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REVIEW PAPER

Phytoremediation Potential of Aquatic Macrophyte, *Azolla*

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Abstract Aquatic macrophytes play an important role in the structural and functional aspects of aquatic ecosystems by altering water movement regimes, providing shelter to fish and aquatic invertebrates, serving as a food source, and altering water quality by regulating oxygen balance, nutrient cycles, and accumulating heavy metals. The ability to hyperaccumulate heavy metals makes them interesting research candidates, especially for the treatment of industrial effluents and sewage waste water. The use of aquatic macrophytes, such as *Azolla* with hyper accumulating ability is known to be an environmentally friendly option to restore polluted aquatic resources. The present review highlights the phytoaccumulation potential of macrophytes with emphasis on utilization of *Azolla* as a promising candidate for phytoremediation. The impact of uptake of heavy metals on morphology and metabolic processes of *Azolla* has also been discussed for a better understanding and utilization of this symbiotic association in the field of phytoremediation.

Keywords *Azolla* · Bioaccumulation · Biosorption · Phytoremediation · Toxicity

INTRODUCTION

Rapid industrialization, urbanization, and population in the last few decades have added huge loads of pollutants in the water resources (CPBC 2008). Such unprecedented pollution in aquatic ecosystems needs eco-friendly cost-effective remediation technology. A large number of industries including textile, paper and pulp, printing, iron–steel, electroplating, coke, petroleum, pesticide, paint, solvent, and pharmaceutical etc. consume large volumes of water and organic chemicals which differ in their composition and toxicity. The discharge of effluents from these industrial units to various water bodies (rivers, canals, and lakes etc.) leading to water pollution is a matter of great concern, especially for developing countries like India. Developed countries have water pollution problems mainly due to industrial proliferation and modern agricultural technologies, which are mainly addressed through improving wastewater treatment techniques. However, the lack of technical knowhow, weak implementation of environmental policies, and limited financial resources has given rise to serious challenges.

Among various water pollutants, heavy metals are of major concern because of their persistent and bio-accumulative nature (Rai et al. 1981; Lokeshwari and Chandrappa 2007; Chang et al. 2009; Yadav et al. 2009). Water is an indispensable part for the sustenance of mankind and the increasing awareness about the environment, especially aquatic ecosystems have attracted the attention of researchers worldwide. A definite need exists to develop a low cost and eco-friendly technology to remove pollutants particularly heavy metals, thereby improving water quality. Phytoremediation offers an attractive alternative. Among these, *Azolla,* a free-floating, fast growing, and nitrogen fixing pteridophyte seems to be an excellent candidate for removal, disposal, and recovery of heavy metals from the polluted aquatic ecosystems (Arora et al. 2006; Umali et al. 2006).

In India, where most of the developmental activities are still dependent upon water bodies, heavy metal pollution is posing serious environmental and health problems (Sánchez-Chardi et al. 2009; Siwela et al. 2009). Heavy metals are metallic chemical elements with a high atomic weight and density much greater (at least five times) than water. They are highly toxic and cause ill effects at very low concentrations e.g. mercury (Hg), cadmium (Cd), arsenic

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(As), chromium (Cr), thallium (Tl), and lead (Pb). They are added to the aquatic system, either naturally by slow leaching from soil/rock to water or through anthropogenic sources. In recent times, anthropogenic inputs, such as discharge of untreated effluent (waste water), have contributed to the predominant causation. A survey carried out by Central Pollution Control Board (2008) reported that ground water in 40 districts from 13 states of India i.e. Andhra Pradesh, Assam, Bihar, Haryana, Himachal Pradesh, Karnataka, Madhya Pradesh, Orissa, Punjab, Rajasthan, Tamil Nadu, Uttar Pradesh, West Bengal, and five blocks of Delhi is contaminated with heavy metals. Lokeshwari and Chandrappa (2007) reported the bioavailability of heavy metals in Dasarahalli tank located in Bangalore (India) as Zn > Cd > Ni > Fe > Cu > Pb > Cr and warned the high health risks to human beings, due to the ability of these metals to enter the food chain.

PHYTOREMEDIATION

Many conventional technologies—chemical precipitation, ultrafiltration, chemical oxidation and reduction, electrochemical treatment, reverse osmosis, coagulation-flocculation, and ion exchange etc. are used to clean heavy metal pollutants (Volesky 2001; Rai 2009). Each of the remediation technology has specific benefits and limitations (EPA 1997) but in general none of them is cost-effective (Volesky 2001; Rai 2009). Many studies have been conducted to improve the water quality through natural means to overcome this problem. Boyd (1970), Stewart (1970), Wooten and Dodd (1976), and Conwell et al. (1977) were among the pioneers to demonstrate the nutrient removal potential of aquatic plants. Seidal (1976), Wolverton and McDonald (1976), and Wolverton and Mckown (1976) experimentally proved the importance of aquatic plants in removing organic contaminants from aquatic environments. Thereafter, this approach is emerging as an innovative tool, because plants are solar-driven and thus make this technology a cost-effective mode, with great potential to achieve sustainable environment.

The term "phytoremediation" comes from the Greek *φυτο* (phyto) = plant, and Latin "remedium" = restoring balance, or remediation; consists of mitigating pollutant concentrations in contaminated soils, water or air with naturally occurring or genetically engineered plants that have ability to accumulate, degrade or eliminate metals, pesticides, solvents, explosives, crude oil, and its derivatives etc. (Flathman and Lanza 1998; Prasad and Freitas 2003). The main objective behind the development of phytoremediation technologies is their eco-friendly and cost-effective nature. Other advantages and limitations of phytoremediation are compared in Table 1. The limitations of phytoremediation can be overcome using plants having high biomass, faster growth rate, and ability to adapt with wide rage of environmental conditions. In this respect, the water fern *Azolla* has many advantages. The free-floating habitat, ability to grow in N-deficit sites, known potential to tolerate wide range of pollutants, and accumulation of different heavy metals from contaminated sites reflect exploration a more promising candidate in future for phytoremediation (Arora et al. 2006; Umali et al. 2006). This review is an attempt to gather information available till date regarding phytoaccumulation potential of aquatic macrophyte *Azolla,* emphasizing its strengths and need for in-depth research related to its exploitation at commercial level.

AQUATIC MACROPHYTES AND THEIR POTENTIAL TO ACCUMULATE HEAVY METALS

Aquatic macrophytes include a diverse group of photosynthetic organisms, large enough to be seen with the naked eye. It includes aquatic spermatophytes (flowering plants), pteridophytes (ferns), and bryophytes (mosses, hornworts, and liverworts). However, Schwarz and Haves (1997) also included the charophytes (*Chara* spp. and

Table 1 Advantages and limitations of phytoremediation

Advantages	Limitations
Cost- effective and eco-friendly technology as compared to traditional process both in situ and ex-situ	Process is effective with respect to the surface area covered and limited by the depth reached by the roots
It uses naturally inherent potential of naturally occurring plants and microbes to clean polluted sites. Help in preserving the natural state of environment	The response of plant and microbe varies under different growth conditions (i.e. climate, temperature, light intensity, altitude etc.)
It can be used to treat sites with more than one pollutant	Success of phytoremediation depends upon the tolerance plants used to treat pollutant.
After phytoremediation, the hyperaccumulating plants can be used for retrieval of the precious heavy metals as bio-ores	There exists a possibility of heavy metals re- entering the environment, because of their biodegradable nature

Nitella spp.) as aquatic macrophytes. Chambers et al. (2008) described that aquatic macrophytes are represented in seven plant divisions: Cyanobacteria, Chlorophyta, Rhodophyta, Xanthophyta, Bryophyta, Pteridophyta, and Spermatophyta. These aquatic macrophytes are usually classified into four groups depending upon their growth forms: Group 1 includes emergent macrophytes i.e. plants rooted in soil and emerging to significant heights above the water e.g. *Phragmites australis, Typha latifolia* etc. Group II includes floating leaved macrophytes/plants that occur on submerged sediments at water depths from about 0.5–3.0 m and include mainly angiosperms e.g. *Potamogeton pectinatus* etc. The third group comprises submerged macrophytes or plants primarily growing completely below the surface of water including mosses, charophytes, a few pteridophytes (*Ceratophyllum demersum*), and many angiosperms (*Myriophyllum spicatum, Vallisneria spiralis*, and *Hydrilla* sp. etc.). Group IV comprises free-floating macrophytes representing plants that are nonrooted to substratum, highly diversified group in habitats and forms e.g. *Eichhornia crassipes, Salvinia* sp., *Azolla* sp., and *Lemna* sp. etc.

Aquatic macrophytes are more suitable for wastewater treatment than terrestrial plants because of their faster growth and larger biomass production, relative higher capability of pollutant uptake, and better purification effects due to direct contact with contaminated water. They also play an important role in the structural and functional aspects of aquatic ecosystems by altering water movement regimes (flow and wave impact conditions), providing shelter to fish and aquatic invertebrates and serving as a food source, and altering water quality by regulating oxygen balance, nutrient cycle, and accumulating heavy metals (Srivastava et al. 2008; Dhote and Dixit 2009). Their ability to hyperaccumulate heavy metals make them interesting research candidates especially for the treatment of industrial effluents and sewage waste water (Mkandawire et al. 2004; Arora et al. 2006; Upadhyay et al. 2007; Mishra et al. 2009; Rai 2010a).The potential of aquatic marcophytes for heavy metal removal has been investigated and reviewed extensively (Brooks and Robinson 1998; Cheng 2003; Prasad and Freitas 2003; Suresh and Ravishankar 2004; Srivastva et al. 2008; Dhir et al. 2009a; Dhote and Dixit 2009; Marques et al. 2009; Rai 2009). Table 2 summarizes the recent literature on phytoremediation potential of some macrophytes. It clearly pointed out some generalizations such as existence of a wide variation with respect to the amount of heavy metal accumulation by different plants indicating that phytoremediation potential of aquatic plants is dependent upon the tolerance level and toxicity of plant genera or species employed in a particular study. Secondly, within a particular plant genus and/or species, there exists a difference in accumulation potential for the same heavy metal. The existing variation is because the phytoremediation potential is regulated by environmental factors like chemical speciation and initial concentrations of the metal, temperature, pH, redox potential, salinity, and interaction of different heavy metals among each other. Aravind et al. (2009) reported that supplementation of heavy metal Zn in growth media containing Cd, resulted in decrease in accumulation of Cd in *Ceratophyllum demersum* indicating the existence of metal–metal interactions (Zn and Cd). Boulé et al. (2009) found much lower Cu uptake and tolerance levels in *Lemna minor* from the noncontaminated area compared to another ecotype (*L. minor*) from uranium-polluted mine. Aquatic macrophytes have ability to concentrate heavy metals in their roots, shoots as well as leaves. However, the accumulation of heavy metals is much higher in roots of these plants (Mishra et al. 2009; Paiva et al. 2009; Mufarrege et al. 2010). V.K. Mishra and Tripathi (2008) compared the phytoremediation potential of three aquatic macrophytes and concluded that *Eichhornia crassipes* was more efficient in removal of heavy metals (Fe, Zn, Cu, Cr, and Cd) followed by *Pistia stratiotes* and *Spirodela polyrrhiza.* Rahman et al. (2008) reported that external supplementation of ethylenediaminetetraacetic acid (EDTA) in the growth medium of *Spirodela polyrhiza* increased the uptake of heavy metal, As(V) and As(III). A very interesting feature revealed by studies on model plants is that the key step of hyperaccumulation does not rely on the novel genes but on the differential regulation and expression of common genes in hyperaccumulator and nonhyperaccumulator plants.

BIOACCUMULATION AND BIOSORPTION

Both the living and dead biomass of aquatic macrophytes can be used for removal of heavy metal contaminants from the aquatic ecosytems (Umali et al. 2006; Rai 2008; Mashkani and Ghazvini 2009; Mishra et al. 2009). Based on the state of biomass, the term “Bioaccumulation” is defined as the phenomenon of uptake of heavy metals by living cell, whereas “biosorption” refers to passive uptake of pollutants by dead/inactive biological material or material derived from biological sources. Other differences among both the methods are summarized in Table 3. In general, the use of living biomass may not be a viable option for the continuous treatment of highly toxic contaminants as the bioaccumulation of these heavy metals is closely connected with their toxicity, affecting plant and its growth. Once the toxicant concentration becomes too high after long duration of treatment, the amount of toxicant accumulated will attain a saturation level (Eccles 1995). Beyond this point, the plant metabolism gets interrupted, resulting in death of the organism. However, the

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Table 2 Recent literature on macrophytes known for their potential to accumulate heavy metals

Plants	Heavy metals	Accumulation (dry weight basis)	Reference
Eichhornia crassipes	Hg	119 ng Hg g^{-1}	Molisani et al. (2006)
	Cd	3992 μg Cd g^{-1}	K.K. Mishra et al. (2007), S. Mishra et al. (2007)
	Cu	314 μg Cu g^{-1}	Hu et al. (2007)
	Cr, Cd, Ni	2.31 mg Cr g^{-1}	Verma et al. (2008)
		1.98 mg Cd g^{-1}	
		1.68 mg Ni g^{-1}	
	Cr	1258 μg Cr g^{-1}	Paiva et al. (2009)
Elodea densa	Hg	177 ng Hg g^{-1}	Molisani et al. (2006)
Eleocharis acicularis	Fe, Pb, Zn, Mn, Cr, Cu, Ni	59 500 μg Fe g^{-1}	Hoang Ha et al. (2009)
		1120 μg Pb g^{-1}	
		964 μg Zn g^{-1}	
		388 μg Mn g^{-1}	
		265 μg Cr g^{-1}	
		235 μg Cu g^{-1}	
		47 μg Ni g^{-1}	
Lemna gibba	Ur, As	897 μg Ur g^{-1}	Mkandawire et al. (2004)
		1022 μg As g^{-1}	
	Zn	4.23–25.81 mg Zn g^{-1}	Khellaf and Zerdaoui (2009)
Lemna minor	Ti	221 μg Ti g^{-1}	Babic et al. (2009)
	Cu	400 μg Cu g^{-1}	Boule et al. (2009)
	Pb	8.62 mg Pb g^{-1}	Uysal and Taner (2009)
Elodea canadensis	Ni	>3500 μg Ni g^{-1}	Maleva et al. (2009)
Pistia stratiotes	Hg	0.57 mg Hg g^{-1}	Mishra et al. (2009)
		215 ng Hg g^{-1}	Molisani et al. (2006)
		83 μg Hg g^{-1}	Skinner et al. (2007)
	Cr, Cd, Ni	2.50 mg Cr g^{-1}	Verma et al. (2008)
		2.13 mg Cd g^{-1}	
		1.95 mg Ni g^{-1}	
	Cr, Ni, Zn	> 9 mg Cr g^{-1}	Mufarrege et al. (2010)
		> 10 mg Ni g^{-1}	
		> 12 mg Zn g^{-1}	
Egeria densa	Cd, Cu, Zn	70.25 mg Cd g^{-1}	Pietrobelli et al. (2009)
		45.43 mg Cu g^{-1}	
		30.40 mg Zn g^{-1}	
Salvinia auriculata	Pb	191 ng Hg g^{-1}	Molisani et al. (2006)
		494 μg Pb g^{-1}	Espinoza-Quiñones et al. (2009)
Salvinia minima	Cd, Pb;	11 262 μg Cd g^{-1}	Olguin et al. (2002)
		7705 μg Pb g^{-1}	
	Pb	14 000 μg Pb g^{-1}	Sánchez-Galván et al. (2008)
Salvinia natans	Cr	7.40 mg Cr g^{-1}	Dhir et al. (2009b)
Ceratophyllum demersum	As	525 μg As g^{-1}	K.K. Mishra et al. (2007), S. Mishra et al. (2007, 2008), V.K. Mishra et al. (2008)
	Cd	1293 μg Cd g^{-1}	
	Cd, Zn	143 μg Cd g^{-1}	Aravind et al. (2009)
		57 μg Zn g^{-1}	
Potamogeton pusillus	Cu	162 μg Cu g^{-1}	Monferran et al. (2009)

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Table 2 continued

Plants	Heavy metals	Accumulation (dry weight basis)	Reference
Vallisneria spiralis	Cr, Cd, Ni	2.85 mg Cr g^{-1} 2.62 mg Cd g^{-1} 2.14 mg Ni g^{-1}	Verma et al. (2008)
	Hg	158 µg Hg g^{-1}	Rai and Tripathi (2009)
Myriphyllum triphyllum	Cd	17 µg Cd g^{-1}	Sivaci et al. (2004)
Typha aungustifoila	Cr, Zn, Cu	20 210 µg Cr g^{-1} 16 325 µg Zn g^{-1} 7,022 µg Cu g^{-1}	Firdaus-e-Bareen and Khilji (2008)
Typha latifolia	Zn, Ni, Cu	340 µg Zn g^{-1} 55 µg Ni g^{-1} 50 µg Cu g^{-1}	Sasmaz et al. (2008)
Sagittaria montevidensis	Hg	62 mg Hg g^{-1}	Molisani et al. (2006)
Wolffia globosa	As	>1000 µg As g^{-1}	Zhang et al. (2009)
Spirodela polyrhiza	As	7.65 n mol As g^{-1}	Rahman et al. (2007)
Mentha sp.	Fe	378 µg Fe g^{-1}	Arora et al. (2008)

Table 3 Differences between bioaccumulation and biosorption

Characters	Bioaccumulation	Biosorption
Biomass type	Living	Dead
Commercial applicability	Relatively less applicable because living material require additions of nutrients and other inputs	More applicable
Cost	Usually high	Low
Maintenance/ storage	External energy is required to maintained culture in active growth phase	Low maintenance required and easy to store.
Selectivity	Better	Less effective than bioaccumulation which can be improved by modification/processing of biomass
Sensitivity	Nutrient dependent	Nutrient independent
Temperature	Severely affect the process	Does not affect the process because biomass is dead
Metal location	Inter and intracellular	Extracellular
Degree of uptake	Active process	Passive process
Rate of uptake	Slower	Very fast
Desorption	Not possible	Possible
Regeneration and use	Since metal is intracellularly accumulated, the chances are very limited	High possibility of biosorbent regeneration, with possible reuse for a number of cycles.

biosorption depends on the type of plant genera/species used and conditions of performed process: temperature, pH, biomass concentration, and metal ions concentration, which are flexible and can be easily altered. The binding of metal ions by the biomass is a two-step process where initially the metal was taken up onto the surface of the cell (biosorption) followed by the bioaccumulation inside the cell due to the metal uptake metabolism. Hence, the first stage can be performed by both, living and dead biomass (Saygideger et al. 2005).

AZOLLA—A BETTER MACROPHYTE FOR PHYTOREMEDIATION

Azolla is a small aquatic fern belonging to Phylum-Pteridophyta, Class Polypodiopsida, Order Salviniales, Family Azollaceae with a monotypic genus (Wagnar 1997; Pabby et al. 2003b, c, Pabby et al. 2004b; Sood and Ahluwalia 2009). This fern represents the only example of Pteridophyte harboring symbiotic association with diazotrophic, nitrogen-fixing cyanobacteria, and bacteria residing in leaf

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cavities (Sood et al. a, b; Sood and Ahluwalia 2009). This three partner association remains together during vegetative and reproductive phase of life history, thereby excluding the need of re-inoculation, hence proving its potential over and above other biofertilizers (Carrapico 2010). Apart from its agronomic potential, this association has diversified applications as food and feed supplements, weed suppressors, larvicide, utility in biogas and hydrogen production, and waste water treatment (Ahluwalia and Pabby 2002; Pabby et al. 2004b), which is illustrated in Fig. 1. Increasing environmental awareness and concern has attracted scientific community to extend its exploitation more vigorously in the area of phytoremediation because the fern can hyperaccumulate variety of pollutants such as heavy metals, radionuclides, dyes, and pesticides etc. from aquatic ecosystems along with other macrophytes (Padmesh et al. 2006; Rai and Tripathi 2009; Mashkani and Ghazvini 2009; Sood et al. 2011). This fern has many features that prove it as a better plant system than many other macrophytes, which include:

Fast Growth Rate

The ability of *Azolla* to grow rapidly, doubling its biomass in 2–4 days, is the most important attribute, along with its free-floating nature (Arora and Saxena 2005; Kathiresan 2007; Sood et al. 2008a). Both these qualities help in its easy harvest for disposal or recovery of heavy metals from biomass.

Nitrogen-Fixing Ability

Nitrogen is among the most important macronutrient required for the synthesis of nucleic acids, proteins, phospholipids, and many secondary metabolites which play an important role in the overall growth of the plants (Amtmann and Armenguad 2009). The ability of *Azolla* to fix atmospheric nitrogen allows this fern to grow successfully in aquatic habitats lacking or having low levels of nitrogen (Pabby et al. 2001, 2003b; Sood et al. 2007). This may help *Azolla* to proliferate in polluted waters as well.

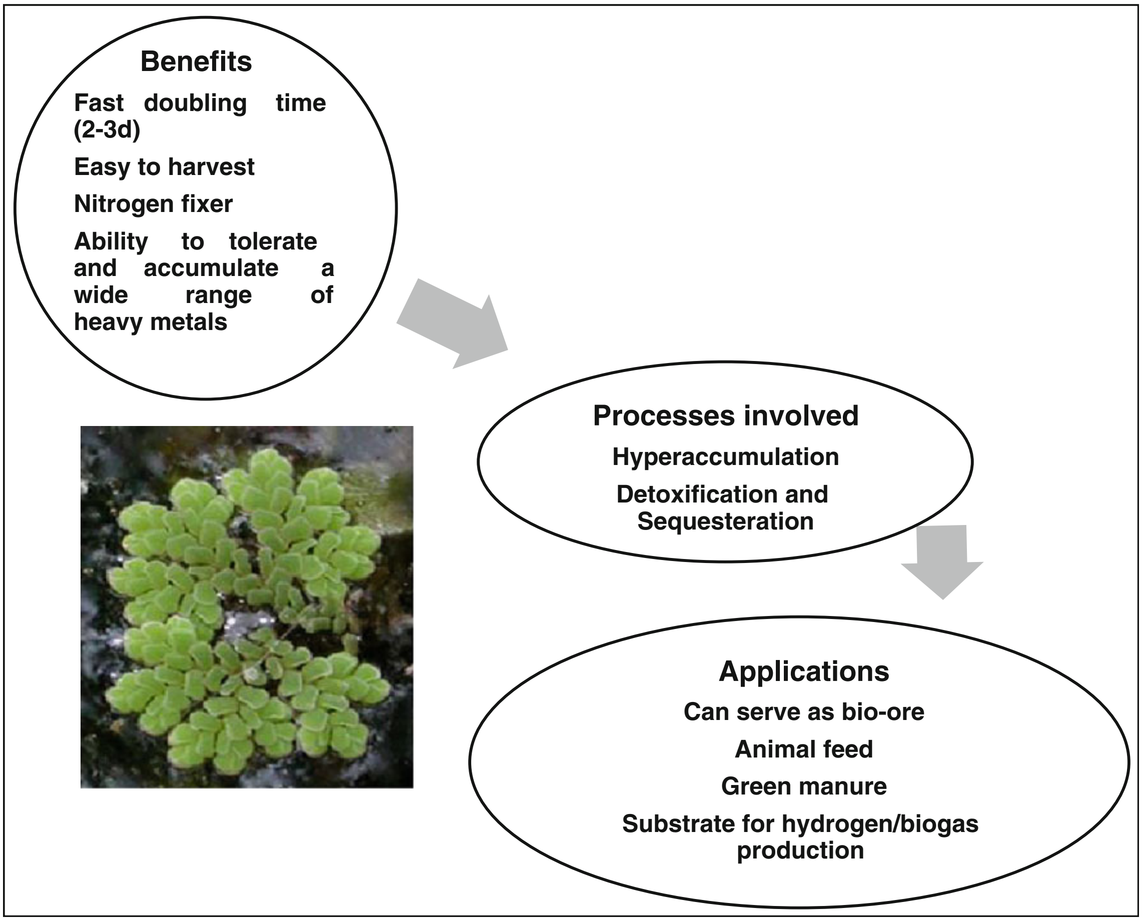

Fig. 1 Benefits of *Azolla* in phytoremediation

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Biomass Disposal

The removal of heavy metal contaminants by aquatic macrophytes presents the problem of plant biomass disposal so as to prevent recycling of accumulated metals (Rai 2009). The water content in *Azolla* fronds varies between 90 and 94% (Serag et al. 2000). Therefore after drying, its volume reduces drastically, hence solving the problem of disposal to a much greater extent. The dry *Azolla* biomass can easily be transported for recovery of the heavy metal.

PHYTOREMEDIATION ABILITY OF *AZOLLA*

Both living and dead biomass of *Azolla* have been exploited for the removal of heavy metals from industrial effluents and sewage water (Bennicelli et al. 2004; Upadhyay et al. 2007; Rai 2008; Mashkani and Ghazvini 2009). Bioaccumulation potential of different species of *Azolla* for various heavy metals is summarized in Table 4.

Rai (2008) reported that *A. pinnata* removed 70–94% of heavy metals (Hg and Cd) from ash slurry and chlor-alkali effluent in Singrauli region of U.P. (India) and the concentration of these heavy metals ranged between 310 and 740 mg Kg^{-1} (μg g^{-1}) dry mass in tissues of *Azolla* (Table 4). Zhang et al. (2008) showed large variation in bioaccumulation potential of As among 50 strains of *Azolla* grown hydroponically in a growth chamber that ranged from 29 to 397 mg Kg^{-1} (μg g^{-1}) dry mass. Further among eight tested strains, concentration of As was the highest (284 mg Kg^{-1} or 284 μg g^{-1} DW) in the frond of *A. caroliniana* and lowest in *A. filiculoides* (54 mg Kg^{-1} or 54 μg g^{-1} DW). Benaroya et al. (2004) compared the Pb content in *A. filiculoides* and its isolated apoplast. The Pb content was 0.37, 2.3, and 1.8% of the dry weight after 2, 4, and 6 days of growth, respectively, in the whole plant, while the isolated *Azolla* apoplast contained 0.125, 1.22, and 1.4% Pb, respectively. Sela et al. (1989) reported that *A. filiculoides* also accumulate heavy metals such as Cd, Cr, Cu, and Zn and their content was 10,000, 1990, 9000,

Table 4 Literature on heavy metal bioaccumulation by various *Azolla* spp

Azolla spp.	Heavy metal	Initial concentration of heavy metal	Duration of experiment (days)	Heavy metal accumulated (dry weight basis)	Reference
A. pinnata	Hg	3.0 mg l^{-1}	13	667 μg Hg g^{-1}	Rai (2008)
	Hg	10.0 μg l^{-1}	21	450 μg Hg g^{-1}	Mishra et al. (2009)
	Hg	3.0 mg l^{-1}	6	940 μg Hg g^{-1}	Rai and Tripathi (2009)
	Cd	3.0 mg l^{-1}	13	740 μg Cd g^{-1}	Rai (2008)
	Cd	10.0 mg l^{-1}	7	2759 μg Cd g^{-1}	Arora et al. (2004)
	Cr(III)	3.0 mg l^{-1}	13	1095 μg Cr g^{-1}	Rai (2010b)
	Cr(VI)	20.0 μg l^{-1}	14	9125 μg Cr g^{-1}	Arora et al. (2006)
	Ni	500 mg l^{-1}	7	16 252 μg Ni g^{-1}	Arora et al. (2004)
A. caroliniana	As	80.0 μg l^{-1}	7	>120 μg As g^{-1}	Zhang et al. (2008)
	Pb	1.0 mg l^{-1}	12	416 μg Pb g^{-1}	Stepniewska et al. (2005)
	Cd	1.0 mg l^{-1}	12	259 μg Pb g^{-1}	Stepniewska et al. (2005)
	Cr(VI)	1.0 mg l^{-1}	12	356 μg Cr g^{-1}	Bennicelli et al. (2004)
	Cr(III)	1.0 mg l^{-1}	12	964 μg Cr g^{-1}	Bennicelli et al. (2004)
	Hg	1.0 mg l^{-1}	12	578 μg Hg g^{-1}	Bennicelli et al. (2004)
A. filiculoides	As	80.0 μg l^{-1}	7	>60 μg As g^{-1}	Zhang et al. (2008)
	Cr(VI)	20.0 μg l^{-1}	14	12 383 μg Cr g^{-1}	Arora et al. (2006)
	Cr(III)	9.0 mg l^{-1} (ppm)	4	1904 ppm	Sela et al. (1989)
	Cd	9.0 mg l^{-1} (ppm)	4	10 441 ppm	Sela et al. (1989)
	Cd	10.0 mg l^{-1}	7	2608 μg Cd g^{-1}	Arora et al. (2004)
	Ni	9.0 mg l^{-1} (ppm)	4	8814 ppm	Sela et al. (1989)
	Ni	500 mg l^{-1}	7	28 443 μg Ni g^{-1}	Arora et al. (2004)
	Cu	9.0 mg l^{-1} (ppm)	4	9224 ppm	Sela et al. (1989)
	Zn	9.0 mg l^{-1} (ppm)	4	6408 ppm	Sela et al. (1989)
A. microphylla	Cr(VI)	20.0 μg l^{-1}	14	14 931 μg Cr g^{-1}	Arora et al. (2006)
	Ni	500 mg l^{-1}	7	21 785 μg Ni g^{-1}	Arora et al. (2004)
	Cd	10.0 mg l^{-1}	7	1805 μg Cd g^{-1}	Arora et al. (2004)
A. imbricata	Cd	0.5 μg l^{-1}	9	183 μg Cd g^{-1}	Dai et al. (2006)

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and 6500 ppm, respectively (Table 4). Arora et al. (2004, 2006) compared *A. filiculoides* with *A. microphylla* and *A. pinnata* for its phytoaccumulation potential of Cd, Cr, and Ni grown in a polyhouse. They recorded that Cd, Ni, and Cr content (ppm) in tissues was in the following order: *A. microphylla* > *A. filiculoides* > *A. pinnata*; *A. pinnata* > *A. microphylla* > *A. filiculoides* and *A. pinnata* > *A. filiculoides* > *A. microphylla,* respectively. The BCF (Bio concentration factor) of heavy metals (Cd, Cr) recorded by Arora et al. (2004, 2006) for *A. filiculoides* was also much higher than BCF values reported by Sela et al. (1989). Jafari et al. (2010) observed the highest bioconcentration potential of Pb^{2+}, Cu^{2+}, Mn^{2+}, and Zn^{2+} was 94% in *A. microphylla*, 96% in *A. filiculoides*, 71% in *A. pinnata*, and 98% in *A. microphylla,* respectively. Another species of *Azolla, A. caroliniana* also has potential to bioaccumulate Hg and Cr (III and VI). The heavy metal contents in tissues of *A. caroliniana* ranged from 71 to 964 mg kg^{-1} or 964 µg g^{-1}dm; the highest level (964 µg g^{-1}dm) was observed for or Cr(III) suggesting that *A. caroliniana* has the capacity to take up these heavy metals (75–100%) from municipal wastewater (Bennicelli et al. 2004). In another investigation with *A. caroliniana*, the amount of Pb and Cd was brought down to 90 and 22%, respectively, in the growth media supplemented with these heavy metals. The content of heavy metal Pb was up to 416 kg^{-1} or 416 µg g^{-1} dry weight (Table 4) and that of Cd was up to 259 mg Cd Kg^{-1} or 259 µg g^{-1} dry weight in fronds of *A. caroliniana* (Stepniewska et al. 2005). Jain et al. (1989, 1990) reported that the presence of one heavy metal in solution influenced negatively the uptake of other heavy metal. They found that the concentration of Pb decreased in *A. pinnata* when equal concentration of Zn was supplemented along with Pb (Jain et al. 1990). Similar findings were observed by Gaumat et al. (2008) under mixed (Pb + Fe) treatment with *A. pinnata* as compared to single Fe treatment.

The bioaccumulation potential of *Azolla* spp. for various heavy metals has been compared with other aquatic macrophytes by many workers (Upadhaya et al. 2007; S. Mishra et al. 2008; V.K. Mishra et al. 2008; Mishra et al. 2009; Rai and Tripathi 2009; Rai 2010a). Mallick et al. (1996) reported that *Lemna minor* was more efficient in accumulating Zn and Cr than *Azolla pinnata* in Ni,whereas both macrophytes showed preference for Zn followed by Ni and Cr. S. Mishra et al. 2008; V.K. Mishra et al. 2008 observed that the concentrations of Cu, Cd, Mn, Pb, and Hg were higher in *Eichhornia crassipes* than in *Azolla pinnata, Lemna minor,* and *Spirodela polyrrhiza* collected from a site of the man-made reservoir Govind Ballabh Pant Sagar, India. Mishra et al. (2009) compared the mercury (Hg) removal capacities of two aquatic macrophytes, *Pistia stratiotes* and *Azolla pinnata* from coal mining effluent. Both the macrophytes reduced mercury level in the effluent via rhizofiltration and the removal rate of *Pistia stratiotes* and *A. pinnata* was 80 and 68%, respectively. They concluded that *P. stratiotes* was a better accumulator of mercury, with higher removal efficiencies than *A. pinnata.* However, Rai and Tripathi (2009) recorded higher percentage removal (80–90%) and Hg accumulation (940 mg Kg^{-1} or 940 µg g^{-1} dry mass) of *Azolla* sp. than *Vallisneria spirallia* in polluted water of G.B. Pant Sagar located in Singrauli Industrial Region, India. During field phytoremediation experiments conducted in the same region, Rai (2010a) recorded a marked reduction in concentrations of nine heavy metals (Cu, Cr, Fe, Mn, Ni, Pb, Zn, Hg, and Cd). The decrease in heavy metal content ranged from 25 to 67.90% at Belwadah site (with *Eichhornia crassipes* and *Lemna minor*), 25 to 77.14% at Dongia Nala site (with *E. crassipes, L. minor and Azolla pinnata*), and 25 to 71.42% at Ash pond site (with *L. minor and A. pinnata*) receiving effluents from Thermal Power Plant, chlor-alkali industry and coal mine, respectively. Gaur et al. (1994) compared accumulation of heavy metals Cd, Cr, Co, Cu, Ni, Pb, and Zn by *Spirodela polyrhiza* (L.) Schleid and *Azolla pinnata.* They concluded that the order of metal accumulation was Ni > Zn > Co = Cd > Cu > Pb > Cr in *A. pinnata* and Ni > Zn > Co > Cu > Cd > Pb > Cr in *S. polyrhiza.* In secondary treated sewage waste water, Upadhyay et al. (2007) reported the sequence order of percent removal of heavy metals by *Eichhornia crassipes*, *Pistia stratiotes*, *Lemna minor*, *Azolla pinnata*, and *Spirodela polyrhiza* was Fe > Cr > Cu > Cd > Zn > Ni and among these aquatic macrophytes, *E. crassipes* showed the highest removal capacity.

A direct comparison of findings of other macrophytes with *Azolla* species (as given in Table 4) is not possible due to difference in initial concentration of heavy metal and biomass of *Azolla* employed for particular investigation along with duration of the experiment, details of experimental design. Table 4 reveals the tremendous phytoaccumulation potential existing within and among *Azolla* spp.

BIOSORPTION OF HEAVY METALS BY *AZOLLA*

Azolla biomass, in dead or pretreated form, has been used for biosorption of heavy metals Cs, Sr, Pb, Zn, Ni, Cu, Au, Cd, and Cr by various workers (Cohen-Shoel et al. 2002; Rakhshaee et al. 2006; Umali et al. 2006; Nedumaran and Velan 2008; Mashkani and Ghazvini 2009). Table 5 summarizes the results of heavy metal biosorption by various workers. The bioaccumulation of heavy metals by *Azolla* spp. is known to exhibit a concentration-dependent relationship (Stepniewska et al. 2005; Arora et al. 2006; Dai et al. 2006; Rai and Tripathi 2009). Zhao and Duncan

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Table 5 Literature on heavy metal biosorption by *Azolla filiculoides*

Type of biosorbent	Metal	Operating conditions		Uptake (mg g^{-1})	% removal	Reference
		Temp. (°C)	pH			
Native	Cs	30	8	70 mg g^{-1}	NA	Mashkani and Ghazvini (2009)
Chemically modified (ferrocyanide *Azolla)*				130 mg g^{-1}		
Chemically modified (hydrogen peroxide *Azolla*)				195 mg g^{-1}		
Native	Sr	30	9	117 mg g^{-1}	NA	
Chemically modified (ferrocyanide *Azolla*),				168 mg g^{-1}		
Chemically modified (hydrogen peroxide *Azolla*)				212 mg g^{-1}		
Chemically modified (treated with KCl)	Sr	NA	NA	33 mg g^{-1}		Cohen-Shoel et al. (2002)
Native	Au	NA	2	98 mg g^{-1}	98.2%	Umali et al. (2006)
Pre-treated	Pb	NA	7	186 mg g^{-1}	NA	Khosravi et al. (2005)
Semi-intact *Azolla* (biomass soaked in distilled water),	Cd			95 mg g^{-1}		
	Ni			54 mg g^{-1}		
	Zn			48 mg g^{-1}		
Super-activated *Azolla* (activated at pH 10.5 and then using $CaCl_2/MgCl_2/NaCl$)	Pb	NA	10.5	271 mg g^{-1}	90%	
	Cd			111 mg g^{-1}	83%, 87%	
	Ni			71 mg g^{-1}	76%	
	Zn			60 mg g^{-1}		
Dead activated *Azolla*	Pb	25 ± 0.5	6.0 ± 0.2	92 mg g^{-1}		
	Cd			47 mg g^{1}		
	Ni			26 mg g^{1}		
	Zn			25 mg g^{-1}		
Native	Au	NA	2	NA	99.9%	Antunes et al. (2001)
Modified (Milled-sieved *Azolla)*	Cu	NA		364 µmol g^{-1}	NA	Fogarty et al. (1999)
Modified (Epichlorohydrin-immobilised *Azolla*)				320 µmol g^{-1}	NA	
Native	Zn	NA	6.2	30 mg g^{-1}	NA	Zhao et al. (1999)
Native	Ni	NA	7	28 mg g^{-1}	NA	Zhao and Duncan (1998a)
Native	Zn	NA	6.2	31 mg g^{-1}	NA	Zhao and Duncan (1998b)
Native	Pb	NA	3.5–4.5	93 mg g^{-1}	95%	Sanyahumbi et al. (1998)
Native	Cr (VI)	32	2	120 mg g^{-1}	NA	Zhao and Duncan (1997)

(1997, 1998a, b) investigated the removal of sexivalent Cr, Ni, and Zn by *A. filiculoides* from aqueous solution and from electroplating rinse effluent. The batch adsorption experiments (Table 5) showed that the maximum adsorption capacity of *A. filiculoides* for Cr^{6+} was 20.2 mg g^{-1} at pH 2 and temperature of 32°C (Zhao and Duncan 1997). The maximum uptake of Ni was found to be 27.9 mg g^{-1} at 60% saturation of the biomass, whereas in batch experiments it was 43.3 mg g^{-1} (Zhao and Duncan 1998a). Zhao and Duncan (1998b) used *A. filiculoides* in fixed-bed sorption column for removal of Zn. The sorption capacity of 31.3 mg g^{-1} was observed at pH 6.2. In another study, the maximum zinc uptake by *A. filiculoides* in batch systems and column was found to be 45.2 and 30.4 mg g^{-1} at pH 6 and 6.2, respectively (Zhao et al. 1999). The nonviable biomass of *A. filiculoides* also removed 93 mg g^{-1} Pb from solution (Sanyahumbi et al. 1998) (Table 5). Lead removal remained at approximately 90% between 10 and 50°C and biomass concentration had little effect on lead removal (Sanyahumbi et al. 1998). Cohen-Shoel et al. (2002) found that the Sr^{2+}-binding capacity of the control *Azolla* biofilter without prewash was 23 mg g^{-1} while that of the 4 liters KCl-treated *Azolla* was 32.8 mg g^{-1} (Table 5). Mashkani and Ghazvini (2009) conducted biosorption batch experiments to determine the Cs and Sr binding ability of native and chemically modified biomass derived from *A. filiculoides.* They observed the best Cs and Sr removal results when *A. filiculoides* was treated by

$MgCl_2$ and H_2O_2 at pH 7 for 12 h and washed by NaOH solution at pH 10.5 for 6 h. They suggested that pretreatment of *Azolla* modified the surface characteristics, which in turn, improved the biosorption process (Mashkani and Ghazvini 2009). Fogarty et al. (1999) compared Cu removal efficiencies of pre-treatment and immobilization of *A. filiculoides* biomass. They observed that epichlorhydrin-immobilized *Azolla* showed greater removal of Cu when compared with milled-sieved *Azolla* and untreated *Azolla,* and *Azolla*-based systems have biosorption capacities greater than comparable biomass systems and in line with commercial sorbent exchange values (Fogarty et al. 1999). Khosravi et al. (2005) compared heavy metal (Pb, Cd, Ni, and Zn) removal capacity of activated, semi-intact, and inactivated *A. filiculoides* wastewater. They reported maximum uptake capacities of these metal ions were 271, 111, 71, and 60 mg g^{-1}, respectively, using the activated *A. filiculoides* by NaOH at pH 10.5 and then $CaCl_2/MgCl_2/NaCl$ with total concentration of 2 M (2:1:1 mol ratio) separately (Table 5).

The biosorption potential of *Azolla* for uptake of precious metals such as gold has been investigated by various groups (Antunes et al. 2001; Umali et al. 2006). Antunes et al. (2001) demonstrated 99.9% removal of gold from solution with 5 g *A. filiculoides* per liter. They observed that pH had a significant effect on gold removal. Complete removal of gold occurred at pH 2, with 42% removal at pH 3 and 4, and 63 and 73% removal at pH 5 and 6, respectively. The dried milled biomass of *A. filiculoides* removed up to 98.2% of gold from wastewater containing 5 mg per liter gold in batch biosorption process (Table 5), from a gold-plating factory. The gold uptake capacity of the fern biomass was 98 mg g^{-1} (Umali et al. 2006).

In general, a direct comparison of biosorption potential of *Azolla* data with other macrophytes is not possible, because of the differences in experimental conditions employed (pH, temperature) in various studies. The observed variability when the same *Azolla* sp. was employed for the same metal could also be due to different experimental conditions employed. Apart from the different experimental conditions, this variation may also be the result of the biomass being pretreated or chemically modified to improve the biosorbent characteristics (Table 5). It was surprising to see that only *A. filiculoides* has been exploited for biosorption studies.

HEAVY METAL PHYTOTOXICITY IN *AZOLLA*

The heavy metals introduced into the aquatic system are known to pose high level of toxicities to the aquatic organisms and human beings (Sánchez-Chardi et al. 2009; Siwela et al. 2009). The effect of these toxic metals results in alterations at morphological, physiological/biochemical, and ultrastructural level in aquatic organisms, which can be used as biomonitoring tools for the assessment of metal pollution in aquatic ecosystems (Zhou et al. 2008).

Growth and Development

The literature showed that exposure of heavy metals suppressed the vegetative growth and sporulation in different species of *Azolla* depends on the tolerance of species as well as concentration of the heavy metal (Arora et al. 2004, 2006). The inhibition was invariably maximum at the highest concentration of heavy metals employed (Bennicelli et al. 2004; Arora et al. 2004, 2006; Stepniewska et al. 2005; Rai 2008). Bennicelli et al. (2004) reported that the presence of Hg, Cr(III), and Cr(VI) in growth medium with a concentration of 0.1, 0.5, and 1.0 mg dm^{-3} caused 20–31% inhibition of growth of *A. caroliniana.* This reduction in growth was maximum in the presence of Hg (Bennicelli et al. 2004). The presence of Pb and Cd also decreased growth by 30–37% and 24–47%, respectively, in *A. caroliniana,* when subjected to the above mentioned concentration of these heavy metals (Stepniewska et al. 2005). Rai (2008) working with *A. pinnata,* observed 27–33.9% suppression of growth in the presence of various treatments (0.5, 1.0, and 3.0 mg l^{-1}) of Cd and Hg. The decline in biomass was again highest with Hg (Rai 2008). While comparing tolerance of *A. microphylla, A. pinnata,* and *A. filiculoides* to the presence of Cr, Arora et al. (2006) observed *A. filiculoides* to be the best producing 72% of control biomass. The application of Cd, Cr, Mo, and Mn at a concentration of 3, 6, 5, and 10 μg ml^{-1}, respectively, significantly decreased the sporulation frequency and number of sporocarps per plant in *A. microphylla* and *A. caroliniana* (Kar and Singh 2003).

Biochemical Effects

Toxicity of heavy metals in relation to biochemical parameters—pigments, photosynthesis, and activities of oxidative enzymes have been worked out by only few researchers (Sarkar and Jana 1986; Shi et al. 2003; Dai et al. 2006). Sarkar and Jana (1986) observed that the treatment of *A. pinnata* with As, Pb, Cu, Cd, and Cr (2 and 5 mg l^{-1} each), decreased Hill activity, chlorophyll content, protein and dry weight, and increased tissue permeability with respect to control. The effects were most pronounced with the highest treatment of (5 mg l^{-1}). The harmful effects of the metals were in the order: $Cd > Hg > Cu > As > Pb > Cr$. Shi et al. (2003) showed that increase in concentration of Hg and Cd resulted in a drop in the chlorophyll content, mainly chlorophyll *a* and/ *b*. The photosynthetic O_2 evolution also decreased

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drastically, whereas respiration rate first peaked at 2 mg l^{-1} concentration of heavy metals and declined thereafter. The activities of SOD (Superoxide dismutase), CAT (Catalase), and POD (Peroxidase) also first increased and decreased afterward except the activity of POD, which decreased with the increasing concentration of Cd^{2+} (Shi et al. 2003). Dai et al. (2006) reported change in the color of fronds, a decrease in the contents of chlorophyll and carotenoids in the fronds of *A. imbricata* at higher Cd concentrations, while an increase in content of total phenolics and phenylalanine ammonia-lyase (PAL) activity were detected during Cd treatment. This suggested that the Cd-induced change in color of fronds might be due to the decrease in chlorophyll and carotenoids while the increase in total phenolics and their biosynthesis-related PAL play a role in detoxification of Cd in *A. imbricata*. Rai and Tripathi (2009) found a concentration-dependent decrease in the content of chlorophyll *a*, protein, RNA, and DNA, and nutrient (nitrate and phosphate) uptake was detected in *A. pinnata* because of Hg toxicity. Sánchez-Viveros et al. (2010) reported that heavy metal copper disrupted photosystem II resulting in drop in potential phytochemical yield at higher concentrations in *A. filiculoides* and *A. caroliniana*. They concluded that chlorophyll fluorescence analysis can be used as a useful physiological tool to assess early changes in photosynthetic performance of *Azolla* in response to heavy metal pollution (Sánchez-Viveros et al. 2010).

Effect on the Process of Nitrogen Fixation

Azolla is exploited worldwide as biofertilizer particularly in rice fields due to the biological nitrogen-fixing capacity of its symbiotic heterocystous cyanobionts (Pabby et al. 2003a, 2004a; Sood et al. 2008a, b). A number of abiotic and biotic factors are known to have a pronounced effect on the nitrogen-fixing capacity of this biofertilizer (Pabby et al. 2000, 2001, 2002a, 2002b, 2003b, 2004b). Sela et al. (1989) reported that Cd, Ni, and Zn completely inhibited nitrogenase activity in *A. filiculoides* while Cu and Cr partially suppressed nitrogen fixation. While comparing nitrogenase activity in three species of *Azolla*—*A. microphylla, A. pinnata*, and *A. filiculoides*—grown in medium containing different concentrations (1–20 µg ml^{-1}) of Cr, the nitrogen fixation was not affected at 1–5 µg ml^{-1}, but at higher concentrations ($\geq$10 µg ml^{-1}) it diminished significantly (Arora et al. 2006). Recently, Dai et al. (2009) reported considerable decrease in heterocyst frequency, the activities of nitrogenase and glutamine synthetase, amount of soluble proteins and total nitrogen, with both increase in Cd concentration (above 0.05 mg l^{-1}) and duration of Cd treatment in *A. imbricata*, suggesting that higher cadmium treatment caused the disorder of nitrogen metabolism and reduced the accumulation of nitrogen in *A. imbricata*-*Anabaena azollae* symbiosis.

Ultrastructural Variation and Localization of Heavy Metals

The accumulation of heavy metals is known to result in various types of damage at the ultrastructural level (Sela et al. 1988, 1990; Shi et al. 2003; Gaumat et al. 2008). A localization study by X-ray microanalysis of heavy metals in *A. filiculoides* showed that Cu accumulated at a higher concentration in the root than in the shoot, whereas Cd content was similar in both organs (Sela et al. 1988). Cd ions are highly mobile in *Azolla*, and many of them were detoxified in aggregates containing PO_4 and Ca (Sela et al. 1988). Furthermore, X-ray microanalysis revealed that the content of Cd increased in the inner epidermis, cortex, and bundle cell walls of roots within 77 h. The accumulation of Cd was characterized by the appearance of small dark grains with high content of cadmium, phosphate, and calcium along the epidermal cells (Sela et al. 1990). Ultrastructural observations of *A. imbricata* showed that the extent of damage was much more with higher concentrations and longer duration of incubation with heavy metals—Hg and Cd. The results showed swelling of chloroplast, disruption, and disappearance of chloroplast membrane and disintegration of chloroplasts; swelling of cristae of mitochondria, deformation and vacuolization of mitochondria; condensation of chromatin in nucleus, dispersion of nucleolus and disruption of nuclear membrane (Shi et al. 2003). Benaroya et al. (2004) observed the presence of Pb precipitates in the vacuoles of mesophyll cells of *A. filiculoides* which appeared as dark, electron dense deposits in light and transmission electron microscope in leaf cells of fronds treated with lead. All the observed lead deposits were localized in vacuoles, while larger lead deposits were found in mature leaves than in young leaves. However, no lead deposits were found in cells of the cyanobiont *Anabaena*. In *A. pinnata*, Pb treated fronds showed varied levels of ultramorphological changes such as compactness of fronds, closed stomata, and deposition of epicuticular waxes while Fe-treated fronds did not show these changes. They also observed that Pb-induced ultra-morphological abnormalities were relieved by presence of Fe in the medium under mixed (Pb + Fe) treatment (Gaumat et al. 2008).

Molecular Mechanism of Metal Hyperaccumulation

Hyperaccumualtion of heavy metal involves several steps, such as transport of heavy metal across plasma membrane, translocation of heavy metal, detoxification, and sequestration at cellular and whole plant level (Shah and

Nongkynrih 2007; Rascio and Navari-Izzo 2011). In recent years, the understanding of entry of both essential and nonessential metal ions in plant cells at the molecular level, has greatly advanced. Several plant metal transporters identified so far include ZIP1-4, ZNT1, IRT1, COPT1, Nramp (natural resistance associated macrophage protein), AtVramp1/3/4, and LCT1 on the plasma membrane–cytosol interface; ZAT, CDF (cation diffusion facilitator), ABC type, AtMRP, HMT1, CAX2 seen in vacuoles; RAN1 seen in Golgi bodies. During metal interaction in Cd/Zn hyperaccumulator *Arabidopsis halleri,* a decrease in the uptake of Cd was observed by roots with the increase in Zn concentrations. This clearly demonstrates that Cd influx is largely due to Zn transporters that have a strong preference for Zn over Cd (Zhao et al. 2002). Similarly, the preference of Zn over Ni by some Zn-/Ni-hyperaccumulators supplied with the same concentrations of heavy metals, also strongly suggests that a Zn transporter system might be involved in Ni entry into the roots of *Thlaspi caerulescens* (Assuncao et al. 2008). A better understanding of these metal transporters and their functionality under multi-elemental conditions to achieve removal of heavy metal ions from the contaminated sites holds great potential. Constitutively large quantities of small organic molecules are present in hyperaccumulator roots that can operate as metal-binding ligands. Different chelators contribute to metal detoxification by buffering cytosolic metal concentrations, whereas chaperone specifically delivers metal ions to organelles and metal requiring proteins. In plants, the principal classes of metal chelators include phytochelatins, metallothioneins, organic acids, and amino acids (Shah and Nongkynrih 2007).

There is only one report by Schor-Fumbarov et al. (2005) who characterized metallothioneins from *Azolla filiculoides* (Accession No. AF482470) grown under heavy metal stress. These metallothioneins (MTs) are low molecular weight (4–10 kDa), cysteine-rich, metal-binding proteins that bind metals via the thiol groups of cysteine residues. MT proteins have been classified based on the arrangement of Cys residues; Class I includes primarily mammalian MTs containing 20 highly conserved Cys residues and Class II includes MTs from plants and fungi, as well as invertebrate animals. The plants Class II have been further divided into four types based on the arrangement of cysteine residues in the amino- and carboxy-terminal domains (Cobbett and Goldsbrough 2002). Depending on the MT RNA expression in a number of plant species, type 1 MT genes are expressed more abundantly in roots than leaves, whereas type 2 MT genes are expressed primarily in the leaves. Type 3 includes many MTs identified as being expressed during fruit ripening, and type 4 MTs, exemplified by the wheat Ec protein are expressed only in seeds (Cobbet and Goldsbrough 2002). Schor-Fumbarov et al. (2005) concluded that metalloprotein, AzMT2 in *Azolla filiculoides* showed significant similarity to type 2 MTs in plants and is encoded by the fern genome. The temporal analysis of AzMT2 indicates participation of this MT in both the homeostasis of essential metals as well as detoxification of toxic metals. The lack of research with respect to molecular mechanism involved in phytoremediation potential in *Azolla* may be in part due to existence of complex nature of interaction between the host—*Azolla* and its symbionts—bacteria and cyanobacteria (Sood and Ahluwalia 2009) and ambiguous identity/taxonomic nature of its cyanobacterial symbiont (Sood et al. 2008a, b).

RESEARCHABLE ISSUES AND FUTURE OUTLOOK

An interesting aspect of research on which information is lacking is the evolution of hyperaccumulation and its ecological significance or benefits to hyperaccumulators. Several hypotheses have been put forward some of which provide supporting evidence, while others led to contradictory responses. These include the "inference hypothesis" or elemental allelopathy which postulates that the perennial hyperaccumulators may interfere with the growth of neighboring plants through enrichment of metal in the surrounding environment. Another interesting hypothesis evolves around the elemental defense mechanism, which metal accumulation can serve as a self defense strategy against natural enemies such as pathogens or herbivores. However, in-depth analysis is required in *Azolla* before conclusions can be drawn. Research on these aspects in *Azolla* may prove interesting and enlightening in light of its proliferative nature. *Azolla* possesses all the properties of an ideal plant for use in phytoremediation, such as fast growth rate, high biomass production, moderately extensive root system, easy to harvest and tolerance to a wide range of heavy metals. An integrated approach can be developed using *Azolla* biomass produced during phytoremediation as source for bioenergy production or bio-ore for recovery of marketable amount of precious heavy metal. The cake left after the extraction of heavy metals can be a good source of protein rich feed for animals or can be use a green manure. Much research is still needed on metal transporters and their regulatory genes. This will provide effective strategies to utilize *Azolla* for treatment of wastewater with multi-element contamination. The impact of heavy metal uptake on the overall physiological/biochemical metabolism and their regulation at genetic level represent other promising areas of future research. Armed with a better understanding of this symbiotic association, this environment friendly system can be fruitfully employed in the field of phytoremediation.

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