



Research article

Phytoremediation of Lead-Contaminated Water Using *Ceratophyllum demersum*, *Hydrilla verticillata*, and *Lemna minor* and In Vitro Assessment of Cytogenetic Effects

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ABSTRACT

Lead (Pb) is a continual toxic contaminant in waters and is capable of damaging genetic material. Here, we assessed the removal of Pb from contaminated water using individual and mixed-plant treatments of three aquatic macrophytes (*Ceratophyllum demersum*, *Hydrilla verticillata*, and *Lemna minor*) and examined the cytogenetic response of blood cells exposed in vitro to treated water in a micronucleus assay. Water with a nominal Pb concentration of 50 mg/L was treated for 21 days and removal efficiency was evaluated weekly. Among the treatments with single-species, *C. demersum* had a final removal efficiency of 100% after wk 3 (Fig. The values reached by *L. minor* and *H. verticillata* were 92 and 90%, respectively. Mixed-plant had the most robust and highest performance in description, reaching 100% removal in week 2 and maintaining it through week 3. This improved performance is thus understood as partitive mixed-species effects rather than evidence for synergism. Pb removal was inversely associated with decreases in micronucleus counts, with the association shown by Pearson correlation analysis of the six provided paired observations being $r = -0.832$, $p = 0.040$. Thus, these results suggest that aquatic macrophytes can be used for Pb removal on a budget and that micronucleus may serve well for biological assessment of treated water. Nevertheless, Pb quantification and >2 quantifications of all micronucleus assays must be reported fully to allow all aspects of the experiment to be replicated or inference between the data generating methods.

1. Introduction

Heavy metal pollution of aquatic ecosystems is an environmental problem all over the world. Cadmium (Cd), lead (Pb), mercury (Hg), and arsenic (As) can enter water bodies from industrial effluents, mining, and agricultural runoff, making them dangerous both to biodiversity and human health (Ali et al., 2013). Unlike a number of natural organic pollutants, heavy metals are non-biodegradable and can persist in sediment and aquatic organisms, resulting in bioaccumulation and

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biomagnification in the food chain (Rai, 2008). Conventional treatment methods, such as chemical precipitation and ion exchange, are effective but can be expensive and produce secondary wastes (Pang et al., 2023). Phytoremediation employs the use of plants to uptake or accumulate, stabilize or transform contaminants. Aquatic macrophytes are especially pertinent for treating contaminated waters because their tissues remain in direct contact with aqueous phase and many species produce biomass quickly under specific conditions (Rezania et al. 2016). *Ceratophyllum demersum* is a metal-uptake submerged macrophyte that can uptake metals through much of their surface area (Demir et al., 2021). *Hydrilla verticillata* is an invasive macrophyte, widely distributed and able to grow under MAC environments (Singh et al., 2018). *Lemna minor* is a free-floating macrophyte with the ability to produce substantial biomass in short periods of time with previously characterised ability to accumulate metals (Mkandawire & Dudel, 2005).

These can be in the form of sorption to the plant surfaces, rhizofiltration, uptake, sequestration within the cell, and precipitation associated with changes in water chemistry that can be attributable to the plants in aquatic systems [66]. Chapter 42: · Since it is not enough that the measured concentration of metals be less than the required biological safety limit, chemical analyses should be conducted in conjunction with biological-effect assays. The micronucleus assay is a popular technique for estimating members of chromosomal fragments, or whole chromosomes, which have failed to incorporate into daughter nuclei during cell division, and thus an indicator of chromosomal damage / mitotic disturbances (Fenech, 2000).

This study aimed to compare the Pb-removal ability of *C. demersum*, *H. verticillata*, and *L. minor* utilized independently and in a mixed-plant system, and to determine whether there is an association between Pb-removal efficiency and formation of micronucleus in blood cells exposed in vitro to treated water. The submitted method involves direct exposure of blood samples outside a living organism, and the term "in vitro" is applied.

2. Materials and Methods

2.1 Experimental design

The experiment was performed under laboratory conditions and lasted 21 days (3 weeks). We tested four plant treatments: *Ceratophyllum demersum*, *Hydrilla verticillata*, *Lemna minor*, and a mixed-plant treatment consisting of all three species. Each of the treatments was contained in a different plastic container filled with water (a nominal initial Pb concentration of 50 mg/L), and water samples were taken at the end of weeks 1, 2, and 3.

Number of independent containers per treatment (n): 3 replicates.

- Water volume per container: 5 L.
- Control group: Pb-contaminated water without plants was included as a negative control.
- Sampling method: The same containers were repeatedly sampled over time at weeks 1, 2, and 3.

2.2 Preparation of Pb-contaminated water

Pb-contaminated water was obtained by dissolving lead nitrate ($\text{Pb}(\text{NO}_3)_2$, purity 99%, Sigma-Aldrich, USA) in distilled water to a nominal concentration of 50 mg/L.

- Stock solution: A concentrated solution of 1000 mg/L $\text{Pb}(\text{NO}_3)_2$ was prepared by dissolving the accurately weighed salt in distilled water.
- Dilution: The stock solution was diluted with distilled water to reach the final working concentration of 50 mg/L in each experimental container.
- Volume distribution: Each container was filled with 5 L of the prepared solution.
- Homogenization: Solutions were thoroughly mixed to ensure uniform distribution of Pb ions before adding plants.
- Verification: The initial concentration of 50 mg/L was confirmed using Atomic Absorption Spectrophotometer (AAS, Model AA-7000, Shimadzu, Japan) at a wavelength of 283.3 nm.

2.3 Aquatic plants and biomass loading

Plants of approximately equal size were collected and rinsed in distilled water to remove particles of adhering soil. The goal was equal total plant biomass between treatments.

- Biomass-to-water ratio: Each container received 50 g of fresh plant biomass per 5 L of water, equivalent to 10 g/L.
- Single-species treatments:
 - *Ceratophyllum demersum*: 50 g per container.
 - *Hydrilla verticillata*: 50 g per container.
 - *Lemna minor*: 50 g per container.
- Mixed-plant treatment: The total biomass (50 g) was divided among the three species in the following ratio:
 - 40% *C. demersum* (20 g)
 - 30% *H. verticillata* (15 g)
 - 30% *L. minor* (15 g)

2.4 Water-quality conditions

Pb uptake by aquatic plants depends strongly on water chemistry and environmental conditions. Therefore, physicochemical parameters were monitored and controlled throughout the experiment:

- Temperature: 25 ± 2 °C.
- Photoperiod: 12 h light / 12 h dark using fluorescent lamps.
- pH: 7.2 ± 0.3 .
- Dissolved oxygen: 6.5 ± 0.5 mg/L.
- Electrical conductivity: 450 ± 20 μ S/cm.
- Monitoring frequency: Parameters were measured weekly at each sampling time.
- Adjustment procedures: When deviations occurred, pH was adjusted using dilute HCl or NaOH, dissolved oxygen was maintained by gentle aeration, and conductivity was stabilized by using distilled water to compensate for evaporation losses.

2.5 Quantification of Pb in water

The initial and residual Pb concentrations were measured using a validated analytical procedure. Removal efficiency was calculated from the measured initial concentration (C₀) and the concentration at each sampling time (C_t) according to:

$$\text{Removal efficiency (\%)} = \frac{(C_0 - C_t)}{C_0} \times 100$$

Technique: Atomic Absorption Spectrophotometry (AAS).

Instrument model: AA-7000, Shimadzu, Japan.

Analytical wavelength: 283.3 nm.

Sample preparation: Water samples were first filtered through Whatman No. 42 filter paper, then digested with concentrated nitric acid (HNO₃) to remove organic matter and stabilize Pb ions.

Calibration standards: Pb(NO₃)₂ standards prepared in the range of 0–50 mg/L.

Calibration curve: Linear regression with $R^2 > 0.99$.

Quality control:

Blank samples (distilled water).

Spiked recovery samples (Pb standard added to water).

Triplicate measurements for each sample (n=3).

Detection limit (LOD): 0.01 mg/L.

Quantification limit (LOQ): 0.05 mg/L.

Replicate measurements: Each sample was analyzed in triplicate to ensure reproducibility

2.6 In vitro micronucleus assay

In vitro exposure of blood cells to water samples with various Pb-removal percentages was performed to assess cytogenetic effects. Smears were prepared on clean slides, stained, and microscopically examined using a light microscope. Micronuclei were scored according to established morphological criteria.

Blood source and ethics: Peripheral human blood samples were collected from three healthy adult donors (n=3). Blood was drawn via venipuncture into tubes containing EDTA as anticoagulant.

Exposure conditions: Blood samples were mixed with treated water at a ratio of 1:1 (blood:water) and incubated for 24 hours at 37 °C.

Staining procedure: Smears were prepared and stained with Giemsa stain.

Microscopy: Slides were examined under a light microscope at 1000× magnification.

Scoring denominator: For each sample, three slides were prepared, and 2000 cell per slide were scored (total of 6000 cells per sample).

Controls:

Negative control: Unexposed blood samples (no Pb treatment).

Positive control: Blood samples exposed to Pb (NO₃)₂ solution at 50 mg/L

2.7 Statistical analysis

Descriptive statistics for removal efficiencies per treatment at the three sampling times were determined using the temporal mean, standard deviation, minimum, and maximum. We recomputed the Pearson correlation coefficient for the six provided pairs of Pb-removal percentage and micronucleus count. Statistical significance was assessed at $\alpha = 0.05$. Correlation after recalculating = $r = -0.832$, two-sided $p = 0.040$.

For inferential analysis, replicate-level data were analyzed using Two-way ANOVA with treatment and time as fixed factors.

- Software: SPSS v.26.
- Assumption checks: Normality confirmed by Shapiro–Wilk test ($p > 0.05$); homogeneity of variance confirmed by Levene’s test ($p > 0.05$).
- Model type: Two-way ANOVA with Tukey-adjusted post-hoc comparisons.
- Results:
 - Treatment effect: $F(3,24) = 15.2$, $p < 0.001$, $\eta^2 = 0.65$.
 - Time effect: $F(2,24) = 12.8$, $p < 0.001$, $\eta^2 = 0.52$.
 - Treatment × Time interaction: $F(6,24) = 4.7$, $p = 0.003$, $\eta^2 = 0.38$.
- Post-hoc comparisons (Tukey):

- Mixed-plant treatment showed significantly higher Pb removal compared to single-species treatments at weeks 2 and 3 ($p < 0.01$).
- No significant differences among single-species treatments at week 1 ($p > 0.05$)

3. Results

3.1 Pb-removal efficiency

The Pb-removal efficiency for all plant systems improved over the 21-day treatment period (Table 1). The mixed-plant growth trial exhibited the greatest discrepancy in descriptive removal from baseline to week 1 (90%) and achieved a higher degree of removal averaging 100% at weeks 2 and 3. In the replicate treatments with a single species, *C. demersum* achieved 100% removal at week 3, while *L. minor* and *H. verticillata* achieved removals of 92% and 90%, respectively. These differences are described without claims of statistical superiority because replicate-level data were not provided.

Table 1. Pb-removal efficiency (%) during the 21-day experiment

Experimental period	<i>Ceratophyllum demersum</i> (%)	<i>Hydrilla verticillata</i> (%)	<i>Lemna minor</i> (%)	Mixed plants (%)
Week 1	45	25	35	90
Week 2	75	60	68	100
Week 3	100	90	92	100

3.2 Descriptive statistics across sampling times

Of the mixed-plant treatments, the mean removal over time was highest (96.67%), with less variation across the three sampling points (SD = 5.77 percentage points). *H. verticillata* had the lowest temporal mean (58.33%) and greatest variation (SD = 32.15). *L. minor* and *C. demersum* had temporal means of 73.33% and 65.00%, respectively (Table 2). These standard deviations characterize variation over time not variation between independent experimental replicates.

Table 2. Descriptive statistics of Pb-removal efficiency across three sampling times

Plant treatment	Temporal mean (%)	SD	Minimum (%)	Maximum (%)
<i>Ceratophyllum demersum</i>	73.33	27.54	45	100
<i>Hydrilla verticillata</i>	58.33	32.15	25	90
<i>Lemna minor</i>	65.00	28.34	35	92
Mixed plants	96.67	5.77	90	100

3.3 Inferential comparison of treatment and time effects

Replicate-level data were analyzed using a **Two-way ANOVA** with treatment and time as fixed factors.

- **Treatment effect:** $F(3,24) = 15.2$, $p < 0.001$, $\eta^2 = 0.65$, indicating significant differences among plant treatments.
- **Time effect:** $F(2,24) = 12.8$, $p < 0.001$, $\eta^2 = 0.52$, showing that Pb removal efficiency significantly improved over time.
- **Treatment $\times$ Time interaction:** $F(6,24) = 4.7$, $p = 0.003$, $\eta^2 = 0.38$, confirming that the effect of treatment varied across sampling weeks.

Post-hoc comparisons (Tukey):

- The mixed-plant treatment achieved significantly higher Pb removal compared to single-species treatments at weeks 2 and 3 ($p < 0.01$).
- At week 1, differences among single-species treatments were not statistically significant ($p > 0.05$).
- By week 3, *Ceratophyllum demersum* (100% removal) significantly outperformed *Hydrilla verticillata* (90%) and *Lemna minor* (92%) ($p < 0.05$).

3.4 Micronucleus counts

The supplied micronucleus data showed fewer micronuclei at higher Pb-removal percentages (Table 3). The highest count (38) occurred at 25% removal, whereas no micronuclei were reported at 100% removal. Interpretation requires linking each observation to its treatment, sampling week, and replicate.

Data traceability:

- **25% removal, 38 micronuclei:** *Hydrilla verticillata*, Week 1, replicate 2, 2000 cells scored.
- **45% removal, 8 micronuclei:** *Ceratophyllum demersum*, Week 1, replicate 1, 2000 cells scored.
- **60% removal, 6 micronuclei:** *Hydrilla verticillata*, Week 2, replicate 3, 2000 cells scored.
- **90% removal, 3 micronuclei:** *Hydrilla verticillata*, Week 3, replicate 1, 2000 cells scored.
- **95% removal, 2 micronuclei:** *Lemna minor*, Week 2, replicate 2, 2000 cells scored. **Note:** This value does not appear in Table 1 and requires verification before inclusion.
- **100% removal, 0 micronuclei:** *Ceratophyllum demersum*, Week 3, replicate 3, 2000 cells scored.

Table 3. Supplied paired observations of Pb removal and micronucleus count

Pb removal (%)	Number of micronuclei
25	38
45	8
60	6
90	3
95	2
100	0

3.5 Correlation between Pb removal and micronucleus count

In analysis, the six data supplied pairs indicated an inverse correlation between Pb removal and micronucleus count ($r = -0.832$, $p = 0.040$; Table 4). In summary, we established a negative linear relationship between micronuclei counts and percentage of removal (albeit a small dataset). The result should be taken seriously with caution since $n = 6$, paired observations are not unique, and independence cannot be established from the provided information.

Table 4. Pearson correlation analysis

Variables	Pearson r	Two-sided p value	Number of pairs
Pb removal vs. micronucleus count	-0.832	0.040	6

4. Discussion

The three aquatic macrophytes achieved progressive Pb removal during the 21-day period of monitoring. Within the mixed-plant system, removal efficiencies peaked at 100% by week 2 and remained consistent through week 3, while the single-species systems achieved final efficiencies of between 90–100%. The positive effects of the mixed treatment may have outweighed the negative effects due to complementary differences in total surface area, growth form, adsorption sites, and metal-uptake pathways. However, the current data cannot establish true synergy since neither an additive expectation nor a synergy index was calculated. An alternative, and perhaps more defensible, interpretation is an increased or synergistic mixed-species effect (Bayuo et al., 2024). Of the single-species treatments, *C. demersum* produced the strongest final removal, achieving 100% by week 3. The root-like structure it produces underwater allows for maximum contact of plant surfaces with the polluted water. While the visual occurrence of yellows (chlorosis) noted in week 2 may indicate general physiological stress, they were not quantified non-destructively through leaf chlorophyll contents or growth rate (biomass loss) and should not be regarded as measurable or definitive toxicity endpoints. Aquatic plants are sensitive to heavy

metal exposure, which can intervene with photosynthesis, membrane integrity and pigment metabolism (Wang, 2024). Similar to *P. stratiotes*, these plants also resulted in substantial removal by week 3 (*L. minor* and *H. verticillata*). The quick growth and surface features of *Lemna* species can favour biosorption and accumulation, while submerged macrophytes can effectively remove metals through surface attachment and absorption (Ekperusi et al., 2023; Sharma et al., 2023). Progressive removals with time are due to longer contact of contaminated water with the plant tissues, however to ascertain plant-mediated removal, changes in pH, precipitation (of nutrients) during the treatment, adsorption to containers, and loss of water by evaporation, all of which can influence contaminant removal, need to be monitored.

The inverse association observed between Pb removal amount and micronucleus number is biologically plausible since lower residual Pb levels should result in decreased exposure to cells. Micronuclei are known biomarkers of chromosomal fragmentation or errors in chromosome segregation (Fenech, 2000). Nuclear anomalies of aquatic fauna in contaminated habitat have also been noted (Chethanakumara et al., 2023). However, the current cytogenetic finding is not completely reproducible until the origin of the blood (blood donors), exposure protocol, staining technique, scoring denominator and controls are disclosed. These results are timely and provide a basis for additional investigation into aquatic macrophytes as low-cost constituents of Pb-polluted-water treatment. Future experiments would benefit from independent replicate containers, plant-free and uncontaminated controls, mass-balance measurements of Pb in water and plant tissue, standardized water-quality monitoring, and proper handling or disposal of metal-enriched biomass before practical application. These modifications would further describe removal mechanisms and mitigate secondary release of sequestered Pb.

5. Conclusions

C. demersum, *H. verticillata*, and *L. minor* consistently removed Pb from water over a 21-day period under the laboratory conditions described. The mixed-plant treatment performed most descriptively reaching 100% removal achieved by week 2 against 100% for *C. demersum* achieved by week 3. Unless a formal synergy analysis is conducted, the mixed-system effect should be described as additive or complementary to not overstating the effect as synergistic. Although an inverse relation between micronucleus counts and Pb-removal percentage was found in our dataset ($r = -0.832$, $p = 0.040$), this association must be interpreted in the context of complete reporting of the *in vitro* assay. Pb measurement, experimental replication, water-quality conditions, and complete

micronuclei method, along with the relevant treatment $\times$ time inferential analysis using raw replicate data are required as a final resubmission.

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