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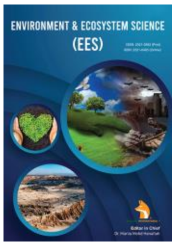

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RESEARCH ARTICLE

PHYTOACCUMULATION OF LEAD (PB) IN WASTEWATER BY *AZOLLA* SP.

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ABSTRACT

Laboratory chemical wastewater contains the heavy metals such as Pb and Cr. The removal of Pb from such water in the absence and presence of Cr and other metals using phytoremediation technique with Azolla was studied. A hyperaccumulator Azolla aquatic plant species was grown in a solution of Pb and/or Cr under three experimental settings and the relative growth rate of the plant, the bioconcentration factor and the Pb removal efficiency were measured for a period of 21 days under controlled laboratory environment. In the first experimental setting, the Azolla was planted in Pb solutions of concentrations 2.5 ppm, 5.0 ppm, and 10.0 ppm. A removal efficiency of Pb up to 98% was reported in the solution of 2.5 ppm Pb concentration. In the second experimental setting, when the Pb was mixed with Cr, a decrease in the relative growth rate of Azolla was observed, yet with an increase in the Pb removal efficiency. In the third experiment series, two concentrations (2.5 ppm and 5.0 ppm) of Fe, Mg and Ca in the solution were used in addition to Pb and Cr. Here, a decreased relative growth rate with increasing exposure days, and a rising trend in metal removal efficiency and bioconcentration factor were observed. The results revealed a promising potential of Azolla sp in the phytoremediation of Pb in solution. Further, the Azolla sp was found more effective in removing Pb when it is present individually rather than in a solution with Cr.

KEYWORDS

Phytoremediation, Azolla sp, Lead (Pb), Relative growth rate (RGR), Metal remove efficiency (MRE), Bioconcentration factor (BCF), Translocation Factor (TF)

1. INTRODUCTION

The environmental accumulation of heavy metal lead (Pb) poses severe consequences such as soil, water and food chain contamination and ecosystem disruption. (Hazrat et al., 2019). In contemporary waste management, physical and chemical treatments are commonly employed. Yet, acknowledging the advantages of eco-friendly approaches, the introduction of plant-based and biological methods, collectively known as phytoremediation, has gained prominence. Phytoremediation offers a cost-effective and environmentally sound means of remediating contaminated soil and water through the use of plants (Shehata et al., 2019). This approach utilizes naturally occurring or genetically engineered plants, referred to as hyperaccumulator plants, to cleanse polluted environments. Through hyperaccumulation, heavy metal pollutants are absorbed by plant roots, either concentrating them in plant tissues or transforming them into less harmful forms (Sumreen, 2019).

Azolla, commonly known as a water fern, has emerged as a noteworthy hyperaccumulator for heavy metals in aquatic environments. Extensively studied by various researchers such as, Azolla's unique ability to accumulate and stabilize metals within its roots and shoots makes it a promising candidate for phytoremediation (Akhtar et al., 2020; Anand et al., 2017; Khosravi et al., 2005; Sarwar et al., 2017; SELA et al., 1989). Its growth pattern, characterized by branching fern-frond-like plants with simple roots, contributes to its effectiveness. Azolla's multifaceted applications, including agro-environmental settings, bio-fertilization, and water quality improvement, highlight its versatility (Akhtar et al., 2023; Yao et al., 2018).

Azolla's efficacy in removing pollutants from wastewater, including metals such as Cr, Cu, Ag, As, Cd, and Ti, Pb, Ni is well-documented (Herath et al., 2023; Khosravi et al., 2005; Sheel et al., 2013; SELA et al., 1989). Research demonstrates high metal absorption efficiency, ranging from 74% to 97.7% for metals such as Cr, Cu, Pb, and Zn. Notably, Azolla has shown promising growth rates in laboratory studies, achieving metal absorption efficiencies of 86% to 98% for metals such as Cr, Ni, Cd, and Pb. However, when multiple metal ions are considered simultaneously, the presence of one metal ion may influence the uptake and accumulation of the other, and their interactive effects may play an important role (Taghilou et al., 2023). In order to understand the impact of Cr and other metals on the phytoaccumulation of Pb, in the present study we studied the removal of Pb by Azolla when Pb is present in a solution with and without Cr, Fe, Mg and Ca.

2. MATERIALS AND MATERIALS

2.1 Plant acquisition and acclimatization

Azolla species were procured from a reputable source. Healthy mature plants were selected for the experiments, and adhering mud particles and epiphytic species were removed. To adapt to the experimental laboratory conditions those plants were grown in four plastic trays (38cm × 28cm × 7cm) containing tap water with Albert solution for 14 days. A set of favorable conditions was maintained such as pH 4.5 – 7, and temperature 25 – 30 °C with indirect light intensity.

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2.2 Preparation of stock solution

Individual stock solutions were meticulously prepared at 100.0 ppm for the metal Pb being studied. Experimental solutions were selected based on a few factors such as less toxicity and plant availability.

2.3 Experiment 1: Assessment of phytoremediation efficiency of *Azolla* in individual metal solutions

Based on the stock solutions, concentration series (2.5 ppm, 5.0 ppm, 10.0 ppm) for the metal solution was systematically prepared, drawing from the method established by (Anand et al., 2017). Distinct experiments for selected metal were executed, with each metal solution being contained within separate beakers (1000.0 mL). With each metal solution, alongside a controlled quantity (10.0 mL) of Albert solution for nutrient supplementation. Control experiments involving the introduction of *Azolla* into deionized water was performed under the same environmental conditions to serve as reference points. The healthier plant's adherent water was removed using filter papers for 30 minutes. A standardized amount as an initial weight of healthy *Azolla* (1.0 g) was introduced into the experimental and control.

The experiment was run for 14 days. The solution level was checked daily, and deionized water was added based on necessity, to compensate for the water loss through evaporation and transpiration. After 7 days the nutrient Albert solution (10.0 mL) was added into the experimental and control. On the $14^{th,}$ the day of the experiment, plants in each replicate were harvested, rinsed with deionized water to remove any ions adhering to the plant's surface, and dried out by blotting on two filter papers. Finally, weight was measured for every replicate of the experiment and control. Experimental samples were filtered with Whatman No.42 filter paper and analyzed by Atomic Absorption Spectrometer (iCE™ 3300 AAS) for Pb. Plant samples undergo wet digestion and then filtered with Whatman No.42 filter paper twice time and heavy metals concentration was measured by AAS.

2.4 Experiment 2: Assessment of phytoremediation efficiency of *Azolla* when Pb is present with Cr

In this second phase, two experimental setups were performed.

- Control experiments were conducted to assess the introduction of *Azolla* into separate Pb solutions with varying concentrations based on their higher BCF value. (Selected based on one-way ANOVA ($p<0.05$). The Higher BCF was 12.263 in 5.0 ppm concentration.
- Phytoremediation capability of Pb by *Azolla* sp when Pb introduced with another strong heavy metal Cr with concentrations 5.0 ppm and 2.5 ppm respectively.
- The experimental laboratory waste solution was formulated by mixing the Pb, Cr, Fe, Mg, and Ca in different concentrations.
- The elemental concentration of other metals used two experimental set ups are shown in Table 01.

Table 1: Concentration of metals in the solution

Metal	Pb	Cr	Fe	Mg	Ca
Setup 1: Treatment (ppm)	5.0	2.5	2.5	2.5	2.5
Set up 2: Treatment (ppm)	5.0	2.5	5.0	5.0	5.0

The experimental setups were maintained separately for both the 7^{th} and 14^{th} day experiments and replicates were maintained for the accuracy of the analysis. The solution levels were examined daily, and deionized water was added as needed to compensate for water loss due to evaporation and transpiration. After the initial 7 days of the experiment, a nutrient solution consisting of Albert's solution (10.0 mL) was added to the control group. Plant harvests in each replicate were conducted on the 7^{th} and 14^{th} days of the experiment. Subsequent analysis procedures were consistent with those outlined in Experiment 1.

2.5 Atomic absorption spectrometry (AAS) analysis

- Experimental solutions

Experimental samples were filtered by Whatman No. 42 twice to take a clear solution without particles to prevent interference in AAS analysis. The solution was homogenized before analysis for accurate results.

- Plant materials

The wet acid digestion method was followed for the plant heavy metal analysis. Plants were oven-dried at 65°C for 24 hours. Plant particles were ground and Concentrated HNO_3 (5.0 mL) was added to 1.0 g of oven-dried plant materials and allowed to stand overnight. Heat (160 - 200°C) carefully on a hot plate until the production of red NO_2 fumes (brownish) has ceased. If remain any residue add 5.0 mL of HNO_3 (make sure the brownish color gas disappears). Heat again and allow it to evaporate to a small volume. Transfer the sample to a 50-mL flask and dilute to volume with deionized water (Perkin Elmer Corporation, 1996)

2.6 Calculations

I. Relative growth rate (RGR)

Where ln W1(g) is the Initial fresh weight, ln W2(g) is the Final fresh weight and Day is the experimental days (Beretta et al., 2014; Greco and Freitas, 2002).

$$Relative\ Growth\ Rate\ (gDay^{-1}) = \frac{\ln W2 - \ln W1}{Day} \quad (1)$$

II. Metal removal efficiency (MRE)

where C_1 and C_0 are respectively initial and remaining concentrations of metal in medium (mgL^{-1}) (Abu Bakar *et al.*, 2013).

$$\%\ Efficiency = \frac{C_0 - C_1}{C_0} \times 100 \quad (2)$$

III. Bio concentration factor (BCF) (Lenaduwa Lokuge, 2016)

$$BCF = \frac{Concentration\ of\ metals\ in\ dried\ plant\ tssues\ (mg/kg^{-1})}{Initial\ concentration\ of\ metal\ in\ the\ experimental\ solution\ (mg/L^{-1})} \quad (3)$$

IV. Translocation factor (TF) (Parnian et al., 2016)

$$TF = \frac{Concentration\ of\ metals\ in\ dried\ plant\ shoot\ (mg/kg^{-1})}{Concentration\ of\ metals\ in\ dried\ plant\ root\ (mg/kg^{-1})} \quad (4)$$

2.7 Statistical analysis

Statistical analysis using ANOVA (SAS_JAM Pro 17 and Minitab 19) was conducted to determine the mean values of *Azolla* RGR, MRE, BCF, and TF at three different concentrations (2.5 ppm, 5.0 ppm, and 10.0 ppm). The significance level was set at $P < 0.05$ to identify whether the means were significantly different or not. This analysis aimed to identify the optimum concentration for the variables under investigation.

ANOVA and Tukey comparison were run to check the statistical difference among the means. For the same Tukey-Kramer multiple comparison test of significance was carried out which suggested the variation among the column means is significant or not at different levels of significance.

3. Results

Table 2 summarizes the results obtained in each of the experiment discussed in Section 2.

Table 2: Summery of the results obtained in the experiments.

	Phase – 1			
	Control	Pb -2.5 ppm	Pb 5.0 ppm	Pb - 10.0 ppm
RGR ($gDay^{-1}$)	0.084 ± 0.009[a]	0.079 ± 0.010[a]	0.075 ± 0.004[a]	0.085 ± 0.002[a]
MRE (%)	*	98.82 ± 1.02[a]	95.65 ± 4.46[a]	97.13±1.40[a]
BCF	*	9.76 ± 3.56[b]	12.26 ± 1.03[a]	4.53 ± 1.79[b]
TF	*	0.34 ± 0.09[ab]	0.48 ± 0.15[a]	0.26 ± 0.97[b]

	Phase 2 - 14 Days			
	Pb 5.0 ppm*	Pb + Cr *	Mixed 2.5*	Mixed 5.0*
RGR (gDay-1)	0.07 ± 0.01de	0.06 ± 0.01e	0.08 ± 0.01cd	0.09 ± 0.01c
MRE (%)	98.31 ± 0.85ab	99.81 ± 0.34a	99.74 ± 0.45a	100.0 ± 0.00a
BCF	13.72 ± 0.57a	5.12 ± 0.68c	4.87 ± 0.16c	5.00 ± 0.23c
TF	0.40 ± 0.01a	0.18 ± 0.03b	0.27 ± 0.03ab	0.31 ± 0.07ab

	Phase 2 - 7 Days			
	Pb 5.0 ppm*	Pb + Cr *	Mixed 2.5*	Mixed 5.0*
RGR (gDay-1)	0.11 ± 0.01b	0.08 ± 0.01d	0.15 ± 0.01a	0.16 ± 0.01a
MRE (%)	96.72 ± 0.24bc	94.27 ± 0.87cd	93.04 ± 0.11d	95.51 ± 1.92cd
BCF	10.94 ± 0.13b	3.16 ± 0.61d	2.87 ± 0.50d	3.32 ± 0.16d
TF	0.22 ± 0.01b	0.29 ± 0.07ab	0.25 ± 0.09ab	0.31± 0.05ab

Note: Different small letters denoted significant difference ($P < 0.05$) among treatments.

Phase 2 – 7 & 14 days all factors are statistically tested (one-way ANOVA, Tukey comparison P<0.005)

Pb 5.0 ppm* - Pb 5.0 ppm as control, Pb + Cr * - Pb 5.0 ppm & Cr 2.5 ppm

Mixed 2.5* - Pb 5.0 ppm, Cr 2.5 ppm, Ca 2.5 ppm, Fe 2.5 ppm and Mg 2.5 ppm

Mixed 5.0* - Pb 5.0 ppm, Cr 2.5 ppm, Ca 5.0 ppm, Fe 5.0 ppm and Mg 5.0 ppm

3.1 Relative Growth Rate

During Pb absorption, Azolla exhibits a noteworthy RGR within 10 days, specifically 0.085 ± 0.002 g/day at a concentration of 10.0 ppm. Statistically, no significant differences were observed in mean RGR values (P<0.05), implying consistent growth across different concentrations of Pb solution. Where at a concentration of 5.0 ppm, RGR reached 0.11 $gDay^{-1}$ on the 7th day and 0.075 $gDay^{-1}$ and 0.07 $gDay^{-1}$ on the 10th and 14th days, respectively. *Azolla* sp remarkable ability to double its biomass within 3 to 5 days is highlighted, reaching maximum surface area coverage within 7 days, with an initial biomass of 1.0 g increasing to an average of 3.0 g by the end of phase 1. When Pb Mixed with Cr azolla reached the highest RGR in the experiment after the 7th day it was 0.16 $gDay^{-1}$.

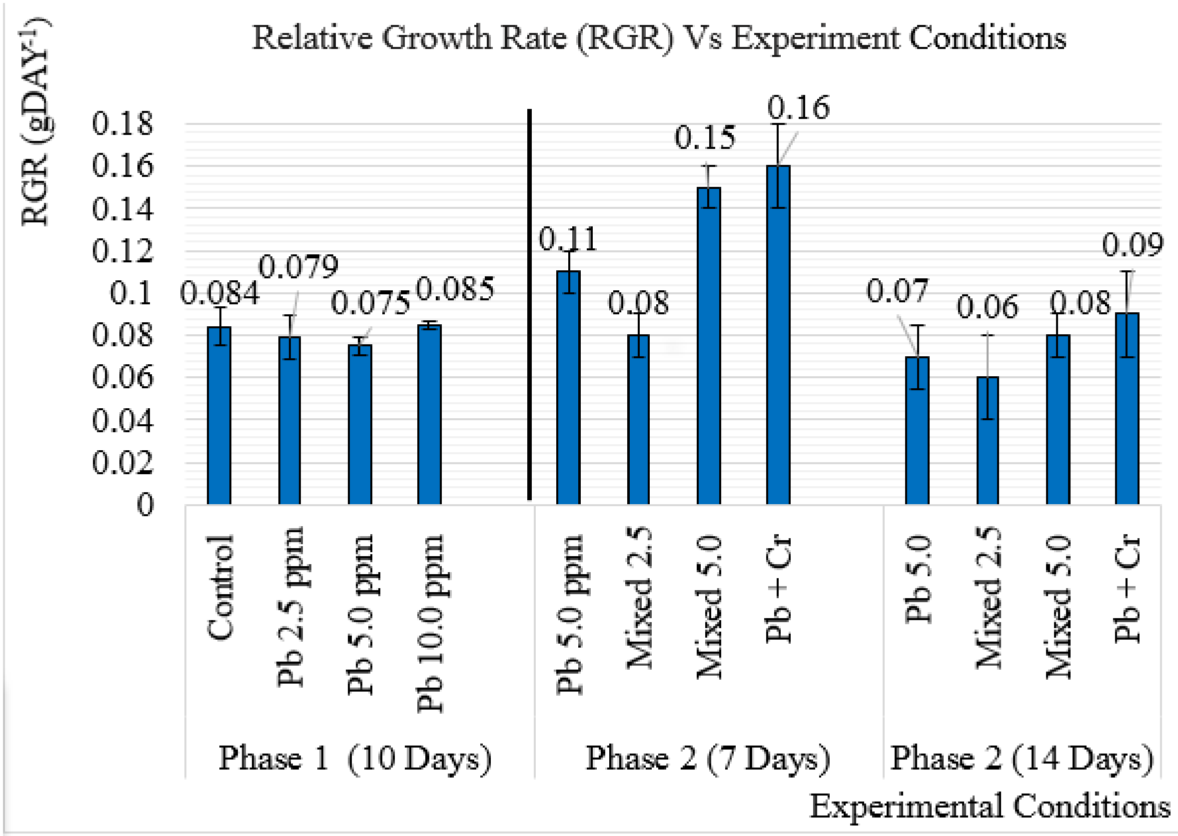

Figure 1: Relative Growth Rate (RGR) Vs Experiment Conditions

3.2 Metal Removal Efficiency

In phase 1, when *Azolla* was introduced into a different concentration series of Pb solution, the MRE remained almost the same without any deviation according statistically. the experimental phase 2 - 7, and 14 days, the introduction of *Azolla* into a separate Pb solution for 7 and 14 days resulted in a 2% increase in metal removal efficiency, with an overall efficiency ranging from 96% to 98% at the concentration 5.0 ppm. Metal removal efficiency increase when exposure days are increasing.

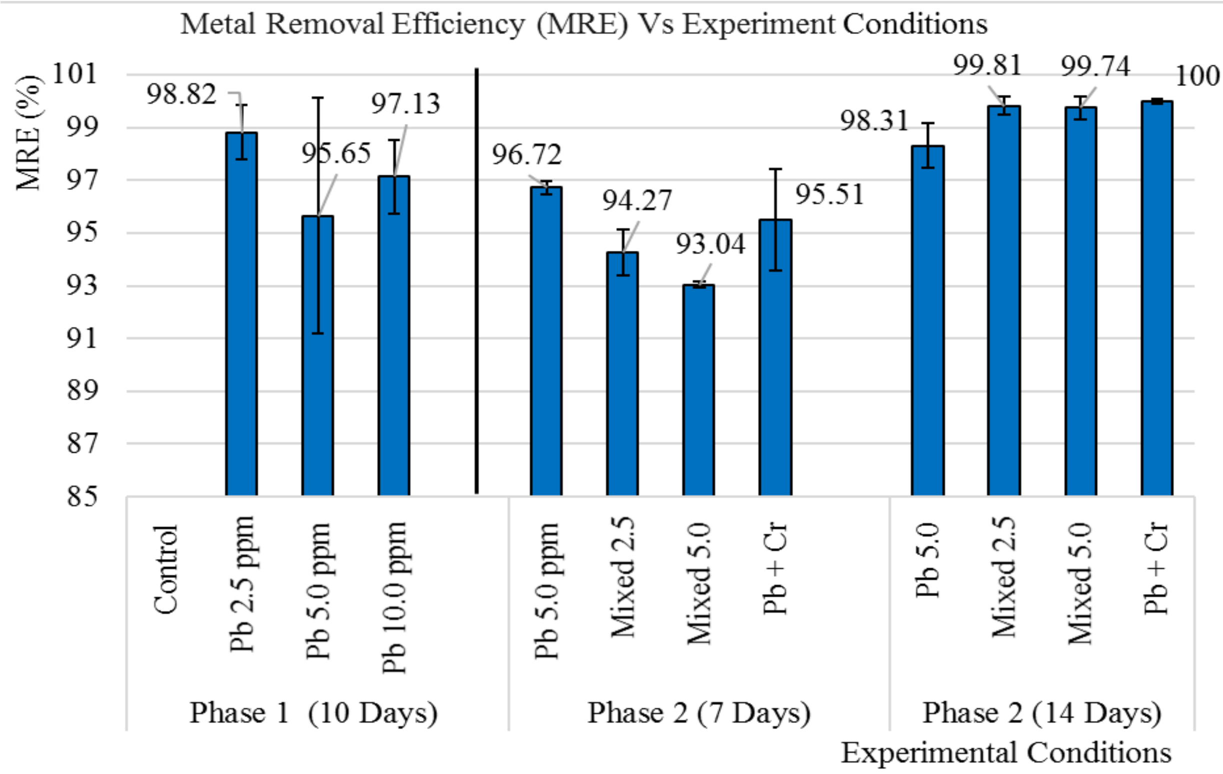

Figure 2: Metal Removal Efficiency (MRE) Vs Experiment Conditions

3.3 Bio Concentration Factor

At a concentration of 5.0 ppm, the BCF value was very high at 12.26 ± 1.03, while at concentrations of 2.5 ppm and 10.0 ppm, the BCF values reduced by 9.76 ± 3.56 and 4.54 ± 1.79, respectively. During the Phase 2 experiment, it was observed that the BCF value increased when the exposure days were increased from 7 to 14 days, with values of 10.94 ± 0.13 and 13.72 ± 0.57, respectively. The mean BCF for a concentration of 2.5 ppm in phase 1 is lower than that of 5.0 ppm.

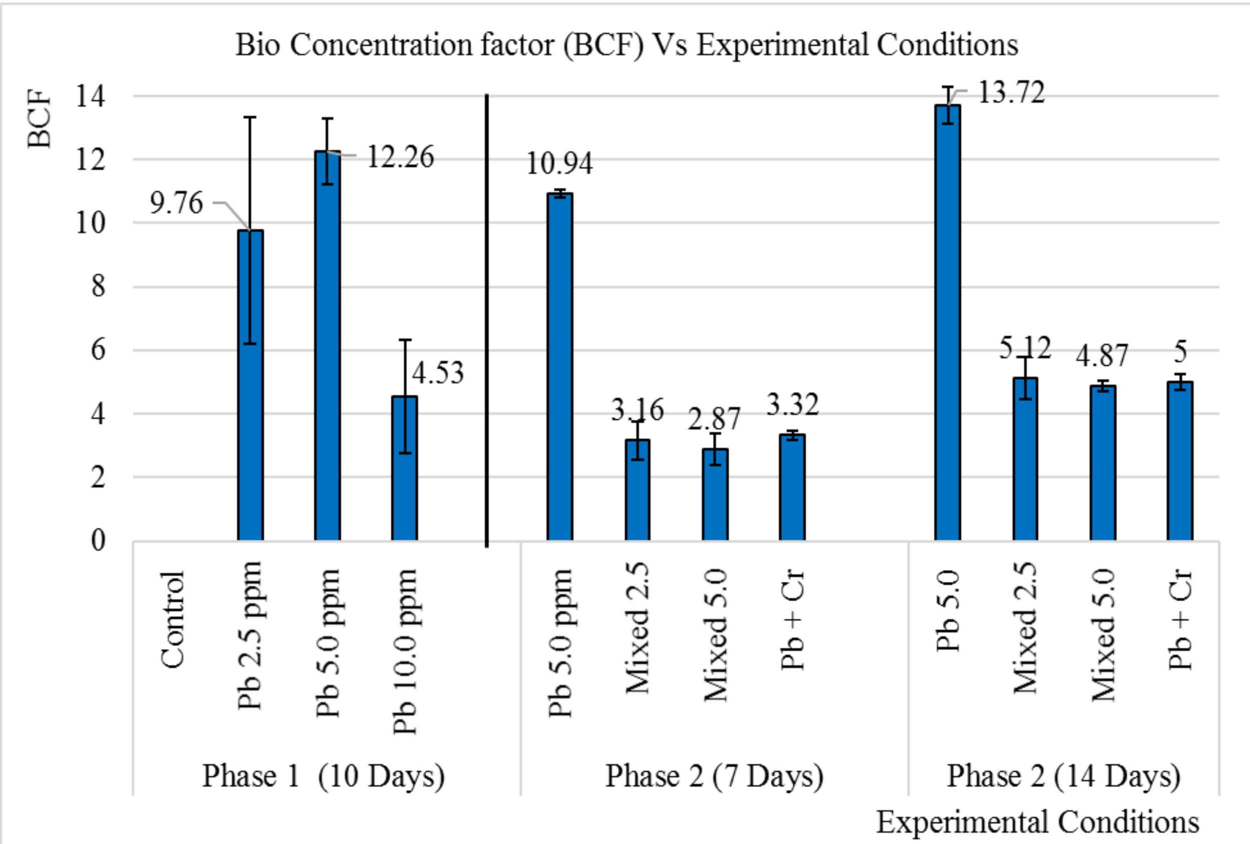

Figure 3: Bio Concentration factor (BCF) Vs Experimental Conditions

3.4 Translocation Factor

In Phase 1, the TF values of 2.5 ppm, 5.0 ppm, and 10.0 ppm showed significant differences ($P<0.05$) among them, with respective values of 0.34 ± 0.09, 0.48± 0.15, and 0.26 ± 0.97. Phase 2, the experimental solution containing 5.0 ppm of Pb demonstrated a significant increase in translocation when the exposure period was extended from 7 to 14 days. The TF of Pb in combination with Cr decreased similarly as the BCF.

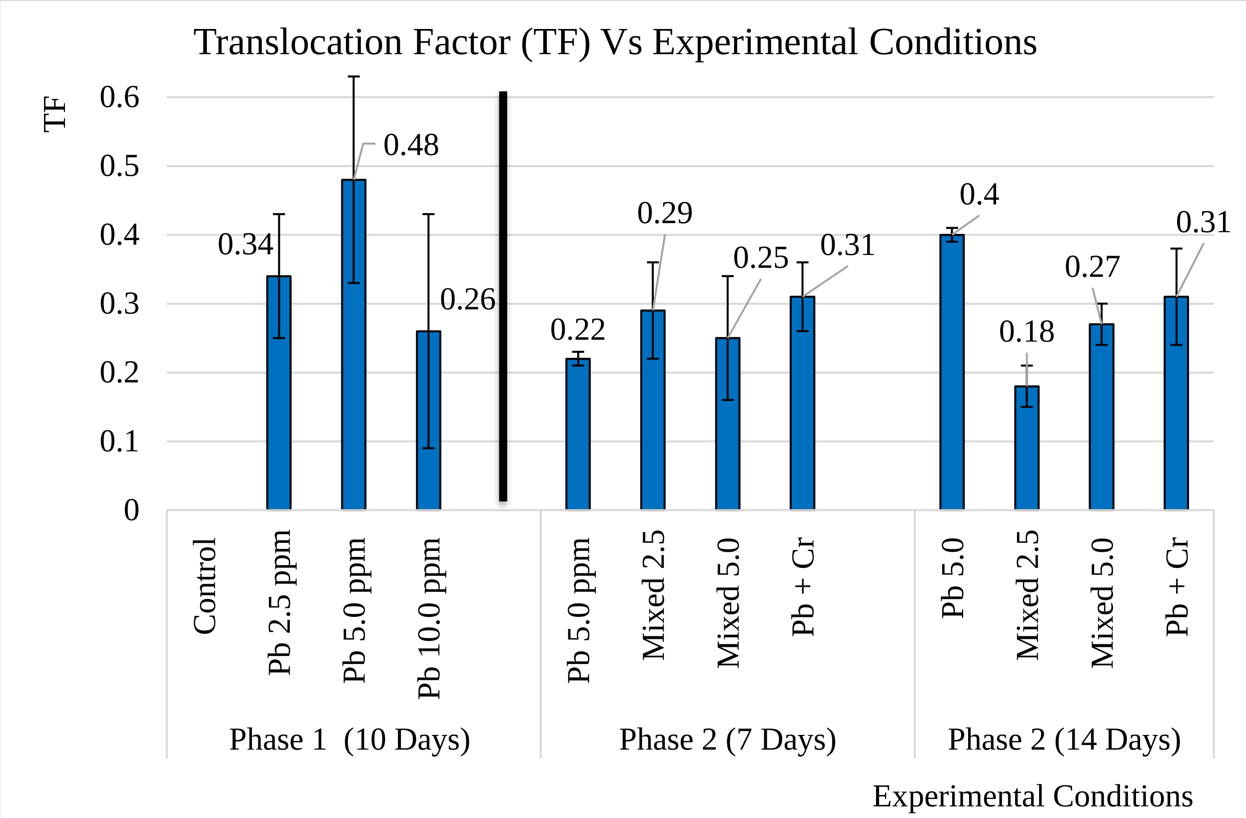

Figure 4: Translocation Factor (TF) Vs Experimental Conditions

4. Discussion

4.1 RGR

The Relative Growth Rate (RGR) is a crucial metric for assessing and comparing the growth performance of plants (Turnbull et al., 2008). Heavy metals, when absorbed by plants, often have adverse effects on germination, root growth, and biomass production. However, Azolla, in contrast, shows an ability to absorb lead (Pb) without significant negative impacts. In a 10-day experiment with a 10.0 ppm Pb concentration, Azolla exhibited a noteworthy RGR of 0.085± 0.002 g/day, with consistent growth across different Pb concentrations.

A study by Mandakini et al., 2016 reports Azolla sp reaching an RGR of 2.40 gDay-1, 2.13 gDay-1, and 1.54 gDay-1 within 7 days of the experiment in Pb solutions with concentrations of 2 ppm, 6 ppm, and 10 ppm, respectively. This differs from our findings, where at a concentration of 5.0 ppm, RGR reached 0.11 gDay-1 on the 7th day and 0.77 gDay-1 and 0.07 gDay-1 on the 10th and 14th days, respectively. Azolla sp remarkable ability to double its biomass within 3 to 5 days is highlighted, reaching maximum surface area coverage within 7 days, with an initial biomass of 1.0 g increasing to an average of 3.0 g by the end of phase 1. The study's findings indicate that the beakers could accommodate approximately 3.0 g of *Azolla sp*, contrasting with, who used larger surface area glass aquarium tanks in a greenhouse setting (Lenaduwa Lokuge, 2016). Environmental conditions influence *Azolla* growth, as demonstrated in the table and graphical illustration showing how exposure days affect RGR. Study by Lenaduwa Lokuge, (2016) state that RGR increases with exposure days, but this research, limited by surface area, indicates a plateau after the 7th day.

The maximum growth of Azolla within its designated surface area was achieved after a 7-day experiment, with no significant increase in biomass after the 7th day. Additionally, the presence of Pb and chromium (Cr) adversely affected Azolla growth, with Cr being a particularly strong inhibitor. Cr can cause various damages, affecting crop growth rate, productivity, grain quality, chlorosis, root tips' browning, seed germination, photosynthesis, water, nitrate, nutrient uptake, ATPase activity, and Fe (II) deficiency(Coker et al., 2018; Khan et al., 2023).

All plants, including Azolla, require a combination of macro and micro essential nutrients for optimal growth. Azolla relies on nutrients such as phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), iron (Fe), manganese (Mn), molybdenum (Mo), and boron (B)(Sadeghi et al., 2013). When exposed to a chemical mixture containing essential nutrient metals and Pb, Azolla exhibited a consistent RGR. This indicates that Azolla growth is influenced by the interplay between heavy metals and essential nutrients, emphasizing the importance of understanding this interaction for plant growth and sustainability. The study's findings contribute to the broader understanding of how different factors affect plant growth and highlight the potential of Azolla in mitigating the impact of heavy metals.

4.2 Metal Removal Efficiency

Metal Removal Efficiency (MRE) is a crucial measure for assessing the effectiveness of a system or method in absorbing heavy metals from water and soil. A 100% efficiency indicates complete absorption by the plants. In the initial phase, introducing Azolla to different concentrations of Pb solution resulted in consistent MRE, with statistically insignificant differences between concentrations of 2.5 ppm and 5.0 ppm. Previous studies reported varying MRE values for Azolla, ranging from 79.7% to 93%, attributed to environmental factors, solution concentrations, and Azolla varieties by (Hazrat et al., 2019; Lenaduwa Lokuge, 2016; Taghilou et al., 2023b).

In contrast, during experimental phase 2 with durations of 7 and 14 days, Azolla displayed a 2% increase in MRE, reaching an overall efficiency of 96% to 98%. Statistical analysis showed no significant difference in means for the two durations. However, extending exposure from 7 to 14 days led to a significant increase in MRE, aligning with findings from indicating that longer exposure periods enhance metal removal efficiency (Naghipour et al., 2018).

Naghipour et al.'s research emphasized the influence of biomass and contact time on MRE. Increasing biomass from 0.4g to 0.8g over 10 days substantially improved MRE, ranging from 60.9% to 91.25%. Similarly, extending contact time from 10 to 15 days with a constant biomass of 0.8g further enhanced MRE to 91.25% to 95.41%. In the current study, a biomass of 1.0g was employed, and exposure days were extended by 7 to 14, revealing significant differences in MRE. All experimental conditions demonstrated higher MRE compared to prior studies, highlighting the importance of optimizing both biomass and contact time for efficient metal removal using Azolla. These findings contribute valuable insights for refining strategies in utilizing Azolla for effective heavy metal remediation.

4.3 Bio Concentration Factor

BCF is the how much heavy metals uptake into the plant biomass compare with available heavy metals present into the solution (Herath et al., 2023). BCF is used to measure the heavy metal accumulation efficiency in plants; BCF values >1 indicate a potential heavy metal hyperaccumulator species

(Maiti et al., 2021).

In phase 1 of the experiment, significant differences were observed in BCF values. At a concentration of 5.0 ppm, the BCF value was very high at 12.26 ± 1.03, while at concentrations of 2.5 ppm and 10.0 ppm, the BCF values reduced by 9.76 ± 3.56 and 4.54 ± 1.79, respectively. At a concentration of 2.5 ppm, the Pb concentration was insufficient for efficient absorption into the plants, while at a concentration of 10.0 ppm, metal stress affected Pb absorption. According to a study by M. S. , BCF increased significantly with an increase in concentration and exposure days (Akhtar et al., 2023).

The BCF in this study exhibits an increase as the duration of exposure to the substance increases, as indicated by the mean value in phase 2. However, the mean BCF for a concentration of 2.5 ppm in phase 1 is lower than that of 5.0 ppm. Other studies, such as the one conducted also support the notion that the *Azolla* acquires certain essential metals through additional experiments by (Akhtar et al., 2023; Akhtar et al., 2020). In this study, the growth of *Azolla* is hindered by the lower availability of essential metals, thus making it reliant on Pb for its growth. Consequently, when a lower concentration of Pb is sufficient for growth, it automatically reduces the BCF. Similarly, the higher concentration of 10.0 ppm proves to be intolerable for the plants compared to 2.5 ppm, resulting in lower BCF values in *Azolla*. The optimal concentration, as demonstrated by the BCF values in the phase 2 experiment, is 5.0 ppm.

During the Phase 2 experiment, it was observed that the BCF value increased when the exposure days were increased from 7 to 14 days, with values of 10.94 ± 0.13 and 13.72 ± 0.57, respectively. When Pb was introduced with another heavy metal Cr, the mixed metal composition showed a lower BCF value compared to Pb in its separate form. The combination of Pb and Cr exhibited a synergistic effect, indicating that they were less toxic when present separately, but when mixed, they showed a higher toxic level than before. Similar results were observed when Pb was mixed with other metals such as Ca, Fe, and Mg, where the targeted metal absorption was reduced due to the dominance of the other metal in that place. *Azolla* was found to absorb more of these metals than Pb. Although *Azolla* showed higher RGR and MRE compared to other factors in the mixed metal composition, the opposite was observed in the BCF case. The BCF values did not significantly change when the exposure days were increased from 7 to 14 days, indicating that the means were significantly the same.

4.4 Translocation Factor

The measurement of heavy metal transfer from one organ to another can be achieved through the utilization of TF (Takarina and Pin, 2017). In this study, the TF of *Azolla* is primarily evaluated in terms of the metal concentrations found in its shoot and the root. The ratio between the concentration of the shoot and root is examined to determine whether the absorbed metals are translocated from the *Azolla* roots to the shoots when this ratio is greater than 1.

In Phase 1, the TF values of 2.5 ppm, 5.0 ppm, and 10.0 ppm showed significant differences ($P<0.05$) among them, with respective values of 0.34 ± 0.09, 0.48± 0.15, and 0.26 ± 0.97. The TF exhibited a similar pattern to the BCF in Phase 1. It is possible that there was insufficient metal in the 2.5 ppm solution, while metal stress was observed in the 10.0 ppm solution.

In Phase 2, the experimental solution containing 5.0 ppm of Pb demonstrated a significant increase in translocation when the exposure period was extended from 7 to 14 days. The TF of Pb in combination with Cr decreased similarly as the BCF. Additionally, plant observations in this experiment indicated that plant discoloration occurred, suggesting chlorophyll degradation due to a synergistic effect. This phenomenon of chlorophyll degradation has been previously observed in studies where heavy metal concentrations were high, as reported by (Sheel et al., 2013).

The addition of various metals to Pb has been found to enhance the RGR, particularly when compared to Pb mixed with Cr. Furthermore, the TF also exhibits a significant increase in composition mixture as the exposure days increase. A study conducted by Naghipour *et al* (2018) also supports these findings, highlighting the substantial increases observed with prolonged exposure. Based on the daily observations, it was observed that after the 10th day of exposure, certain changes were noted in the Azolla. The color of the *Azolla* transformed from dark green to a purplish-yellow hue, while some shoots exhibited brownish dead parts. Additionally, the roots became detached from the shoots. These observations were recorded and analyzed as part of the daily observation data. The TF twofold mechanism enhances metal absorption while enhancing the translocation of metals from roots to shoots.

5. Conclusions

Phytoremediation is a highly effective and environmentally friendly approach used to remove heavy metals from contaminated sites, such as soil, water, and air. This method involves the utilization of specific hyperaccumulator plants that can absorb and detoxify targeted pollutants. Among the hyperaccumulator plants, *Azolla* stands out as an excellent option for the removal of toxic pollutants, including fluoride, ammonium, nitrogen phosphorous, dyes, and heavy metals. *Azolla* possesses unique characteristics that contribute to its high phytoremediation potential, such as the structure of its roots and shoots, its adaptability to different environments, and its symbiotic relationship with *Azolla anabaena*.

In a recent study, *Azolla* was proposed as a viable phytoremediation solution for the recovery and management of heavy metals in laboratory waste, even at lower concentrations. The efficiency of *Azolla* was particularly notable when dealing with Pb as a standalone metal. However, even in the presence of other heavy metals and metal compositions, *Azolla* still demonstrated significant removal capabilities. The study revealed that providing the optimum concentration for plant growth resulted in higher relative growth rates and a dark green appearance with increased chlorophyll content. Conversely, at higher concentrations, no significant growth was observed, and the plants exhibited signs of chlorophyll degradation, plant decomposition, and detachment of roots. Overall, the findings of this study emphasize the potential of *Azolla* phytoremediation for effectively managing heavy metal contamination, especially in scenarios where lead is present alongside other metals. However, careful consideration of concentration levels is necessary to ensure optimal plant growth and successful remediation.

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