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Screening of *Azolla caroliniana* for Metal Related Bio-Potential Factors

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Abstract: Heavy metals are widespread environmental contaminants that raise significant concerns due to their toxic, persistent, and non-biodegradable nature. Understanding the biological interactions of metals within plants is essential for the phytoremediation process, as it sheds light on the plants' ability to absorb metals, their movement within plant tissues, and their accumulation in above-ground biomass. This study aims to analyze the Bioconcentration Factor (BCF), Bioaccumulation Factor (BAF), Metal Enrichment Factor (MEF), and Metal Translocation Factor (MTF) of the floating macrophyte species Azolla caroliniana through a hydroponic bioassay.

The aquatic vascular plant Azolla caroliniana was examined for its capacity to remove heavy metals. A hydroponic bioassay utilizing a synthetic metal solution was conducted from June 2018 to December 2022, exposing Azolla caroliniana to a mixed metal solution to assess its ability to absorb, transfer, accumulate, and enrich heavy metals, thereby evaluating its potential for phytoremediation. The results of this study underscored the metal phytoremediation capabilities and accumulation patterns of ten heavy metals: As, Cd, Cr, Co, Cu, Fe, Mn, Ni, Pb, Sr, and Zn across different plant organs of Azolla caroliniana. The findings revealed that the order of BCF for the metals was Fe>Zn>Mn>Cu>Pb>Cd>Cr>Ni>Co>Sr>As, while the MEF order was Fe>Zn>Cu>Mn>Pb>Cd>Cr>Ni>Co>Sr>As. The ranking of BAF for the studied metals was Fe>Zn>Mn>Cu>Cd>Pb>Cr>Ni>Co>Sr>As, and the MTF order was Ni>Cu>Mn>Cd>Zn>Co>Pb>As>Cr>Fe>Sr. Importantly, Azolla caroliniana demonstrated hyperaccumulation for Zn, Fe, Mn, Cu, Cd, Pb, Ni, Co, Cr, Sr, and As, as indicated by a BCF greater than 1.

Keywords: *Aquatic Macrophytes, Azolla caroliniana, Heavy Metals, Bioconcentration, Biomagnification, Metal Transfer, Metal Enrichment, Phytoremediation.*

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I. INTRODUCTION

Rapid industrialization and urbanization have led to an increase in the emission of toxic heavy metals into the biosphere (Gazso, 2001). Activities such as mining and agriculture have contaminated extensive areas globally (Smith et al., 1996; Shallari et al., 1998). The Earth's crust naturally contains various metals, with their composition differing from one location to another, resulting in spatial variations in surrounding concentrations (Jaishankar et al., 2014). Common heavy metals found in wastewater include arsenic, copper, cadmium, chromium, lead, nickel, and zinc, all of which are highly toxic and pose significant risks to human health and the environment (Lambert et al., 2000). The release of heavy metals in biologically available forms due to human activities can harm or alter both natural and artificial ecosystems (Taylor et al., 1989).

Due to the limited availability of fresh water, farmers are compelled to utilize untreated wastewater as a substitute, which can lead to harmful effects on crops. The ability of plants to absorb heavy metals varies; some species accumulate higher levels of these metals than others, posing different health risks to humans through the food chain. Additionally, industrial wastewater, which is rich in organic matter, is often used for irrigation but is also tainted with heavy metals such as arsenic, cadmium, chromium, cobalt, copper, iron, manganese, nickel, lead, strontium, and zinc. Municipal wastewater represents the largest source available for irrigation, yet it contains a considerable amount of chemical pollutants, including heavy metals. Despite the challenges posed by various metallic species, wastewater continues to be applied to agricultural fields. While careful management can mitigate some of the negative impacts on plants and soil, risks remain. Therefore, it is essential to assess the contamination levels of water from various

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sources and to remove pollutants using appropriate macrophytes. This study will explore the effectiveness of *Azolla caroliniana* in the removal of heavy metals.

Heavy metals such as cadmium (Cd), lead (Pb), cobalt (Co), zinc (Zn), and chromium (Cr) are commonly found in wastewater and exhibit phytotoxicity at both low and high concentrations. When these metals accumulate in sediments, they can enter the food chain through aquatic plants and animals. While trace amounts of certain heavy metals are essential for a healthy life, excessive concentrations can lead to toxicity or poisoning. Recently, there has been a notable increase in cases of heavy metal pollution in the environment, attributed to the toxic nature and persistent presence of these metals in aquatic ecosystems (Tijaniet al., 2005). Heavy metal contamination is a global concern, although the severity and concentration of pollutants vary by region. At least 20 metals are classified as toxic, with half of them being released into the environment, posing significant risks to human health (Akpor and Muchie, 2010). It is crucial to remediate sites contaminated by heavy metals to mitigate these risks. Unlike organic compounds, metals cannot be degraded, and cleanup efforts typically involve their removal. Most conventional remediation methods are expensive and can diminish soil fertility, leading to further negative environmental impacts (Kumar et al., 2016).

Phytoremediation represents a cost-effective, environmentally friendly, and aesthetically pleasing method particularly suited for developing nations such as India. Ongoing research has led to the identification of various effective metal hyperaccumulators for use in phytoremediation and phytomining. Plants possess the ability to absorb non-essential metals, a trait that can be utilized to eliminate pollutant metals from the environment (Salt et al., 1995; Das et al., 1997; Rogers et al., 2000). There is currently significant interest in developing affordable and sustainable technologies for the remediation of soil and wastewater contaminated with hazardous heavy metals (Zayed et al., 1998). Bioremediation technologies that utilize plants have gained attention as viable methods for cleaning up contaminated soil and water (Sadowsky, 1999). Various plant species have been evaluated for their phytoremediation potential, with members of the Lemnaceae and Azollaceae families recognized as effective metal accumulators, making them suitable for reducing water pollution (Horvat et al., 2007; Rai, 2010). Floating and submerged macrophytes are particularly advantageous for the reduction and monitoring of heavy metals (Gupta and Chandra, 1998; Shingadgaon et al., 2018). Previous studies in wastewater treatment have demonstrated that aquatic macrophytes can partially accumulate or absorb trace metals found in wastewater (Chandra et al., 1993; Shingadgaon and Chavan). These macrophytes absorb heavy metals through their root systems and store them in a bound form, resulting in treated effluent that is less harmful to aquatic ecosystems. In areas contaminated with metals, plants are employed to stabilize and extract these metals from soil and groundwater through processes such as phytoextraction, rhizofiltration, and phytostabilization (Shingadgaon and Chavan; Kumar et al., 2019).

➢ *Necessity of the Current Study:*

There is a pressing requirement for alternative, cost-effective, and efficient methods to remediate heavily polluted industrial sites. Phytoremediation, which involves the use of plants to restore contaminated soil, water, and air, has emerged as a low-cost, non-invasive, and socially acceptable approach to mitigating environmental pollutants (Boyajian and Carreira, 1997; Singh et al., 2003). For developing nations like India, the potential of aquatic macrophytes is particularly significant, especially in regions where many shallow ponds and marshlands present unsuitable conditions for conventional fish farming and agriculture (Mohan Ram, 1978). Different plant species exhibit varying capacities to accumulate elements in their roots, stems, and/or leaves. Consequently, identifying the most effective trace element accumulator and the specific organ that absorbs the highest concentration of trace elements will be highly beneficial (Baldantoni et al., 2004).

The management of heavy metal pollution through contemporary machinery is prohibitively costly for many developing nations, such as India, which may struggle to afford the substantial expenses associated with such treatments (Rai and Tripathi, 2007; Rai, 2008). This research aims to explore the potential of *Azolla caroliniana* in remediating various heavy metals and its effectiveness in purifying contaminated aquatic environments. The findings will contribute to the advancement of phytoremediation technologies and their application in addressing heavy metal pollution through diverse methods. The primary objective of this study is to evaluate the efficacy of the floating macrophyte *Azolla caroliniana* in the phytoremediation of metal-laden effluents, thereby providing insights into effective remediation strategies. Research involving hyperaccumulator plants, which are known for their ability to absorb heavy metals, is essential for mitigating the environmental impacts of these pollutants.

II. MATERIALS AND METHODS

A. Overview of Azolla caroliniana Macrophyte:

The aquatic fern *Azolla caroliniana* Willd. (Azollaceae) is a diminutive plant prevalent in various regions globally, particularly in tropical climates (Watanbe et al., 1992). A distinctive characteristic of this fern is its symbiotic relationship with the cyanobacterium Anabaena azollae Strasb. (Nostoceae), which has the capability to fix atmospheric nitrogen. Consequently, Azolla species are utilized as green manure, especially in Asian rice paddies (Carrapiço, 2001).

In addition to its primary use, the fern has several other applications (Bennicelli et al., 2004), one of which involves the bioaccumulation of heavy metals. Azolla caroliniana, known for its rapid growth, exhibits a remarkable capacity to absorb and accumulate heavy metals, positioning it as a viable option for the phytoremediation of contaminated water and soil, particularly for metals such as Cu, Cd, Cr, Ni, and Pb. Research has demonstrated that Azolla species can bind various metals, including Zn, Pb, Cu, Cd, Au, Ni, Sr, Cr, and Hg (Gaur and Noraho, 1995; Sanyahumbi et al.,

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1998; Antunes et al., 2001; Cohen-Shoel et al., 2002; Bennicelli et al., 2004). Furthermore, this fern is capable of removing nutrients (Forni et al., 2001) and organic compounds like sulphonamides (Forni et al., 2002). The mosquito/water fern (Azolla) is a small, free-floating species widely found in rice fields, rivers, ponds, and lakes. Its ability to fix nitrogen through its symbiotic association with Anabaena, which inhabits the dorsal cavity of Azolla fronds, has led to its use as green manure to enhance soil fertility and boost rice yields (Wagner, 1997). Reports indicate that Azolla possesses a significant capacity to accumulate hazardous elements such as mercury, cadmium, chromium, copper, nickel, and zinc (Rai, 2008; Rai and Tripathi, 2009), and can effectively remove pollutants from wastewater (Bennicelli et al., 2004; Arora and Saxena, 2005; Rakhshaee et al., 2006).

B. Collection and Sampling of Azolla caroliniana:

Natural water bodies, encompassing both stagnant and flowing waters as well as reservoirs, were examined for the presence of *Azolla caroliniana*. The macrophytes were carefully observed, sampled, and collected from the Marathwada study area without causing any damage. Sampling was conducted using appropriate scientific methods, followed by rinsing with water and careful wrapping in paper. The specimens collected were fresh and green, placed in suitably sized transparent polyethylene bags, with each sample containing a minimum of ten healthy macrophytes. These were replanted within four to five hours of collection to acclimatize in metal-mixed synthetic wastewater within rectangular test chambers measuring 1 meter by 1 meter and 10 cm deep, ensuring a 2 cm freeboard.

Additionally, a separate set of samples was taken to the laboratory, where they were rinsed with gently flowing tap water while following necessary safety protocols, air-dried, and identified using various literature sources for qualitative floristic data. This identification focused on species and community characteristics rather than merely their physical structures and appearances. Photographs and samples were sent to botanical research specialists in the relevant fields for verification and confirmation of the identified information, including essential details, before drawing final conclusions, thus providing an authentic and supportive second opinion. The macrophyte *Azolla caroliniana* was selected for this study due to its local abundance, facilitating collection from any area within the study region for further research.

C. Studies on Metal Accumulation Potential:

Macrophytes were selectively pruned by removing those that were either infected or unhealthy. The remaining healthy specimens were then transferred to a laboratory-scale test bath. These test baths, made from small plastic containers, were intended to evaluate the macrophytes' adaptability to the new environmental conditions. The chosen healthy macrophytes were exposed to the local climatic conditions for a sufficient period to ensure they acclimatized and reached full growth in a synthetic wastewater test bath containing metals. In this research, the acclimatization phase was established at one month. Growth persisted for an additional month in a synthetic wastewater test bath mixed with metals, using stock solutions at concentrations of 100 ppm. The bath solution was freshly prepared to contaminate tap water, facilitating the periodic creation of the synthetic wastewater test bath, along with a control group that did not contain synthetic wastewater. Various metal salts, including $Na_3AsO_4 \cdot 12H_2O$, $CuSO_4 \cdot 8H_2O$, PbO, $FeSO_4 \cdot 7H_2O$, $CdSO_4 \cdot 8H_2O$, $NH_4SO_4 \cdot NiSO_4 \cdot 6H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, $Sr(OH)_2$, and $MnCl_2 \cdot 4H_2O$, were utilized according to established standard procedures (AOAC, 1975; APHA, 1980; Echem, 2014; Smith, 1983). Following the designated growth period, the macrophyte plants were harvested and analyzed to determine their capacity for metal accumulation, with adjustments made to account for the control group results using normal water. This assessment aimed to evaluate their potential and suitability for application in phytoremediation processes. The complete methodology employed for assessing heavy metal absorption capabilities is outlined below:

The plant materials of *Azolla caroliniana* were initially rinsed with tap water before being transported for laboratory analysis. They underwent a second wash with gently flowing tap water, followed by a rinse with double distilled water to eliminate any dirt and impurities. The macrophyte samples were then air-dried and cut with stainless steel scissors to separate the shoots, roots, stems, and leaves. Each of these components was oven-dried at 60 °C until a constant weight was achieved, then ground in a pestle and mortar to create a homogeneous mixture, which was subsequently stored for analysis. For the determination of metal content, 500 mg of each plant material was digested using H_2O_2 and H_2SO_4. The resulting digested aliquot was analyzed for various heavy metals using appropriate methods. The heavy metals from the prepared aliquot were examined using Atomic Absorption Spectroscopy (AAS) and Gas Chromatography (GC) in other laboratories, based on sample and metal-specific rates, or through suitable methods referenced from credible literature or research publications, depending on the availability of necessary facilities for the required analysis. The methods employed for analysis encompassed a variety of techniques, including the Cobalt by cobaltous pyridine method as outlined by Nicolaysen (1941), the Iron (Fe) analysis via the Dichromate method, and the Zinc (Zn) assessment using the EDTA Complexometry-Back Titration method (Tazul and Ahemad, 2013). Manganese (Mn) was analyzed using Volhard’s method, while Copper (Cu) was determined through Sodium Thiosulphate titration, with confirmation provided by the Spectrophotometric method (Ahmed and Zannat, 2012). Lead (Pb) was assessed using the EDTA Complexometric method, and Manganese (Mn) was also analyzed via the Periodate Oxidation Method. Chromium was evaluated using the Diphenylcarbazide Spectrophotometric method (IBM, 2012), and Cadmium (Cd) was analyzed through a spectrophotometric approach (Ahamed and Chowdhury, 2004). Additionally, Chromium (Cr) was assessed using the Diphenylcarbazide Method (Yarbro, 1976), and Cobalt (Co) was analyzed through a colorimetric method (Hobart, 1920).

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A series of samples was processed and digested utilizing an automated program within the NuWav-Ultra Microwave Digestion Extraction System. The analysis of metal contents, including arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), strontium (Sr), and zinc (Zn), was conducted in a separate laboratory. This was achieved using the Shimadzu Atomic Absorption Spectrometer model 6300 and the Agilent 725 ICP-OES instrument for verification purposes, as necessary. The majority of the results were corroborated through random cross-checks against recognized standard methods to ensure both analytical simplicity and precision, as cited in numerous studies (Bendix and Grabenstetter, 1943; Kimura and Murakani, 1951; Sandall, 1965; Hackley et al., 1968; Loftberg, 1969; Rubeska, 1969; Baker et al., 1971; James and MacMohan, 1971; Song et al., 1976; Sarma et al., 2005; Soomro and Menon, 2009; Ahemad and Roy, 1969; Soomro and Shar, 2014; Wei, 2014).

D. Chemicals and Reagents

All chemicals, reagents, and solvents used in this study were of analytical reagent grade or exhibited the highest purity levels, and they were freshly prepared before use. Doubly distilled water was consistently utilized throughout the experimental processes. The glassware was thoroughly cleaned by soaking in acidified solutions of potassium permanganate ($KMnO_4$) or potassium dichromate ($K_2Cr_2O_7$), followed by treatment with concentrated nitric acid (HNO_3) and multiple rinses with doubly distilled water. Calibration curves, serving as standard references for solutions with known metal concentrations, were created. These curves were then employed to determine the concentrations of substances in unknown samples. The calibration curves for heavy metals were specifically utilized in analyzing the concentrations within the test samples.

E. Evaluation of Factors Influencing Heavy Metal Mobility Potential:

The ability of *Azolla caroliniana* macrophytes to promote the transfer of heavy metals from contaminated substrates into their root systems, along with their capacity to accumulate these metals in various plant tissues, was examined. This analysis included the movement of metals from the roots to the above-ground, harvestable portions of the plant and assessed the potential for metal accumulation. Various metrics were utilized in this evaluation, such as the bio-concentration factor (BCF), bioaccumulation factor (BAF), metal translocation factor (MTF), and metal enrichment factor (MEF). These indicators were employed to assess the feasibility of utilizing *Azolla caroliniana* macrophytes for phytoremediation, providing valuable insights into their effectiveness in rehabilitating metal-contaminated environments.

➢ *Bioaccumulation Factor (BAF):*

The bioaccumulation factor (BAF) measures the extent to which a substance, especially a heavy metal, accumulates in an organism or biological system. In the context of heavy metals, the BAF is expressed as the ratio of the concentration of the heavy metal found in the organism to that in the surrounding environment. The Bioaccumulation Factor (BAF) can be calculated using this formula:

- BAF = [Concentration of heavy metal in aerial parts] / [Concentration of heavy metal in the source]
  For specific metal, it can be expressed as;
- BAF_{Metal} = [Metal content in aerial parts] / [Metal Concentration in Source]
  $= C_{AP}/C_{WW}$

Where BAF_{Metal} stands for bioaccumulation factor for a specified metal, C_{AP} represents specific Metal Concentration in aerial parts expressed in mg/kg and C_{WW} represents Metal Concentration in growth environment-source like wastewater expressed in mg/kg.

➢ *Bioconcentration Factor (BCF):*

The bioconcentration factor (BCF) is characterized as the ratio of the total concentration of metals present in plant roots to that found in the surrounding environment, which may consist of contaminated soil or wastewater (Elkhatib et al. 2001; Gonzalez & Gonzalez-Chavez 2006; Yoon et al. 2006). The BCF serves to measure the extent to which a chemical, especially heavy metals, accumulates within an organism or biological system from its environment. This metric reflects the capacity of plants to uptake metals from the soil (Kamari et al., 2014). The BCF is calculated using the following equation:

- BCF = [Concentration of heavy metal in the organism] / [Concentration of heavy metal in water]

The bioconcentration factor (BCF) for macrophytes was evaluated using the formula proposed by Demina et al. (2009), expressed as follows: B.C.F = Concentration of the element in the plant / Concentration of the element in the water. The bio-concentration factor (BCF) for a particular metal is determined using the equation below:

- BCF_{Metal} = [Metal Concentration in root] / [Metal Concentration in Source]
  = CR / CWW

Where BCF_{Metal} stands for bio-concentration factor for a specified metal, C_R represents specified Metal Concentration in root expressed in mg/kg and C_{WW} represents Metal Concentration in growth environment-source like wastewater expressed in mg/kg, wherein for this particular study the metal refers to any of the all eleven metals studied.

➢ *Metal Enrichment Factors (MEF)*

Metal Enrichment Factors (MEF) were utilized to evaluate the extent of metal contamination in sediment, following the methodology established by Buat-Menard and Chesselet (1979). A widely recognized method for assessing anthropogenic influence involves the computation of a normalized enrichment factor (EF), which is derived from metal concentrations obtained from uncontaminated background levels, as noted by Salomons and Forstner (1984), Dickinson et al. (1996), and Hornung et al. (1989).

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The calculation of the EF aims to minimize the variability in metal concentrations that may arise from differing source ratios, thus functioning as a valuable analytical tool. This method normalizes the measured concentrations of heavy metals against a reference metal, such as iron (Fe) or aluminum (Al), as described by Ravichandran et al. (1995).

- MEF_{Metal}=[Metal content in only shoot]/ [Metal Concentration in Source]
  = C_{OS}/C_{WW}

Where MEF_{Metal} stands for specific metal enrichment factor, C_{OS} represents specified Metal content in only shoot expressed in mg/kg and C_{ww} represents Metal Concentration in growth environment-sources like wastewater expressed in mg/kg.

- *Metal Translocation Factor (MTF):*

The metal translocation factor (MTF) is defined as the ratio of the total metal concentration in the shoots to that in the roots (Mocko and Waclawek 2004; Yoon et al. 2006; Sanghamitra et al. 2012). This factor indicates the relative concentration of metals in the shoots compared to the roots. An MTF value exceeding 1 suggests that the plant is proficient in transporting metals from the root system to the shoots (Rezvani and Zaefarian, 2011). The metal transfer factor serves as a valuable metric for assessing the mobility of metals from their origin to macrophytes. The MTF for particular metals can differ considerably depending on the species of macrophytes and the prevailing environmental conditions. Important factors that affect MTF include the physical and chemical characteristics of the source, the behavior of trace metals in both the source and the macrophytes, and fluctuations in environmental conditions. The transfer factor from soil to plants is calculated by determining the ratio of metal concentration in the plants to that in the source (Kumar et al., 2015; Akande and Ajayi, 2017; Ogoko, 2015).

- MTF_{Metal} = [Metal Content in only shoot] / [Metal Concentration in root]
  =C_{OS}/C_R

Where MTF_{Metal} stands for specific metal translocation factor, C_s represents Metal content in aerial parts expressed in mg/kg and C_r represents Metal Concentration in root expressed in mg/kg. It is also called as shoot-root quotient and may be denoted as MTF in general.

III. RESULTS AND DISCUSSIONS

A defining feature of vascular plants is their ability to accumulate metals and other elements beyond their physiological requirements (Epstein, 1972). This trait has prompted researchers to investigate the capacity of various aquatic plants to absorb pollutants, including nutrients, from both raw and treated sewage as well as other aquatic environments (Dymond, 1948; Sutton and Ornes, 1975; Sutton and Ornes, 1977; Wolverton and McDonald, 1978; Sioey et al., 1978). One of the most prevalent aquatic vascular plants found in freshwater littoral zones worldwide is the Mosquito Fern (Azolla caroliniana Willd). In these shallow aquatic ecosystems, it frequently serves as the dominant macrophyte species (Ornes and Wildman, 1979). Despite Azolla's relatively limited root system, its potential as a biological filter for effluent treatment has yet to be evaluated. Specifically, there is a lack of information regarding the extent to which Water Fern can absorb metals like cadmium from wastewater and its significance within aquatic food webs. Understanding the role of metals in aquatic ecosystems is crucial for fisheries and wildlife management, as well as for setting legal limits for these metals in natural surface and groundwater.

Various pollutants are present in wastewater originating from multiple sources, including industrial discharges, trade effluents, and municipal sewage, which ultimately contaminate water bodies. Among these pollutants, both organic and inorganic substances, particularly heavy metals, pose significant risks to aquatic life and human health as they can enter the food chain through bioaccumulation. Elevated concentrations of trace metals in plant tissues can have harmful toxic effects on animals, especially when consumed. For example, zinc and copper are trace metals essential for healthy growth in small amounts. While they are necessary for the growth of plants and the nutrition of animals and humans, excessive levels can result in phytotoxicity in plants and zootoxicity in animals (Osundiya et al., 2014; Shingadgaon and Chavan, 2019).

The potential of macrophytes to absorb heavy metals from contaminated growth media into their roots, as well as their capacity to accumulate these metals in various plant parts and translocate them from roots to the harvestable aerial portions, was assessed using the bioconcentration factor (BCF), bioaccumulation factor (BAF), metal translocation factor (MTF), and metal enrichment factor (MEF). This evaluation aims to determine the feasibility of utilizing native macrophyte species for phytoremediation, providing valuable insights for the remediation of metal-contaminated sites.

Heavy metals have emerged as pervasive pollutants on a global scale, raising considerable environmental issues due to their ability to persist in non-degradable forms. These metals can exert toxic effects on ecosystems by entering the food chain, thereby posing multiple risks to human health (Chopra et al., 2009; Roberts, 1999; WHO, 2011). Their presence is detectable in contaminated aquatic environments, from the sediments at the bottom to the surface waters. The behavior of heavy metals in both water and wastewater is affected by several factors, including sediment composition, water chemistry, salinity, redox potential, and pH levels (Connell et al., 1984). This scenario underscores the need for the use of floating or free-floating macrophytes to remediate water and wastewater across different depths.

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A. Uptake Potentials of Metals in Azolla caroliniana:

The aquatic macrophyte *Azolla caroliniana* demonstrated differences in metal concentrations, indicating varying abilities to absorb each metal. This observation aligns with earlier studies (Freitas et al. 2004; Nouri et al. 2009; Nazareno & Buot 2015; Jones and Buot, 2017). Table 1 provides a comprehensive overview of the metal concentrations found in the roots, shoots, and aerial components of *Azolla caroliniana*.

Table 1: Concentration of Metals in Various Sections of the *Azolla caroliniana* Species

Macrophyte species	Metal	Conc. in roots (µg/g)	Conc. in shoots (µg/g)	Conc. in aerial parts (µg/g)
Azolla caroliniana	As	127	67	54
	Cd	2424	1423	1153
	Cr	1324	684	523
	Co	328	176	111
	Cu	3218	2375	1756
	Fe	5754	2855	2287
	Mn	3877	2353	1768
	Ni	366	278	175
	Pb	2768	1478	1109
	Sr	210	97	56
	Zn	4753	2768	2166

The roots exhibited the highest levels of iron (Fe), recorded at 5754 µg/g, followed by zinc (Zn) at 4753 µg/g. Manganese (Mn) was measured at 3877 µg/g, while copper (Cu) was found at 3218 µg/g. Lead (Pb) concentrations were at 2768 µg/g, cadmium (Cd) at 2424 µg/g, and chromium (Cr) at 1324 µg/g. The lowest concentrations of arsenic (As), strontium (Sr), cobalt (Co), and nickel (Ni) in the roots were 127 µg/g, 210 µg/g, 328 µg/g, and 366 µg/g, respectively. Figure 1 illustrates a comparative analysis of metal concentrations across the roots, shoots, and aerial parts of this macrophyte species. The order of metal accumulation in the roots of *Azolla caroliniana* was established as Fe > Zn > Mn > Cu > Pb > Cd > Cr > Ni > Co > Sr > As.

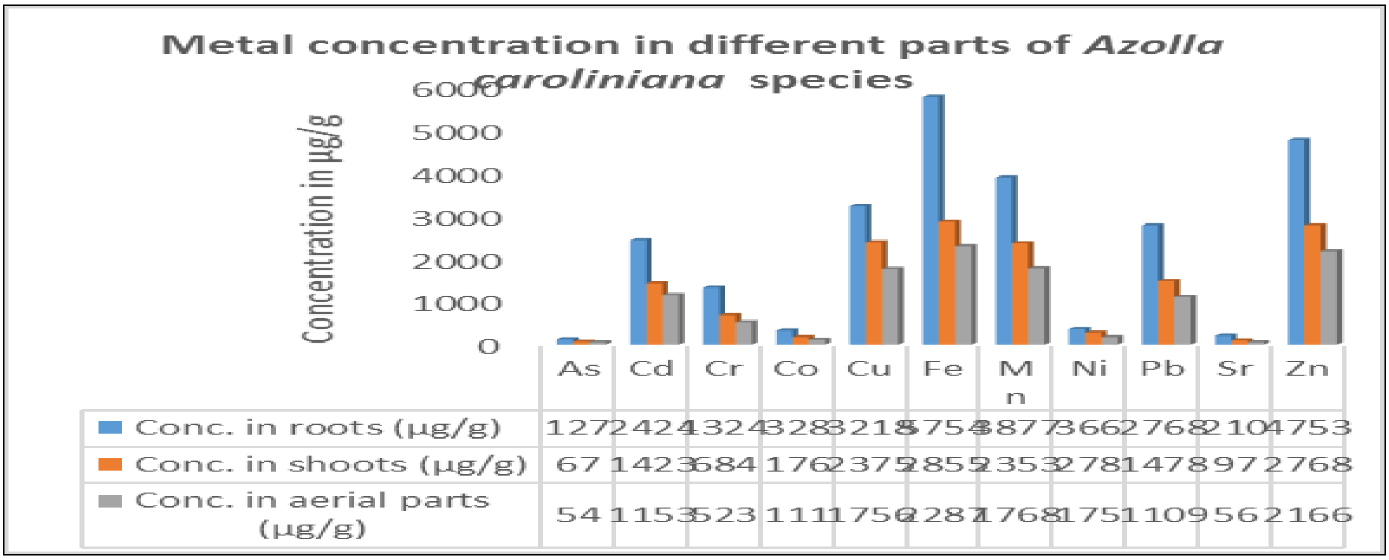

Fig 1: Comparative Levels of Heavy Metals in Various Sections of *Azolla Caroliniana*.

In comparison, the concentration of metals in the shoots of *Azolla caroliniana* was generally less than that found in the roots for all metals assessed. The highest level of iron (Fe) detected in the shoots was 2855 µg/g, followed by zinc (Zn) at 2768 µg/g, copper (Cu) at 2375 µg/g, and manganese (Mn) at 2353 µg/g. Additional metal concentrations in the shoots included lead (Pb) at 1478 µg/g, cadmium (Cd) at 1423 µg/g, chromium (Cr) at 684 µg/g, nickel (Ni) at 278 µg/g, cobalt (Co) at 176 µg/g, strontium (Sr) at 97 µg/g, and arsenic (As) at 67 µg/g, which represented the lowest concentration among the measured metals in the shoots. The sequence of metal accumulation in the shoots varied from that in the roots, with the order being Fe > Zn > Cu > Mn > Pb > Cd > Cr > Ni > Co > Sr > As.

The accumulation of metals in the aerial parts of *Azolla caroliniana* was observed to be lower than in both the roots and shoots, as shown in Table 1. Among the metals, iron had the highest concentration in the aerial parts, recorded at 2287 µg/g, followed by zinc at 2166 µg/g, manganese at 1768 µg/g, copper at 1756 µg/g, cadmium at 1153 µg/g, lead at 1109 µg/g, chromium at 523 µg/g, nickel at 175 µg/g,

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cobalt at 111 µg/g, strontium at 56 µg/g, and arsenic at 54 µg/g, which was the lowest concentration among the metals accumulated in the aerial parts. The ranking of metal accumulation in the aerial portions of *Azolla caroliniana* is as follows: Fe > Zn > Mn > Cu > Cd > Pb > Cr > Ni > Co > Sr > As.

The Metal Transport Factor (MTF) denotes the internal capability of a plant to transport metals, as outlined by Nouri et al. (2009). According to Yoon et al. (2006), both the Bioaccumulation Factor (BCF) and MTF are essential for evaluating a plant's effectiveness in metal phytoremediation. The BCF quantifies a plant's ability to accumulate metals within its roots, whereas the MTF assesses the plant's capacity to transfer these metals from the roots to its above-ground structures. Plants with BCF values below one are considered ineffective for phytoextraction (Yoon et al. 2006). In contrast, plants that demonstrate both BCF and MTF values greater than one (BCF>1, MTF>1) are regarded as suitable for phytoextraction. Furthermore, plants exhibiting a BCF greater than one and an MTF less than one (BCF>1 and MTF<1) are acknowledged for their potential in phytostabilization. A hyperaccumulator plant is defined by having either a BCF or MTF exceeding one, along with total metal accumulation surpassing 1000 mg kg−1 for Cu, Co, Cr, or Pb, or exceeding 10000 mg kg−1 for Fe, Mn, or Zn (Kabata-Pendias 2011).

In this study, the bioconcentration factor, metal enrichment factor, bioabsorption factor, and metal transfer factor for each metal tested were evaluated for the aquatic plant *Azolla caroliniana*, as detailed in Table 2, with a comparative graphical representation provided in Fig. 2.

Table 2: Bioconcentration Factor (BCF), Bioaccumulation Factor (BAF), Metal Enrichment Factor (MEF), and Metal Translocation Factor (MTF) for the Free-Floating Macrophyte Species *Azolla caroliniana* in a Phytoremediation Bioassay

Macrophyte Species	Metal	BCF	MEF	BAF	MTF
Azolla caroliniana	As	1.27	0.67	0.54	0.5276
	Cd	24.24	14.23	11.53	0.5870
	Cr	13.24	6.84	5.23	0.5166
	Co	3.28	1.76	1.11	0.5366
	Cu	32.18	23.75	17.56	0.7383
	Fe	57.54	28.55	22.87	0.4962
	Mn	38.77	23.53	17.68	0.6069
	Ni	3.66	2.78	1.75	0.7596
	Pb	27.68	14.78	11.09	0.5340
	Sr	2.10	0.97	0.56	0.4619
	Zn	47.53	27.68	21.66	0.5824

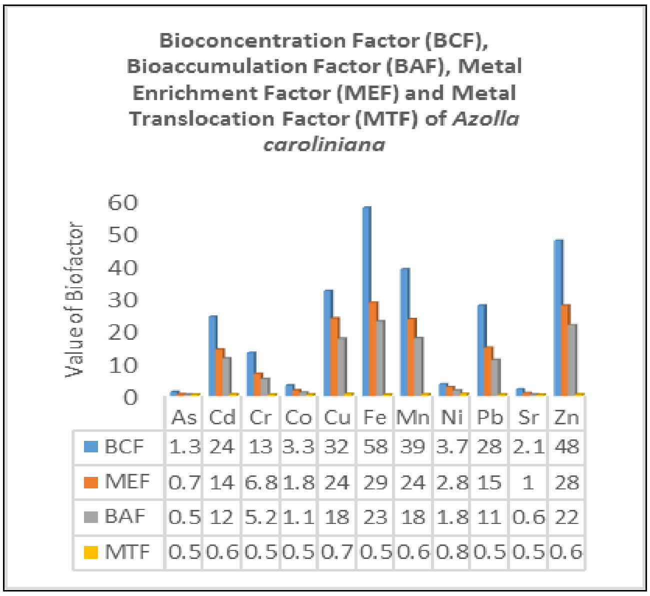

Fig 2: A Comparative Overview of BCF, BAF, MEF, and MTF in Azolla Caroliniana

The bioconcentration factor (BCF) in *Azolla caroliniana* for iron (Fe) was found to be the highest at 57.54, followed by zinc (Zn) with a BCF of 47.53, and manganese (Mn) at 38.77. Copper (Cu) recorded a BCF of 32.18, while lead (Pb) had a BCF of 27.68. Cadmium (Cd) showed a BCF of 24.24, and chromium (Cr) had a BCF of 13.24. Nickel (Ni) was measured at 3.66, cobalt (Co) at 3.28, strontium (Sr) at 2.10, and arsenic (As) at 1.27. The BCF values examined in this study are illustrated in Fig. 3. The metals are ranked according to their bioconcentration factors as follows: Fe > Zn > Mn > Cu > Pb > Cd > Cr > Ni > Co > Sr > As.

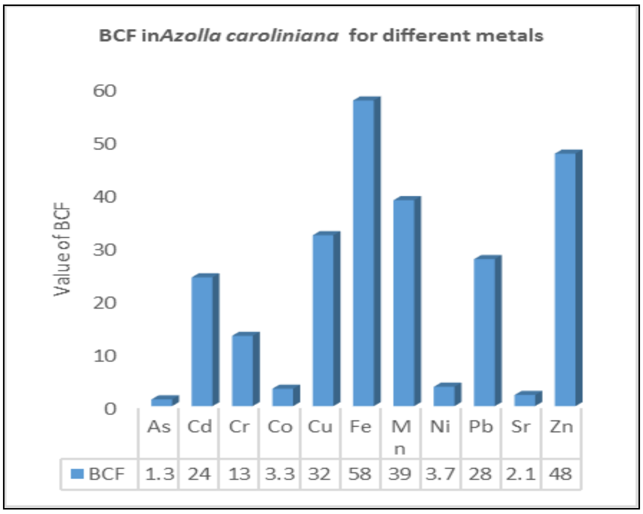

Fig 3: Comparisons of BCF Values of Metals in *Azolla caroliniana*

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The metal enrichment factor (MEF) serves as a valuable metric for evaluating the extent of heavy metal accumulation in environmental substrates such as soil or sediment. This assessment is achieved by comparing the concentration of a specific metal in a sample to that of a reference element, which aids in identifying potential sources of pollution. Figure 4 illustrates the comparative MEF values for *Azolla caroliniana* across all metals analyzed. The MEF indicates the ability of macrophytes to absorb metals, with iron (Fe) exhibiting the highest MEF at 28.55 among the metals studied. This is followed by zinc (Zn) with an MEF of 27.68, copper (Cu) at 23.75, manganese (Mn) at 23.53, lead (Pb) at 14.78, cadmium (Cd) at 14.23, chromium (Cr) at 6.84, nickel (Ni) at 2.78, cobalt (Co) at 1.76, strontium (Sr) at 0.97, and arsenic (As) at 0.67. The ranking of MEF values for the examined metals is as follows: Fe > Zn > Cu > Mn > Pb > Cd > Cr > Ni > Co > Sr > As.

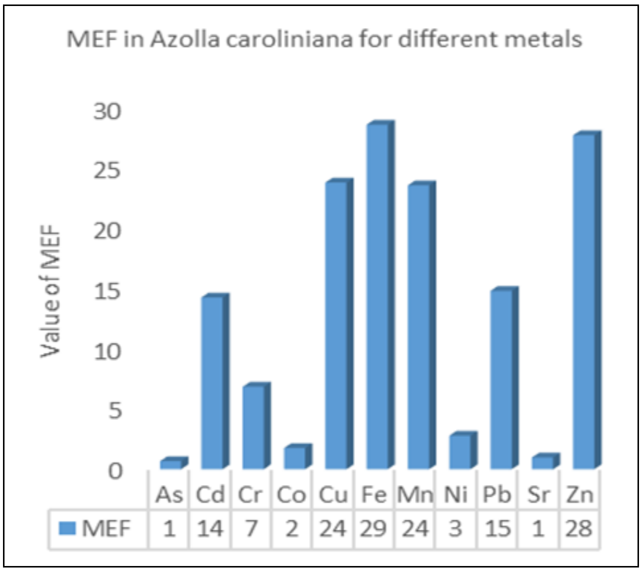

Fig 4: Comparison of Metal Enrichment Factors in *Azolla Caroliniana* across Various Studied Metals

The bioaccumulation factor (BAF) measures the degree to which a substance accumulates within an organism relative to its concentration in the surrounding environment, taking into account all possible exposure routes. It is determined by the ratio of the concentration of the substance in the organism (Cb) to its concentration in the water phase (Cw). Figure 5 illustrates the comparison of BAF values for the metals examined. In *Azolla caroliniana*, the highest BAF was observed for iron (Fe), with a value of 22.87, followed closely by zinc (Zn) at 21.66, manganese (Mn) at 17.68, copper (Cu) at 17.56, cadmium (Cd) at 11.53, lead (Pb) at 11.09, chromium (Cr) at 5.23, nickel (Ni) at 1.75, cobalt (Co) at 1.11, strontium (Sr) at 0.56, and arsenic (As) at 0.54. The ranking of BAF values for the metals analyzed is as follows: Fe > Zn > Mn > Cu > Cd > Pb > Cr > Ni > Co > Sr > As.

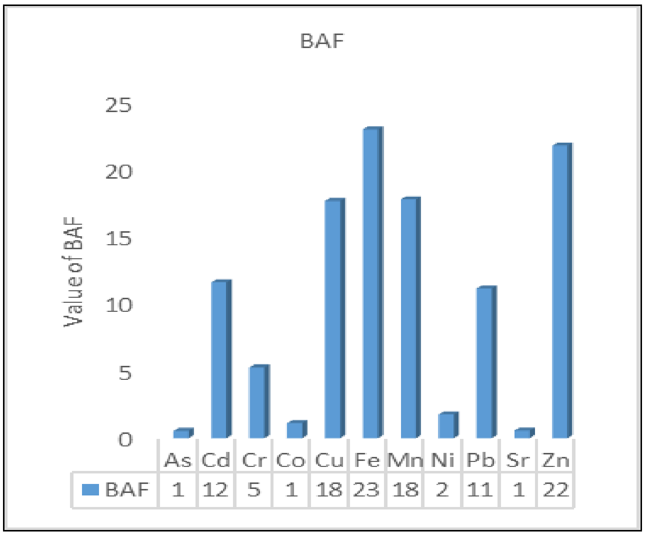

Fig 5: Comparison of Bioaccumulation Factors in *Azolla caroliniana* for Various Metals Examined

Metal Transfer Factor (MTF), also known as Transfer Factor (TF), serves as an indicator of the movement of metals from the growth environment into plants. MTF quantifies the ability of crop plants to transport accumulated heavy metals from one organ to another within the plant. The process governing MTF is intricate, influenced by various factors including metal concentration, interactions, pH levels, and pollution load. The current research presents a graphical comparison of metal transfer factors in *Azolla caroliniana*, as illustrated in Fig. 6. Unlike the metal enrichment factor (MEF), the MTF displayed a distinct trend. The highest MTF was recorded for nickel (Ni) at 0.7596, followed by copper (Cu) at 0.7383, manganese (Mn) at 0.6069, cadmium (Cd) at 0.5870, zinc (Zn) at 0.5824, cobalt (Co) at 0.5366, lead (Pb) at 0.5340, arsenic (As) at 0.5276, chromium (Cr) at 0.5166, iron (Fe) at 0.4962, and strontium (Sr) at 0.4619. The order of MTF for the metals examined is as follows: Ni > Cu > Mn > Cd > Zn > Co > Pb > As > Cr > Fe > Sr.

Bioconcentration indicated the absorption of a metal by *Azolla caroliniana* directly from a synthetic water bath, whereas bioaccumulation referred to the accumulation of metal following its uptake from the surrounding environment. Enrichment factors assessed the extent of metal transfer from the environment to *Azolla caroliniana*, while transfer factors evaluated the distribution of metals within various parts of the plant. The findings overall imply that the fern *Azolla caroliniana* has potential for the remediation of water contaminated with heavy metals through phytoremediation.

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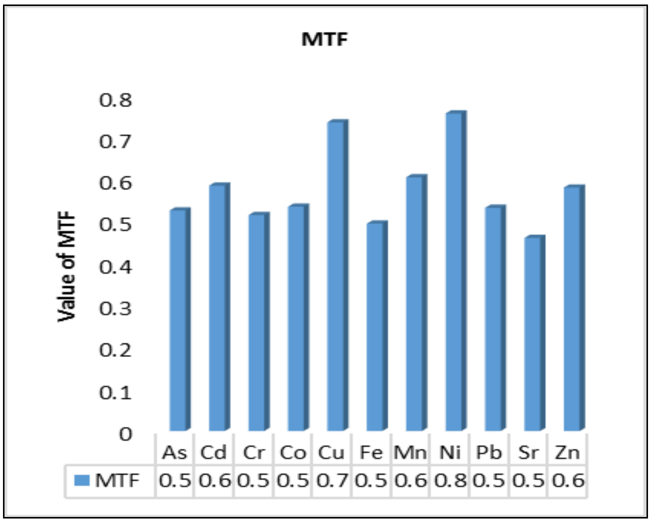

Fig 6: Comparison of MTF for the Metals Studied in *Azolla caroliniana*

IV. CONCLUSIONS

The presence of metals in various parts of the *Azolla caroliniana* plant demonstrates its ability to absorb these metals without exhibiting phytotoxic effects. The results from phytoremediation bioassays indicate that the bioconcentration factor (BCF), bioaccumulation factor (BAF), metal enrichment factor (MEF), and metal translocation factor (MTF) for the macrophyte *Azolla caroliniana* highlight its potential for treating metal-contaminated wastewater. The BCF values for the metals analyzed are ranked as follows: Fe > Zn > Mn > Cu > Pb > Cd > Cr > Ni > Co > Sr > As. The MEF ranking is also: Fe > Zn > Cu > Mn > Pb > Cd > Cr > Ni > Co > Sr > As. For the BAF, the order is Fe > Zn > Mn > Cu > Cd > Pb > Cr > Ni > Co > Sr > As, while the MTF ranking is Ni > Cu > Mn > Cd > Zn > Co > Pb > As > Cr > Fe > Sr. The *Azolla caroliniana* species functions as a hyperaccumulator for all the metals examined in this study, as indicated by BCF values greater than 1 for each metal.

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