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Phytoremediation potential and physiological changes in *Spirodela polyrhiza* L. exposed to different heavy metal concentrations

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Abstract
Spirodela polyrhiza L. a monocotyledonous aquatic macrophyte was investigated for its capacity to phytoremediate heavy metal-polluted water. The plants were grown in Hoagland nutrient solution and exposed to different concentrations of Copper, Lead and Cadmium (0 ppm, 0.5 ppm, 1 ppm, 2 ppm, 4 ppm and 8 ppm) separately for a period of 15 days under *in-vitro* conditions. Metal accumulation, Bio concentration factor (BCF), Relative growth rate (RGR), Tolerance index (TI) and effect on photosynthetic pigments were recorded after the experimental period. Results showed that Cu, Pb and Cd accumulation increases in a concentration dependent manner and was found in the order: Cu>Cd>Pb. Bio concentration factor (BCF) varied at different concentrations of heavy metals tested. Maximum values of Bio concentration factor (BCF) for Cu, Pb and Cd were 780, 405 and 600 respectively at 0.5 ppm concentration. Indicating that the accumulation potential of *Spirodela polyrhiza* L. for Cu is higher than Cd and Pb. Furthermore, BCF decreases with increase in concentration of heavy metals in the medium. RGR, TI and Photosynthetic pigments declined with increasing in concentration of metals in the medium as compared to control. RGR was found maximum in control (0.007 g/g/day), while as in case of Cu, Pb and Cd decline in growth rate was observed at all concentrations. The maximum decline in growth rate was observed in Cd, followed Pb and Cu. Tolerance index (TI) of *Spirodela polyrhiza* L. against Cu, Pb and Cd was found maximum at 0.5 ppm (Cu: 75.17%, Pb: 66.82% and Cd: 61.37%) while as minimum TI was found at 8 ppm concentration (Cu: 37.38%, Pb: 27.21% and Cd: 20.23%), Furthermore TI of *Spirodela polyrhiza* L. against copper was maximum as compared to lead and cadmium. The maximum content of photosynthetic pigments were detected in control plants without treatment, while as decline in photosynthetic pigments were found among all the heavy metals tested. Copper, however, had the least impact on photosynthetic pigments when compared to lead and cadmium. The results suggests that *Spirodela polyrhiza* L. could tolerate heavy metals and can be a good candidate for phytoremediation of polluted water with low and moderate concentration of heavy metals.

Keywords: *Spirodela polyrhiza* L, heavy metals, phytoremediation, photosynthetic pigments

Introduction
Urbanization and industrialization had triggered extreme water pollution by draining effluents directly into water bodies. Among various water pollutants, Heavy metals are of major concern because of their persistent and bioaccumulative nature (Chang *et al*., 2009) [3]. Heavy metal contamination in aquatic and soil environments is a serious environmental problem, which threatens aquatic ecosystems, agriculture and human health (Overesch *et al*., 2007) [25]. Heavy metals occur in the surrounding environment as natural ingredients or from agricultural, industrial and chemical industries. They are biologically non-degradable and have to be removed from the water. Copper, Lead and Cadmium are toxic heavy metals and have a deleterious effect on living organisms (Khellaf and Zerdaoui, 2009) [11]. Although Copper is essential for plant growth and development, Copper is involved in the metabolic processes of plants and is important microelements, which in excess becomes toxic (Teisseire and Vernet, 2000) [32]. Copper plays an important role in photosynthesis and breathing processes of the plant cell (Kanoun-Boule *et al*., 2009) [10]. However, Pb and Cd has no known biological role in plants, and is harmful even in very low doses (Khellaf and Zerdaoui, 2010) [12]. These metals enter the aquatic environment through both natural (weathering of rocks) and anthropogenic (Industrial effluents and agricultural runoffs) activities (Parlak and Yilmaz, 2013) [26]. Copper, Lead and Cadmium cause many alterations in morphological (Such as necrosis, colony disintegration, root break-up) and physiological (Such as photosynthesis, pigment synthesis

and enzyme activity) parameters of aquatic plants (Khellaf and Zerdaoui, 2010; Xing *et al*., 2010) [12, 36]. Aquatic plants are well known to accumulate metals from the environment and to concentrate them within the trophic chains (Miretzky *et al*., 2004) [20]. In other words, using aquatic plants to separate metals from their surrounding waters is extremely efficient, technologically feasible, and cost-effective (Maine *et al*., 2001; Prasad *et al*., 2001; Upadhyay *et al*., 2007) [17, 28, 33]. In this context, plants with a high colonization rate can be viewed as excellent tools for phytoremediation (Prasad *et al*., 2001) [28]. Duckweed plants are common in the aquatic environment, especially in quiescent water bodies and are divided into four genera: Spirodela, Wolffiella, Lemna and Wolffia. There are approximately 40 species worldwide. Duckweed plants are widely distributed in the world from the tropical to the temperate zones, from freshwater to brackish estuaries and throughout a wide range of trophic conditions (Mohan and Hosetti, 1999) [21]. Duckweed has been reported to remove various elements (e.g., As, Cd, Cr, Cu, Ni, Pb, Fe, Au, Pt, U, B, Sr, and Zn) (Wang *et al*., 2002; Oporto *et al*., 2006; Upadhyay *et al*., 2007; Sasmaz and Obek, 2009) [35, 24, 33, 30]. *Spirodela polyrhiza* L. or great duckweed, is a common floristic element of fresh water worldwide and often covers the entire water surface of canals, standing waters surrounding agriculture areas. *Spirodela polyrhiza* L. belongs to family Lemnaceae and is the smallest and fastest growing angiosperm (Ziegler *et al*., 2015) [38]. It is becoming a vital model plant in theoretical and applied research (Acosta *et al*., 2021) [1]. It holds a distinct position because of several advantages including high productivity and tolerance to heavy metal stress (Olguin *et al*., 2005) [23]. The potential of *Spirodela polyrhiza* L. for heavy metal uptake is fast and involves adsorption, ionic exchange and chelation, while biological processes such as intracellular uptake is comparatively slow but help in subsequent translocation of metals from roots to leaves (Sune *et al*., 2007) [31]. *Spirodela polyrhiza* L. is considered as a good accumulator of Cu, Pb and Cd because it shows high bio-concentration factor (BCF) which can reach in the range of 1000 to 2000 in batch systems and 2000 to 3400 in continuous systems (Olguin *et al*., 2005) [23]. The aim of the present study were to investigate the responses of *Spirodela polyrhiza* L. upon exposure to Cu, Pb and Cd stress on metal accumulation, BCF, RGR, TI and changes in pigments (Chlorophyll a, Chlorophyll b, Total chlorophyll and Carotenoids) content.

Materials and Methods

Fresh samples of *Spirodela polyrhiza* L. used in this investigation were collected from Hokersar wetland (34^0 $05^/$ N: 74^0 $12^/$ E) of Kashmir Himalaya. The experiment was conducted in plastic tubs with three replicates for each treatment. Initially 10 mg of plant samples were grown in plastic tubs containing Hoagland nutrient solution of strength 10%, pH: (6.0- 6.5), under Temp: 25±1 °C, with 16hr light and 8 hr darkness. The plants were exposed to different concentrations of Copper ($CuSO_4.5H_2O$), Lead ($Pb(NO_3)_2$) and Cadmium ($CdSO_4$) (0.5 ppm, 1 ppm, 2 ppm, 4 ppm and 8 ppm) separately. Comparison of metal exposed plants was made with untreated (control) plants. The data pertaining to accumulation, bio concentration factor (BCF), relative growth rate (RGR), tolerance index (TI) and pigment content were determined in the fronds of *Spirodela polyrhiza* L. after 15 days of treatment period.

Determination of Heavy metal Accumulation

The analysis of plant samples were carried out using methodology given by Lindsay and Norvell (1978) [14]. The plant samples were first digested using di-acid or tri-acid digestion. The digested samples were then subjected to Atomic Absorption Spectrophotometer (AAS) analysis for presence of elements (Cu, Pb, and Cd) by using appropriate hallow cathode lamp for the element of interest. A mixture of compressed air as oxidant and acetylene as fuel were used.

Determination of Relative growth rate (RGR)

The relative growth rate (RGR) measured the amount of biomass production over time (Hunt, 1978) [9].

RGR (g/g/day) = lnW2 - lnW1/T2 - T1

W1 is initial fresh weight of plant (g) and W2 indicates final fresh weight of plant (g), T1 and T2 are the initial and final times for treatment respectively (day).

Determination of BCF

The bio-concentration factor (BCF) is the ability of heavy metals accumulation (Marchiol *et al*., 2004) [19]. BCF was calculated by dividing the trace element concentration in plant tissue in mg/kg at harvest with initial concentration of the element in the external nutrient solution in mg/L.

BCF = Trace element concentration in plant tissue (mg kg^{-1}) at harvest / initial concentration of the element in solution (mg l^{-1})

Determination of Tolerance index (TI)

The Tolerance index (Ti) determines the ability of plants to grow in the presence of a given concentration of metal (Lux *et al*., 2004) [16]

Ti was calculated as;

Ti (%) = Fresh weight of treated plants grown in metal solution / Fresh weight of plants grown in control solution.

Chlorophyll estimation

The contents of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids were estimated according to the method given by (Hiscox and Isrealstam, 1979; Duxbury and Yentsch, 1956) [7, 6] using dimethyl sulphoxide (DMSO). Hundred mg of fresh leaves were homogenized in 10 ml of DMSO and after keeping it in the oven for half an hour at 40 to 50 °C, the absorbance of solution was noted at 480, 510, 645 and 663 nm using double beam UV-VIS spectrophotometer (UV-570488). The amount of chlorophyll pigments were calculated using the formulae;

$$\text{Chlorophyll } a \text{ mg g}^{-1} \text{ tissue} = \frac{12.3 \times (A_{663}) - 0.86 \times A_{645}}{d \times 1000 \times W} \times V$$

$$\text{chlorophyll } b \text{ mg g}^{-1} \text{ tissue} = \frac{19.3 \times (A_{645}) - 3.6 \times A_{663}}{d \times 1000 \times W} \times V$$

$$\text{Total chlorophyll mg g}^{-1} \text{ tissue} = \frac{20.2 \times (A_{645}) + 8.02 \times A_{663}}{d \times 1000 \times W} \times V$$

$$\text{Carotenoid content mg g}^{-1} \text{ tissue} = \frac{7.6 \times (A_{480}) - 1.49 \times A_{510}}{d \times 1000 \times W} \times V$$

A_{663}, A_{645}, A_{510} and A_{480} are the values of absorbance at the respective Absorbtion spectra.
V = Final volume of chlorophyll extract in DMSO;
W=Fresh weight of sample
d=length of light path (taken as 1)

Statistical Analysis
Statistical analysis was carried out by using Microsoft excel 2010 and Opstat software. Data presented are the means of triplicates. Duncan's multiple range test (DMRT) was performed to determine the significant difference between treatments. Significance was set for $p \leq 0.05$

Results and Discussion
Metal Accumulation and Bio concentration factor (BCF)
The accumulation of Cu, Pb and Cd in *Spirodela polyrhiza* L. after 15 days of treatment period is presented in Table 1. *Spirodela polyrhiza* L. accumulated copper, lead and cadmium in a concentration dependent manner and increases with increase in metal concentration in the medium. The accumulation of copper, lead and cadmium reached maximum at 8 ppm copper, lead and cadmium after 15 days stress period. The maximum values for Cu (1.410 ppm), Pb (0.610 ppm) and Cd (1.066 ppm) were recorded in treatment T_5 (8 ppm) and minimum values were recorded in treatment T_1 (0.5 ppm) for Cu (0.390 ppm), Pb (0.202 ppm) and Cd (0.300 ppm) (Fig. 1). Among the heavy metals the order of accumulation was Cu>Cd >Pb. As for the BCF of different metals is concerned, the highest BCF was shown by Cu (780), Pb (405) and Cd (600) in treatment T_1 (0.5 ppm). The lowest values was shown by Cu (176.25), Pb (76.25) and Cd (133.31) in treatment T_5 (8 ppm) (Fig. 1). The BCF value decreases in all metal treated plants as concentration of metals increased in the medium. The plant with BCF over 1000 is considered as hyperaccumulator accumulator (Rascio and Navarizo, 2011) but in our study BCF was below 1000, hence *Spirodela polyrhiza* L. is a moderate accumulator of heavy metals. Furthermore, *Spirodela polyrhiza* L. showed maximum BCF value for Cu followed by Cd and Pb. The increase in the accumulation by macrophyte (*Spirodela polyrrhiza* L.) may be due to increased number of binding sites for the complexation of heavy metal ions leading to the increased absorption and uptake. The accumulation of metals in aquatic plants is often accompanied by a variety of morphological and physiological changes, some of which directly contribute to tolerance capacity of the plants (Prasad *et al*., 2001; Ding *et al*., 2007) [4, 28]. Similar findings were reported by (Lu *et al.*, 2004; Velichkova *et al*., 2019) [34].

Relative growth rate (RGR) and Tolerance index (TI)
The metals Cu, Pb and Cd cause irreversible damage to duckweed. The present study found that RGR decreased with an increase in Cu, Pb and Cd concentration in the growth medium, However Cu at low concentration 0.5 ppm show minimum reduction in RGR as compared to Pb and Cd. RGR was found maximum in control (0.096 g/g/ day), While as the minimum value of RGR for Cu (-0.263 g/g/ day), Pb (-0.344 g/g/ day) and Cd (-0.503 g/g/day) were recorded at T_5 (8 ppm) concentration, at low concentration of Cu (0.5 ppm), the effect on RGR remained relatively low (Table 1: Fig. 2). The relative growth rate of Lemna sp. decreases with increasing metal concentrations. The reduction in plant growth was due to heavy metal stress and toxicity in plants (Lu *et al*., 2004; Xue *et al*., 2018) [37]. Khellaf and Zerdaoui, 2009 [11]; Leblebici *et al*., 2010 [13] and Bianconi *et al*., 2013 [2] also reported significant reduction in relative growth rate of lemna species under heavy metal stress. The tolerance index (Ti) is the indicator of the ability of plants to grow in the presence of a certain concentration of metal (Lux *et al*., 2004) [16]. The tolerance index of *Spirodela polyrhiza* L. after 15 days of treatment with Cu, Pb and Cd decreased with increase in heavy metals concentration in the medium. The plant showed a decreasing trend of tolerance as the concentration of heavy metals increased. For instance, The TI of Cu has reduced from 75.17% in T_1 (0.5 ppm) to 37.38% in T_5 (8 ppm). In case of Pb the maximum tolerance index 66.82% was recorded in T_1 (0.5 ppm) and minimum 27.21% in T_5 (8 ppm). While as in case of Cd maximum tolerance index (61.37%) was recorded in T_1 (0.5 ppm) and minimum 20.23% in T_5 (8 ppm) (Table 1: Fig. 2). An important indicator of the ability of plants to grow in the presence of a certain concentration of metal is the Tolerance index (Ti). According to Lux *et al*., 2004 [16] plants are considered tolerant if they have TI higher than 60%. In our study, *Spirodela polyrhiza L.* was found to be tolerant to all heavy metals at low concentration (0.5 ppm), But against Cu *Spirodela polyrhiza* L. shows tolerance up to 1 ppm concentration, and the order of tolerance towards different concentrations of heavy metals was: Cu> Pb> Cd. Similar findings were recorded by Khellaf and Zerdaoui, 2009 [11]; Bianconi *et al*., 2013 [2].

Photosynthetic pigments
In the present study, exposure to Cu, Pb and Cd severely affected different photosynthetic pigments (Chlorophyll a, b, Total chlorophyll and Carotenoids) as compared to control (Table 1 and Fig. 3, 4). Photosynthetic pigments are highly sensitive to the oxidative stress induced by environmental changes and heavy metals. As compared to Cu and Pb, Cd provoked more toxic effects on photosynthetic pigments in *Spirodela polyrhiza* L. The amount of Chlorophyll ***a*** was found maximum in control (1.450 mg/g) and lowest was recorded in 8 ppm Cu (0.457 mg/g), followed by Pb (0.258 mg/g) and Cd (0.115 mg/g). The maximum decline in Chlorophyll b was recorded in Cd (0.084 mg/g) followed by Pb (0.155 mg/g) and Cu (0.274 mg/g) at 8 ppm concentration, while as in control without treatment maximum amount of chlorophyll b (1.267 mg/g) was recorded. A Significant decrease in Total chlorophyll was observed in case of Cd at 8 ppm (0.241 mg/g), Similarly Total chlorophyll in case of Pb and Cu treated plants reached a minimum value of 0.361 mg/g and 0.727 mg/g respectively. While as in case of control maximum amount of Total chlorophyll (1.597 mg/g) was recorded**.** Carotenoids were found maximum in control (1.022), whereas the maximum decline in Carotenoids was recorded in Cd (0.163 mg/g) followed by Pb (0.225 mg/g) and Cu (0.300 mg/g) at 8 ppm concentration. Of the three tested metals, cadmium was found to be strongest inhibitor of photosynthetic pigments followed by lead and copper in *Spirodela polyrhiza*. The results indicates that pigments are highly sensitive to heavy metal stress and decreases with increase in heavy metal concentration. The reason is that at high or acute levels of metal concentrations, damage to cells occurs because Reactive oxygen species (ROS) levels exceeds the capacity of the plant cells to cope up, however at chronic dosage damage to cells occurs because of accumulation and subsequent metal toxicity (Hou *et al*., 2007) [8]. Heavy metals induced diminution in chlorophyll content has been attributed to the activation of chlorophyll degradative enzymes and inhibition of enzymes involved in chlorophyll biosynthesis (Parmar *et al*., 2013) [27]. The replacement of Mg^{2+} ion in chlorophyll by heavy metals and lipid peroxidation of chloroplast membrane could also be a major cause for pigment loss in *Spirodela polyrhiza* L.(Teisseire and Vernet, 2000; Mal *et al*., 2002; Doganlar, 2013) [32, 5].

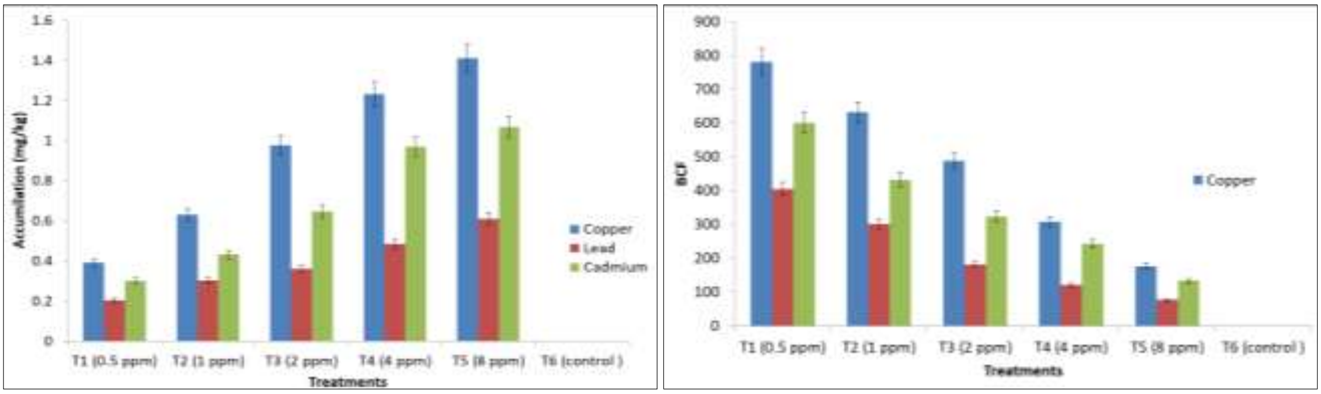

Fig 1: Effect of different concentrations of copper, lead and cadmium on Accumulation and Bioconcentration factor (BCF) of *Spirodela polyrhiza* L. after 15 days of treatment period (pooled data of 2020 & 2021)

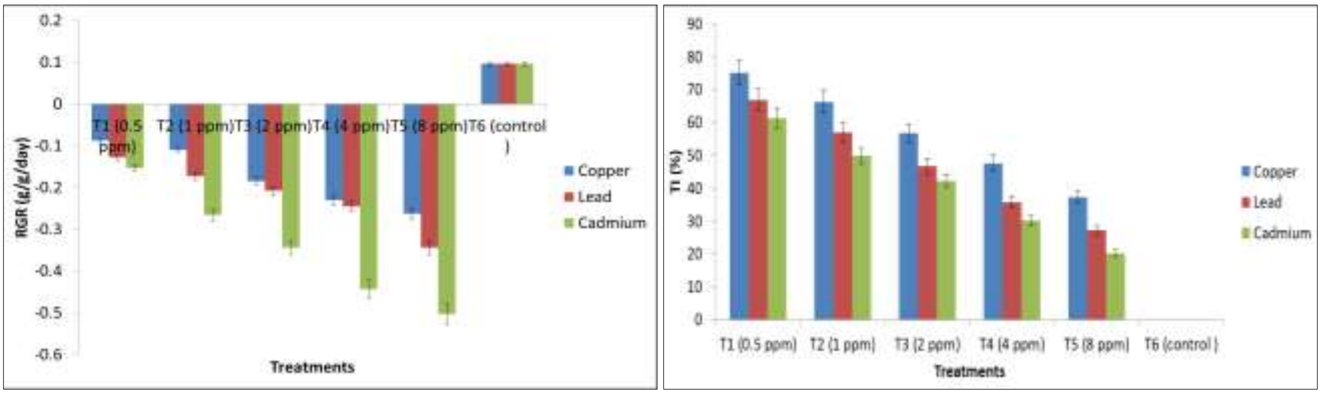

Fig 2:- Effect of different concentrations of copper, lead and cadmium on Relative growth rate (g/g/day) and Tolerance index (%) of *Spirodela polyrhiza* L. after 15 days of treatment period (pooled data of 2020 & 2021)

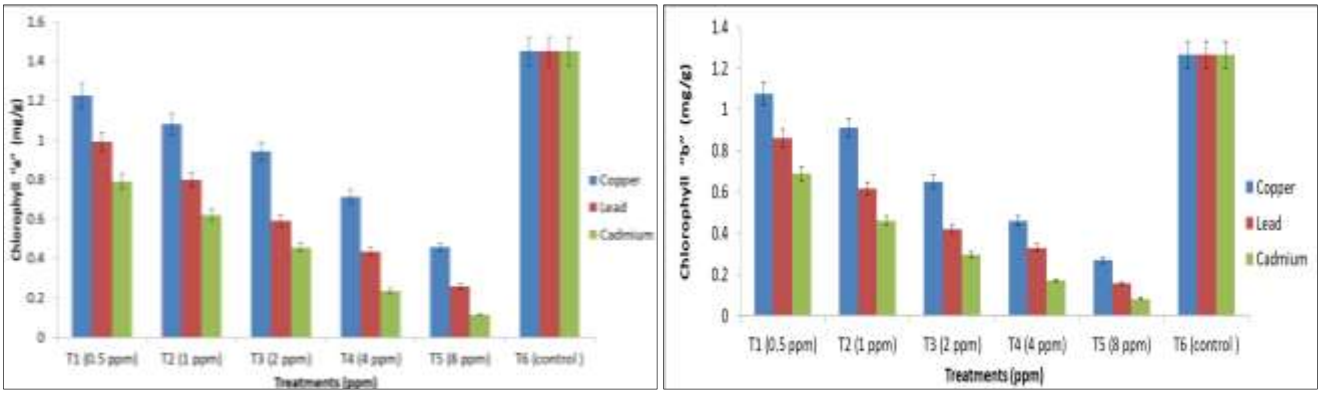

Fig 3: Effect of different concentrations of copper, lead and cadmium on Chlorophyll "a" (mg/g) and Chlorophyll "b" (mg/g) of *Spirodela polyrhiza* L. after 15 days of treatment period (pooled data of 2020 & 2021)

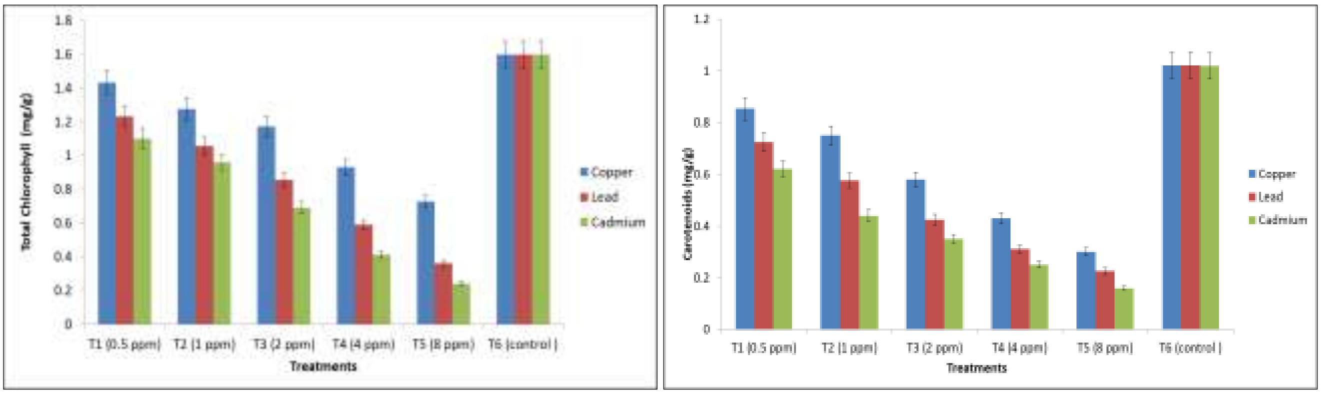

Fig 4: Effect of different concentrations of copper, lead and cadmium on Total Chlorophyll (mg/g) and Carotenoids (mg/g) of *Spirodela polyrhiza* L. after 15 days of treatment period (pooled data of 2020 & 2021)

Table 1: Effect of different concentrations of Copper, Lead and Cadmium on accumulation (mg/kg), bio concentration factor, relative growth rate (g/g/ day), tolerance index (%), chlorophyll "a" (mg/g), chlorophyll "b" (mg/g), total chlorophyll (mg/g) and carotenoids (mg/g) in *Spirodela polyrhiza* L. (pooled data of year 2020 & 2021)

Days	Parameters	Accumulation (ppm)			Bio concentration factor (BCF)			Relative growth rate (g/g/ day)			Tolerance index (% age)			Chlorophyll "a" (mg/g)			Chlorophyll "b" (mg/g)			Total chlorophyll (mg/g)			Carotenoids (mg/g)		
	Heavy metals / Treatments	Copper	Lead	Cadmium	Copper	Lead	Cadmium	Copper	Lead	Cadmium	Copper	Lead	Cadmium	Copper	Lead	Cadmium	Copper	Lead	Cadmium	Copper	Lead	Cadmium	Copper	Lead	Cadmium
15	T1 (0.5 ppm)	0.390^{e}	0.202^{e}	0.300^{e}	780^{a}	405^{a}	600^{a}	-0.087^{b}	-0.128^{b}	-0.153^{b}	75.17^{a}	66.82^{a}	61.37^{a}	1.225^{ab}	0.990^{b}	0.788^{b}	1.077^{b}	0.863^{b}	0.695^{b}	1.430^{ab}	1.230^{b}	1.098^{b}	0.853^{ab}	0.725^{b}	0.621^{b}
	T2 (1 ppm)	0.631^{d}	0.301^{d}	0.431^{d}	631.50^{b}	301^{b}	431.50^{b}	-0.110^{b}	-0.173^{bc}	-0.266^{c}	66.31^{b}	56.94^{b}	49.83^{b}	1.080^{bc}	0.798^{bc}	0.621^{bc}	0.913^{c}	0.615^{c}	0.461^{c}	1.275^{b}	1.055^{bc}	0.960^{b}	0.750^{bc}	0.575^{bc}	0.443^{bc}
	T3 (2 ppm)	0.976^{c}	0.360^{c}	0.646^{c}	488.25^{c}	180^{c}	323.25^{c}	-0.185^{c}	-0.208^{cd}	-0.344^{d}	56.68^{c}	46.68^{c}	42.22^{c}	0.942^{cd}	0.590^{cd}	0.455^{c}	0.650^{d}	0.421^{d}	0.295^{d}	1.171^{bc}	0.855^{c}	0.690^{c}	0.580^{cd}	0.425^{cd}	0.352^{cd}
	T4 (4 ppm)	1.232^{b}	0.485^{b}	0.968^{b}	308^{d}	121.25^{d}	242.12^{d}	-0.230^{cd}	-0.245^{d}	-0.443^{e}	47.58^{d}	35.76^{d}	30.20^{d}	0.712^{de}	0.433^{de}	0.235^{d}	0.464^{e}	0.332^{d}	0.173^{e}	0.930^{cd}	0.588^{d}	0.410^{d}	0.430^{de}	0.310^{d}	0.250^{cd}
	T5 (8 ppm)	1.410^{a}	0.610^{a}	1.066^{a}	176.25^{e}	76.25^{e}	133.31^{e}	-0.263^{d}	-0.344^{e}	-0.503^{f}	37.38^{e}	27.21^{e}	20.23^{e}	0.457^{e}	0.258^{e}	0.115^{d}	0.274^{f}	0.155^{e}	0.084^{e}	0.727^{d}	0.361^{d}	0.240^{d}	0.300^{e}	0.225^{d}	0.163^{d}
	T6 (control)	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	0.096^{a}	0.096^{a}	0.096^{a}	0^{f}	0^{f}	0^{f}	1.450^{a}	1.450^{a}	1.450^{a}	1.267^{a}	1.267^{a}	1.267^{a}	1.597^{a}	1.597^{a}	1.597^{a}	1.022^{a}	1.022^{a}	1.022^{a}
	CD ($p \leq 0.05$%)	0.038	0.026	0.055	19.48	13.26	28.77	0.014	0.019	0.013	2.202	1.935	2.036	0.171	0.176	0.130	0.090	0.083	0.070	0.180	0.179	0.151	0.154	0.140	0.138

Data is given as average of three replicates. Duncan's multiple range test (DMRT) was performed to determine the significant difference between treatments. Similar letters denote values are not significantly different at $p \leq 0.05$.

Conclusion

Based on the findings of the present investigation, It could be concluded that accumulation of *Spirodela polyrhiza* L was maximum for copper as compared to lead and cadmium, Furthermore in *Spirodela polyrhiza* L. maximum BCF value of approximately 780 was recorded at low concentration of Copper (0.5 ppm), While as minimum BCF was recorded in case of Pb and Cd. Hence *Spirodela polyrhiza* L. can be classified as good accumulator species of copper and moderate accumulator of lead and cadmium. All heavy metals show effect on relative growth rate of *Spirodela polyrhiza* L. which result in toxicity but the effect of toxicity was less evident in copper treated plants as compared to lead and cadmium. Tolerance index was found higher in *Spirodela polyrhiza* L. at low concentrations of Cu, Cd and Pb. The decline in photosynthetic pigments was found in all tested metal concentrations. However Cu showed minimum reduction in photosynthetic pigments as compare to lead and cadmium. The present study suggests that *Spirodela polyrhiza* L. could tolerate heavy metals and can be a best option for phytoremediation of polluted water with moderate and low concentration of heavy metals.

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