test (DMRT) at a 95% confidence level.

The decline in biomass observed in both species indicates a response to Cu stress during the phytoremediation process, as reflected by the reduction of Cu concentration in the water. *Azolla microphylla* exhibited a greater decrease in biomass than *Salvinia molesta*, suggesting possible differences in tolerance to Cu exposure between species. These differences in response may be influenced by variations in physiological mechanisms, adaptation capacity to metal stress, and species-specific growth characteristics (Patnaik & Mohanty, 2013).

The effectiveness of phytoremediation is influenced by the ability of plants to maintain growth under metal exposure while simultaneously reducing pollutant concentrations in the environment (Ali et al., 2013). In this study, the reduction of Cu concentration in water accompanied by decreased biomass indicates that Cu exposure exerted stress on plant growth during the phytoremediation process. Nevertheless, both species were able to reduce Cu concentrations in the treatment media, suggesting their potential use as phytoremediators for Cu heavy metal contamination. However, further analysis of metal content in plant tissues is still required to directly confirm the mechanisms of Cu uptake and accumulation.

### 3.4. Effect of Cu Exposure on SOD Enzyme Activity

The SOD enzyme activity values of *Azolla microphylla* and *Salvinia molesta* after 14 days of exposure (Table 3) exhibited fluctuations without significant differences among treatments. Increasing Cu concentrations did not produce a consistent pattern of increase or decrease in SOD activity, indicating that the response of both plant species to Cu stress was not clearly reflected through SOD enzyme activity.

**Table 3.** Mean superoxide dismutase (SOD) enzyme activity after 14 days of phytoremediation treatment using *Azolla microphylla* and *Salvinia molesta*

| Treatment<br>Cu (ppm) | SOD enzyme activity (unit/mL) |                           |
|-----------------------|-------------------------------|---------------------------|
|                       | <i>Azolla microphylla</i>     | <i>Salvinia molesta</i>   |
| 0                     | 90.12±11.73 <sup>a</sup>      | 110.22±12.17 <sup>a</sup> |
| 10                    | 101.98±9.71 <sup>a</sup>      | 107.90±13.81 <sup>a</sup> |
| 25                    | 83.72±13.58 <sup>a</sup>      | 107.15±5.49 <sup>a</sup>  |
| 50                    | 91.28±2.42 <sup>a</sup>       | 117.78±9.74 <sup>a</sup>  |
| 75                    | 94.76±1.71 <sup>a</sup>       | 119.21±5.43 <sup>a</sup>  |
| 100                   | 90.05±5.07 <sup>a</sup>       | 90.94±5.36 <sup>a</sup>   |

Plant responses to heavy metal exposure generally include physiological disturbances such as reduced biomass, altered water balance, and chlorosis, as well as biochemical responses in the form of increased production of reactive oxygen species (ROS) (Parmar et al., 2013). ROS are naturally generated within cells; however, their levels increase markedly under stress conditions, including Cu-induced stress. To neutralize ROS, plants activate antioxidant defense mechanisms, both enzymatic (e.g., SOD, CAT, GPx) and non-enzymatic (Wang et al., 2004; Sheilaadji et al., 2019). SOD acts as the first line of defense by catalyzing the dismutation of superoxide radicals ( $O_2^-$ ) into  $H_2O_2$ , which is subsequently detoxified by other enzymes. Due to its rapid reaction, intracellular SOD levels may fluctuate, resulting in variable measurement outcomes.

The absence of significant differences in SOD activity observed in this study suggests that the level of oxidative stress experienced by the plants may not have reached the threshold required to induce a pronounced increase in enzyme activity. In addition, the plant antioxidant system operates in an integrated manner, allowing other enzymes such as catalase (CAT) and peroxidase (POD) to play a more dominant role in ROS detoxification, particularly in removing  $H_2O_2$ , thereby reducing the need for increased SOD activity (Gill & Tuteja, 2010; Hasanuzzaman et al., 2012).

Several studies have reported that SOD activity in plants exposed to Cu stress may increase during the early phase of stress and subsequently decline after a certain period, as observed in *Medicago sativa* (Chen et al., 2018) and *Hydrilla verticillata* (Srivastava et al., 2006). This finding highlights that changes in SOD activity are dynamic and strongly influenced by exposure duration, metal concentration, and the specific plant organ analyzed. Therefore, observations at day 14 in this study likely represent an adaptation phase in which SOD activity has stabilized following the initial stress response.

Although SOD activity did not show significant differences among treatments, the presence of enzymatic activity still indicates the operation of antioxidant defense mechanisms to maintain cellular stability (Alscher et al., 2002). However, since no nutrients were added to the treatment media, the observed SOD response cannot be conclusively attributed solely to Cu exposure and may also have been influenced by nutrient limitation affecting plant metabolism.

### 3.5. Effect of Cu Exposure on DNA Damage

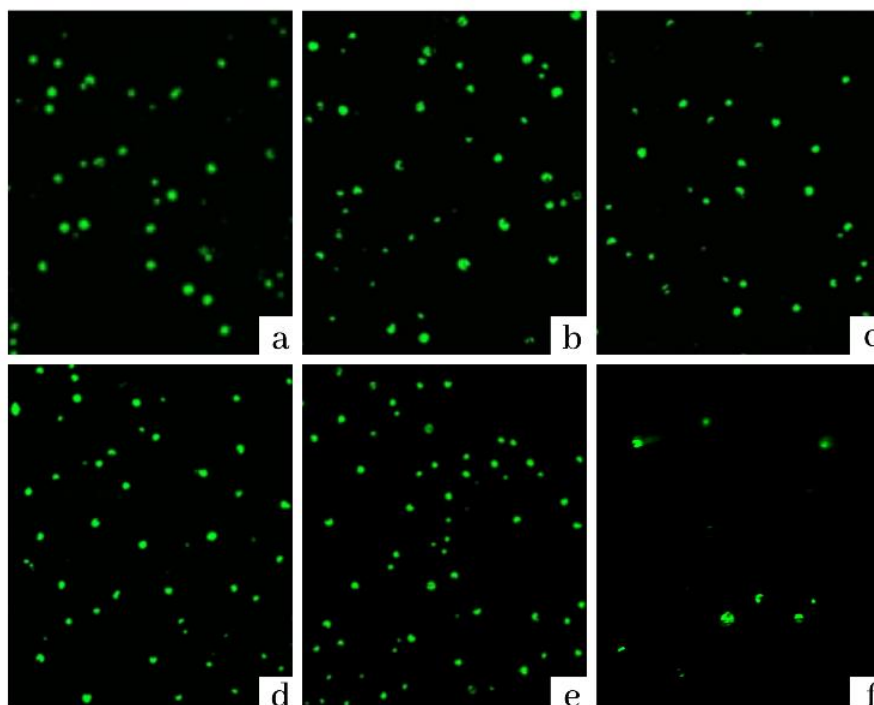
DNA damage analysis in *Azolla microphylla* and *Salvinia molesta* was conducted both qualitatively through the observation of comet morphology and quantitatively using Comet Score software based on three main parameters: tail length, percentage of DNA in the tail (tail intensity), and tail moment (Knopper & McNamee, 2008).

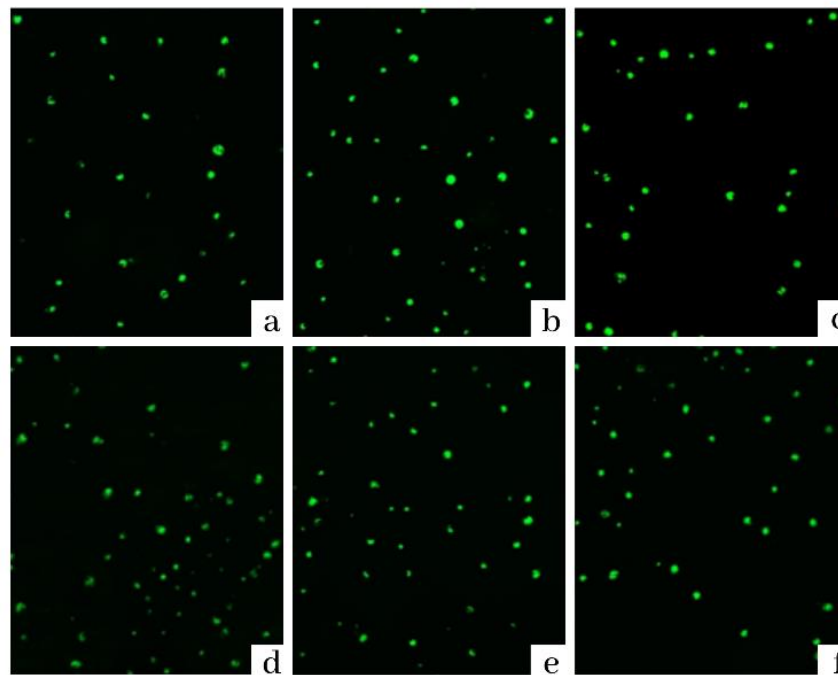
**Table 4.** Mean values of 50 comet assay cells in *Azolla microphylla* and *Salvinia molesta* after 14 days of phytoremediation treatment.

| Treatment<br>Cu (ppm) | Plant                     | Tail Length | Tail Intensity<br>(%) | Tail<br>Moment |
|-----------------------|---------------------------|-------------|-----------------------|----------------|
| Control               | <i>Azolla microphylla</i> | 0           | 2.00±0.95             | 0              |
|                       | <i>Salvinia molesta</i>   | 0           | 2.00±1.53             | 0              |
| 0                     | <i>Azolla microphylla</i> | 0           | 2.00±1.51             | 0              |
|                       | <i>Salvinia molesta</i>   | 0           | 2.00±1.77             | 0              |
| 10                    | <i>Azolla microphylla</i> | 0           | 2.00±1.02             | 0              |
|                       | <i>Salvinia molesta</i>   | 0           | 2.00±1.53             | 0              |
| 25                    | <i>Azolla microphylla</i> | 0           | 2.00±1.08             | 0              |
|                       | <i>Salvinia molesta</i>   | 0           | 2.00±1.49             | 0              |
| 50                    | <i>Azolla microphylla</i> | 0           | 2.00±1.73             | 0              |
|                       | <i>Salvinia molesta</i>   | 0           | 2.00±1.33             | 0              |
| 100                   | <i>Azolla microphylla</i> | 0.02±0.14   | 2.00±1.31             | 0.001±0.01     |
|                       | <i>Salvinia molesta</i>   | 0           | 2.00±1.42             | 0              |

The observation of 50 cells in each treatment indicated that all DNA damage parameters remained at very low levels and showed no significant differences across Cu concentrations, except for the presence of a single comet observed in *Azolla microphylla* at 100 ppm Cu. The *tail intensity* remained constant at 2.00%, which is likely a baseline value resulting from electrophoresis conditions rather than actual DNA damage. According to Knopper and McNamee (2008), increasing metal concentrations are expected to elevate the percentage of DNA in the tail; however, this trend was not observed in either species.

Visualization of the comet assay revealed that most cells retained a round shape without a comet tail (Figures 4 and 5). This indicates the absence of DNA migration due to strand breaks, suggesting that DNA damage did not occur in the majority of treatments. Only *Azolla microphylla* at 100 ppm exhibited a single comet out of 50 observed cells, indicating that high Cu concentration may begin to induce genotoxic effects in a small proportion of cells.

**Figure 4.** Effects of Cu on DNA damage in leaves of *Azolla microphylla* (a) control, (b) 10 ppm, (c) 25 ppm, (d) 50 ppm, (e) 75 ppm, and (f) 100 ppm after 14 days of exposure.



**Figure 5.** Effects of Cu on DNA damage in leaves of *Salvinia molesta* (a) control, (b) 10 ppm, (c) 25 ppm, (d) 50 ppm, (e) 75 ppm, and (f) 100 ppm after 14 days of exposure.

The absence of DNA damage in the majority of cells indicates that both plant species possess effective cellular protection mechanisms. In addition to active DNA repair systems (Mahaputra & Roy, 2002), this condition is also associated with internal detoxification mechanisms in plants, particularly *metal sequestration*. In this process, Cu ions are chelated by compounds such as phytochelatins and metallothioneins, then translocated and compartmentalized into the vacuole, thereby limiting direct interactions between the metal and critical cellular components such as DNA (Clemens, 2006; DalCorso et al., 2008).

The localization of metals within the vacuole plays a crucial role in reducing the concentration of free Cu in the cytosol, thereby suppressing excessive ROS formation and preventing damage to biomolecules, including DNA (Yadav, 2010). Therefore, the low level of DNA damage observed in this study suggests that both species exhibit good tolerance to Cu stress through a combination of detoxification mechanisms and cellular protection strategies.

These findings are also consistent with the previously observed antioxidant activity, where the absence of a significant increase in SOD indicates that oxidative stress remained within a manageable range for the plant defense system. The combined effect of ROS regulation through antioxidant systems and metal isolation within the vacuole enables plants to maintain DNA integrity even under heavy metal exposure.

### 3.6. Effect of Cu Exposure on Water Quality

Water quality measurements in this study included pH, temperature, and dissolved oxygen (DO) as key parameters influencing the survival and functioning of aquatic organisms (Araoye, 2009). The range of values obtained over the 14-day treatment period is presented in **Table 5**.

**Table 5.** Range values of pH, temperature, and dissolved oxygen (DO) measurements during 14 days of phytoremediation treatment using *Azolla microphylla* and *Salvinia molesta*

| Parameters       | <i>Azolla microphylla</i> | <i>Salvinia molesta</i> | Environmental Quality standard |
|------------------|---------------------------|-------------------------|--------------------------------|
| Water pH         | 6.40-8.40                 | 6.30-8,50               | 6.00-9.00                      |
| Temperature (°C) | 24.30-29.00               | 25.00-28.90             | 23.00-29.00                    |
| DO (mg/L)        | 2.60-8.80                 | 2.70-6.60               | >5.00                          |

Source: Perda DIY, 206 & Kep.08/Men.LH/2009

The results indicated that pH and temperature values remained within the permissible quality standards. Temperature tended to remain stable throughout the treatment due to minimal daily

fluctuations ( $<3^{\circ}\text{C}$ ), and therefore showed no significant differences (Chang & Ouyang, 1988). Relatively constant temperature conditions continued to support metal ion uptake by plants (Fitter & Hay, 2001). In contrast to temperature, pH exhibited a significant decrease ( $p < 0.05$ ). Duncan's Multiple Range Test (DMRT) results (**Table 6**) showed that increasing Cu concentrations were correlated with a decrease in medium pH. This reduction in pH is associated with an increase in  $\text{H}^{+}$  ions, which enhances metal solubility (Darmono, 1995). Nevertheless, pH values remained within the range of 7–8, which is considered a stable range for aquatic environments (Palar, 1994).

**Table 6.** Post hoc test results for pH parameter during 14 days of phytoremediation treatment using *Azolla microphylla* and *Salvinia molesta*

| Treatment<br>Cu (ppm) | Posthoc Test Results for pH Parameter |                         |
|-----------------------|---------------------------------------|-------------------------|
|                       | <i>Azolla microphylla</i>             | <i>Salvinia molesta</i> |
| 0                     | 8.10±0.30 <sup>c</sup>                | 8.10±0.30 <sup>d</sup>  |
| 10                    | 8.10±0.40 <sup>c</sup>                | 8.00±0.30 <sup>cd</sup> |
| 25                    | 8.00±0.40 <sup>c</sup>                | 7.90±0.30 <sup>cd</sup> |
| 50                    | 7.90±0.40 <sup>c</sup>                | 7.80±0.40 <sup>c</sup>  |
| 75                    | 7.60±0.30 <sup>b</sup>                | 7.50±0.30 <sup>b</sup>  |
| 100                   | 7.10±0.30 <sup>a</sup>                | 7.00±0.30 <sup>a</sup>  |

Note : Values are presented as mean. Different letters within each column indicate significant differences according to Duncan's Multiple Range Test (DMRT) at a 95% confidence level.

The increased DO values observed in several treatments were likely associated with the photosynthetic activity of aquatic plants, which releases oxygen into the surrounding medium and contributes to improved water quality. In addition, the reduction of Cu concentration during the phytoremediation process may alleviate stress on the aquatic environment and support more favorable conditions for dissolved oxygen balance. However, fluctuations in DO may also be influenced by plant respiration, microbial activity, and changes in medium temperature during the treatment period (Boyd & Tucker, 1998). Some DO values remained below the water quality standard ( $>5$  mg/L), indicating that dissolved oxygen conditions were not fully optimal across all treatments despite the observed increasing trend during phytoremediation. Overall, the presence of *Azolla microphylla* and *Salvinia molesta* may contribute to water quality improvement through enhanced dissolved oxygen levels in the treatment media.

#### 4. Conclusion

Based on the research findings, *Azolla microphylla* and *Salvinia molesta* demonstrated potential as phytoremediators for copper (Cu) heavy metal contamination through their ability to reduce Cu concentrations in the treatment media. *Azolla microphylla* exhibited the highest Cu removal efficiency at 95.32%, while *Salvinia molesta* achieved a reduction of 72.42%. Increasing Cu concentrations tended to decrease the biomass of both plants, indicating the effect of metal-induced stress on plant growth during the phytoremediation process. Superoxide dismutase (SOD) enzyme activity showed no significant differences among treatments, while *comet assay* results indicated no detectable DNA damage in leaf tissues. In addition, the use of both plant species contributed to improved water quality, as indicated by increased dissolved oxygen (DO) levels. These findings suggest that *Azolla microphylla* and *Salvinia molesta* have potential as alternative phytoremediation agents for reducing Cu contamination in aquatic environments. However, further analysis of metal content in plant tissues is required to confirm the mechanisms of Cu uptake and accumulation more specifically.

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