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Fluoride mediated biochemical responses and removal potential in hydroponically grown duckweed (*Spirodela polyrhiza* L. Scheldien)

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Abstract:

The present study was done to investigate the fluoride removal potential of *Spirodela polyrhiza*. The fronds were exposed to 5, 10, 15, 20, 25 50 ppm NaF concentrations for 24, 72, 120 and 168 h and were analysed for its influence on various biochemical parameters viz: photosynthetic pigments (chl a, chl b, total chlorophyll content and carotenoids), carbohydrate content, protein content and malondialdehyde content. The total chlorophyll and protein content of *Spirodela polyrhiza* decreased significantly with increasing fluoride concentration at all the exposure periods. A significant reduction in the level of carbohydrates was observed in a concentration and duration dependent manner. Results revealed that higher concentrations of fluoride caused oxidative damage in the plants as evidenced by increased lipid peroxidation, decreased chlorophyll and protein contents. The observed results also suggested that percentage removal of fluoride increased with longer exposure times. In addition, there is increase in percent decrease in fresh weight and decrease in dry to fresh weight ratio of plant at elevated concentration. Thus, the plant responds to abiotic stress induced by fluoride treatment by showing the alteration in biochemical pathways and regulating cellular homeostasis**.**

Keywords: Biochemical parameters, Homeostasis, Lipid peroxidation, Oxidative damage, *Spirodela polyrhiza*.

INTRODUCTION

Increased industrialization, pace of urbanization, various anthropogenic sources and other geochemical activities has triggered the toxic metal pollution of water resources. Accumulation of metals and their toxic effects throughout the food chain poses serious ecological and health implications in living organisms [1]. Among the non-metals, fluorine is considered as one of the most abundant elements in the earth's crust [2]. Fluorine cannot exist in free elemental state in nature because of its high electronegativity [3]. However, it is released during the manufacture of aluminium, bricks, glass and steel and then combines with most elements forming fluorides which are among the most stable form of all chemical compounds [4]. Fluoride (F^-) exposure for prolonged period generates adverse effects not only in livestock and in human beings but also in plants [5, 6, 7]. Fluoride toxicity disturbs almost all the physiological and biochemical parameters of plants. The disturbance in various cellular processes has been identified that cause deleterious effects on plants including lipid peroxidation, membrane damage, inhibition of protein and inactivation of enzymes, thus affecting overall cell viability. This results in generation of reactive oxygen species (ROS) and altered gene expression [8].

The aquatic ecosystems are more prone to be contaminated by pollutants than terrestrial ones because of their relatively small biomass which may lead to accumulation of heavy metals. Hence, aquatic plants form the first link in relation to metal contents present in aquatic environments [9]. *Spirodela polyrhiza* commonly known as duckweed belonging to a family Lemnaceae is a free floating aquatic macrophyte. The aim of present study was to investigate the response of *Spirodela polyrhiza* upon exposure to fluoride stress with references to changes in pigment (total chlorophyll and carotenoid) content, MDA content, carbohydrate content, protein content, growth parameters and to analyse the fluoride removal efficiency of this plant. This study will also be helpful in understanding the biochemical detoxification strategies that plant adopts against oxidative stress induced by fluoride exposure.

MATERIALS AND METHODS

Plant material and experimental setup

Plants of *Spirodela polyrhiza* were collected from Sewage Treatment Plant of Guru Nanak Dev University, Amritsar, India. Before treatments, plants were kept in 3 % Hoagland culture solution under controlled conditions in a seed germinator (temperature: 25+2°C; light/dark cycle: 16/8 h and light intensity: 115 µmol m^2 s^{-1}) for acclimatisation [10]. After one week of acclimatisation period, healthy fronds were used for experimental purpose. About 5 gms of plant material was taken. The plants were treated with six different concentrations of NaF (5, 10, 15, 20, 25, 50) ppm under controlled conditions (temperature: 25+2°C; light/dark cycle: 16/8 h and light intensity: 115 µmol m^2 s^{-1}). A stock solution of 100 ppm of fluoride was made by dissolving 2.21 gms of anhydrous NaF in 1000 ml millipore water. The different concentrations of fluoride were prepared by diluting 100 ppm stock solution of NaF in 3% Hoagland nutrient medium. The plants were kept in Petri Dishes (100 ml solution) containing six different concentrations of fluoride along with one set of control (3% Hoagland solution). The experiments were performed in triplicates. Plants were harvested after time period of 24, 72, 120 and 168 hours and were analysed for chlorophyll, carotenoids, protein content and carbohydrate content, MDA content, various growth parameters and estimation of fluoride (F^-) content.

Chlorophyll content

Chlorophyll content was determined in acetone extract according to the method given by Arnon (1949) [11]. Fresh plants (100 mg) were homogenised in 3 ml of 80% acetone by using pestle and mortar. The homogenate was centrifuged at 8000 rpm for 10 min at 4^0C and absorbance of chl a and chl b was measured at 663 and 645 nm respectively. The concentrations were expressed as µg/ml.

Total carotenoid content

Carotenoid content was measured by using the protocol given by Lichtenthaler and Wellburn (1983) [12]. The plant material (100 mg) was homogenised in 3 ml of 80% acetone by using pestle and mortar. The homogenate was centrifuged at 8000 rpm for 10 min at 4^0C. The supernatant from the plant extract was used for the analysis of carotenoid content. The absorbance was read at 470 nm the concentration was expressed as µg/ml.

MDA content

The level of lipid peroxidation, expressed as (malondialdehyde) content, was determined as 2-thiobarbituric acid (TBA) reactive species. Plant fresh tissues (1gm) was homogenised in 3 ml TCA (0.1%) and extracted with 3 ml of 0.5% TBA made in 20 % trichloroacetic acid (TCA). Then extract was heated at 95 °C in a boiling water bath for 30 min and then rapidly cooled in ice. After centrifugation at 10,000 rpm for 10 min, the absorbance of the supernatant was taken at 532 nm. Non-specific turbidity was corrected by subtracting the absorbance value taken at 600 nm. The concentration of MDA was calculated using extinction coefficient of 155 m M^{-1} cm^{-1} [13]. The concentration was expressed in µmol/gm fresh weight.

Carbohydrate content

Total carbohydrate content in the plant material was determined using Anthrone method. 100 mg sample was hydrolysed on a boiling water bath for three hours with 5 ml 2.5 N concentrated HCl. After cooling to room temperature, the solution was neutralized with sodium carbonate (Na_2CO_3) until the effervescence ceases. The solution was made upto 100 ml with distilled water and centrifuged. Then 1 ml of supernatant was added to 4 ml of anthrone reagent and then boiled for 8 min on a boiling water bath. After cooling, the absorbance of the solution was measured at 630 nm. The total carbohydrate content present in the sample was expressed in mg/g fresh weight.

Determination of total soluble protein content

Total soluble protein contents of the enzyme extracts were measured according to Bradford (1976) using bovine serum albumin as a protein standard [14]. For plant extract preparation, 1 gm of plant material was homogenised in 3 ml phosphate buffer. The homogenate was centrifuged at 12000 rpm for 20 min at 4^0C. The supernatant was taken in spectrophotometric cuvette containing Bradford reagent and absorbance was read at 595 nm. The total soluble protein content was expressed as mg/g fresh weight.

Estimation of fluoride content

The residual water samples were filtered using Whatman no.42 filter paper immediately after experiment and kept at 0-4 ^{0}C until analysed. The concentration of fluoride (F^-) in residue water sample was measured using ion chromatography with known concentration of fluoride solution for calibration purpose. For the preparation of standards, NaF was used.

Growth parameters

After harvesting, the fronds were cleaned properly with double distilled water. Moisture content was removed by moderately pressing the fronds between two folds of filter paper. Fresh weight of the plant was determined immediately after harvesting at different time intervals. For determining plant dry weight, plants were oven dried at 105^0C for first 20 min for enzymatic deactivation and then dried at 70^0C for 48 h to obtain constant weight (dry weight) [15]. After that, percentage decrease in fresh weight was calculated as:

$$\frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

Dry to fresh weight ratio was calculated as:

$$\frac{Dry\ weight\ (g)}{Fresh\ weight\ (g)}$$

Statistical analyses

All the observed data was subjected to two-way analysis of variance (ANOVA) and significance was determined at 95% confidence levels ($p<0.05$). Mean values and standard error were calculated from the result of three replicates for each of the parameters.

RESULTS AND DISCUSSION

Effect of fluoride on photosynthetic pigments

Results indicated significant reduction ($p<0.05$) in photosynthetic pigments of F^- treated fronds of *Spirodela polyrhiza* **(Table 1)**. Chlorophyll a content showed decreasing trend with increasing fluoride concentrations where maximum decrease of 75% and 77.2% was observed at 50 ppm of fluoride for 72 h and 120 h, respectively **(Fig. 1)**. Similar decreasing trend was also observed in chl b, total chlorophyll and carotenoid content at all the fluoride concentrations and the maximum decline of 80.02%, 77.2% and 84.2% at the time interval of 72 h was recorded at 50 ppm, respectively. Photosynthesis is remarkably influenced by fluoride toxicity at elevated concentrations. Decrease in chlorophyll content may be attributed to decrease in enzymatic activity of protochlorophylide reductase [16]. Reduction in photosynthetic pigment is an indicator of abiotic stress in *Spirodela polyrhiza*. The reduced chlorophyll pigment may be due to inhibition of biosynthesis of chlorophyll or may be due to failure of incorporation of ɣ-aminolevulinic acid with elevation in concentration of fluoride [17]. Reduction in photosynthetic pigment under heavy metal stress was also reported in various aquatic plants such as *Eichhornia crassipes, Potomogeton pusillus and Wolffia arrhiza* [18, 19, 20].

According to Hou *et al*. (2007) the reason behind the reduced photosynthetic pigment content in plants could be due to lipid peroxidation in chloroplast membranes [21]. Hence, chlorophyll concentration in fronds of *Spirodela polyrhiza* was significantly affected by fluoride concentration and found to be exposure period and concentration dependent. Carotenoid is a non-enzymatic antioxidant pigment which protect cell against ROS under

fluoride stress [21]. Previous reports also revealed decrease in carotenoid content due to metal or non-metal toxicity [22**].** Also fluoride is a negatively charged ion, which readily complexed with Mg+ forming MgF^{+} and such complex can slow down the photosynthetic pigment formation [23]. These may be the probable reasons behind the reduction in photosynthetic pigment content with increasing concentration of fluoride.

Effect on protein content

In the present study, decrease in total protein content of *Spirodela polyrhiza* at high concentration of fluoride was found to be statistically significant at ($p<0.05$) **(Table 1).** Maximum decline of 83.2 % in protein content was observed at the time interval of 120 h. The reduction was found to be almost constant at 20 and 25 ppm at the same time interval. However, minimum decrease of 0.66% was observed at lowest fluoride concentration of 5 ppm at 144 h **(Fig. 2 g)**. Mishra *et al*. (2008) reported decrease in protein content in aquatic macrophytes and suggested increase in protein degradation by proteases enzyme or other enzymes that are involved in protein metabolism [24]. Reduction in protein synthesis can be attributed to fluoride ability in modification of free nucleotides and RNA ratio or decrease in biosynthesis of RNA [25].

Table 1. Effect of fluoride on biochemical parameters of *Spirodela polyrhiza* under different time intervals

Time period (h)	Concentration (mg/l)	Biochemical parameters						
		Chl. a (µg/ml)	Chl. b (µg/ml)	Total Chl. (µg/ml)	Carotenoid (µg/ml)	MDA (µmol/g fw)	Carbohydrates (mg/g fw)	Protein (mg/g fw)
24	Control	0.82±0.027	1.35±0.132	2.18±0.159	1.11±0.055	5.54±0.394	33.49±4.924	0.39±0.074
	5	0.65±0.035	1.18±0.085	1.84±0.120	0.99±0.050	7.69±0.155	33.09±8.286	0.30±0.040
	10	0.62±0.025	1.25±0.078	1.88±0.057	0.91±0.032	7.65±0.367	18.39±3.004	0.22±0.048
	15	0.57±0.037	1.20±0.078	1.78±0.059	0.81±0.051	7.87±0.197	16.79±0.512	0.19±0.012
	20	0.58±0.028	1.09±0.071	1.68±0.085	0.75±0.020	7.91±0.352	14.09±8.750	0.12±0.029
	25	0.57±0.036	1.00±0.019	1.58±0.054	0.71±0.018	8.68±0.043	9.19±4.651	0.06±0.007
	50	0.52±0.002	0.94±0.011	1.47±0.014	0.71±0.008	8.81±0.367	6.59±2.306	0.02±0.006
72	Control	1.82±0.084	3.02±0.257	4.58±0.285	2.85±0.134	30.8±0.410	38.19±0.793	1.25±0.056
	5	0.96±0.025	1.50±0.071	2.46±0.047	2.07±0.044	30.9±2.260	35.89±0.655	1.12±0.024
	10	0.77±0.035	1.40±0.024	2.17±0.012	1.16±0.011	35.5±1.797	36.59±1.819	0.82±0.011
	15	0.70±0.015	1.27±0.028	1.97±0.042	1.02±0.003	38.1±0.549	35.69±1.452	0.74±0.014
	20	0.60±0.053	1.09±0.102	1.69±0.152	0.91±0.025	39.2±0.414	35.39±1.22	0.65±0.066
	25	0.49±0.023	0.81±0.078	1.31±0.055	0.68±0.049	39.95±0.919	33.59±1.442	0.56±0.028
	50	0.43±0.032	0.60±0.057	1.04±0.025	0.50±0.042	43.52±1.070	17.29±1.493	0.48±0.014
120	Control	2.28±0.067	3.33±0.118	5.62±0.119	2.84±0.045	29.46±0.675	38.69±4.61	0.94±0.021
	5	1.89±0.140	2.90±0.462	4.79±0.323	2.46±0.294	31.48±1.395	25.79±1.646	0.67±0.101
	10	1.28±0.042	2.43±0.013	3.72±0.045	1.83±0.013	33.20±3.453	25.99±3.81	0.42±0.129
	15	1.23±0.015	2.16±0.113	3.39±0.116	1.65±0.053	35.52±2.284	20.99±4.77	0.40±0.104
	20	0.90±0.017	1.29±0.066	2.20±0.049	1.04±0.025	36.17 ±0.983	19.99±2.75	0.19±0.008
	25	0.74±0.025	1.35±0.018	2.10±0.020	1.04±0.049	36.17±1.534	10.49±3.080	0.19±0.0258
	50	0.50±0.013	1.04±0.031	1.55±0.034	0.79±0.042	37.37±0.043	8.79±2.1	0.15±0.051
168	Control	0.63±0.022	1.20±0.054	1.84±0.049	0.86±0.034	8.25±0.227	21.49±3.609	0.55±0.009
	5	0.53±0.002	0.97±0.018	1.51±0.020	0.72±0.011	12.51±0.949	9.49±3.026	0.55±0.021
	10	0.54±0.025	0.96±0.025	1.50±0.032	0.70±0.021	17.61±0.129	8.59±2.59	0.50±0.007
	15	0.49±0.021	0.92±0.044	1.41±0.066	0.66±0.028	17.38±0.746	8.79±2.16	0.50±0.013
	20	0.48±0.009	0.90±0.038	1.39±0.039	0.65±0.020	18.54±0.324	5.39±1.05	0.36±0.094
	25	0.45±0.009	0.88±0.042	1.33±0.051	0.63±0.014	22±0.258	2.89±1.49	0.32±0.056
	50	0.43±0.021	0.80±0.010	1.23±0.023	0.59±0.004	22.75±0.113	2.49±1.21	0.30±0.054
F ratio								
Time period (3, 56)		441.74*	120.44*	413.77*	295.33*	1041.83*	60.19*	163.38*
Concentration (6, 56)		220.57*	62.05*	199.19*	180.40*	28.23	20.42*	58.10*
Time period × Concentration (18, 56)		47.54*	13.33*	43.68*	35.09*	2.74*	1.73	4.10*
HSD value		0.231	0.653	0.592	0.378	6.263	19.20	0.291

***significant at p≤0.05** Results are presented in Mean ± S.E

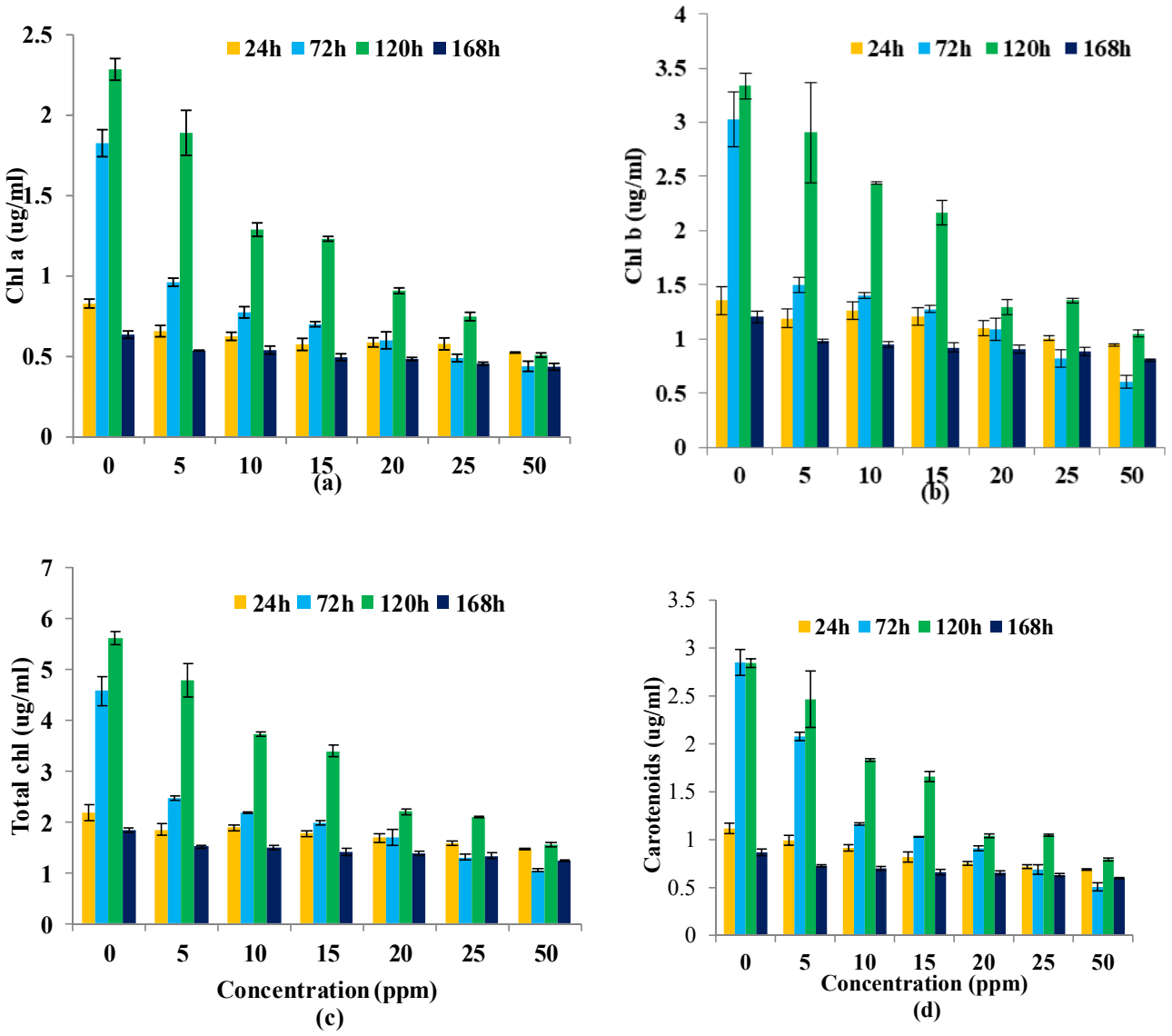


Fig1. Effect of different fluoride concentrations on (a) chl a (b) chl b (c) total chl (d) carotenoids contents of *Spirodela polyrhiza* during different time intervals. Values are mean of triplicates ± SE, n=3.

Table 2. Percent fluoride removal by *Spirodela polyrhiza*

Time period (h)	Percentage removal of fluoride (%age)						
	Control	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm	50 ppm
24	ND	55.50±1.205	24.72±2.541	20.36±3.039	19.78±4.524	18.64±0.588	16.26±1.695
72	ND	60.08±0.981	46.13±2.185	44.37±2.134	43.82±0.577	43.59±3.061	33.17±1.157
120	ND	64.2±1.209	63.23±2.847	59.066±0.41	57.04±0.974	50.20±0.281	52.98±4.978
168	ND	65.64±1.025	60.71±5.578	58.7±2.9810	58.36±0.895	48.85±0.490	52.58±2.312
F-ratio							
Time period (3, 56)	223.127*						
Concentration (6, 56)	281.284*						
Time period × Conc. (18, 56)	11.388*						
HSD value	**12.59**						

***significant at p≤0.05** Data shown are Mean ± S.E of triplicate values ND- Not detected

Table 3. Percent decrease in fresh weight of *Spirodela polyrhiza* with respect to initial weight after fluoride exposure at different time intervals

Concentration (ppm)	24h	72h	120h	168h
Control	0.66±0.441	2.47±1.407	3.16±1.452	4.00±0.288
5	2.33±0.881	3.81±0.438	9.50±1.299	11.33±1.922
10	4.66±0.440	14.05±0.396	21.50±1.607	28.00±2.565
15	9.00±0.500	16.05±1.220	26.83±1.691	30.83±1.166
20	10.33±0.726	19.06±0.845	28.66±0.440	34.00±1.040
25	13.33±0.726	23.06±1.142	33.16±1.092	32.83±1.162
50	15.66±0.726	31.46±1.248	35.5±0.866	39.00±1.892
F-ratio				
Time period (3, 56)	309.87*			
Concentration (6, 56)	291.90*			
Time period x Conc. (18, 56)	10.74*			
HSD value	6.5564			

***significant at p≤0.05** Results are presented in Mean ± S.E

Table 4. Effect of fluoride on dry to fresh weight ratio (DW/FW) of *Spirodela polyrhiza* at different time intervals

Concentration (ppm)	DW/FW (24h) (g)	DW/FW (72h) (g)	DW/FW (120h) (g)	DW/FW (168h) (g)
Control	0.52±0.009	0.57±0.0080	0.497±0.013	0.477±0.036
5	0.33±0.023	0.39±0.0195	0.415±0.023	0.422±0.017
10	0.26±0.025	0.059±0.001	0.231±0.015	0.262±0.016
15	0.24±0.023	0.056±0.002	0.127±0.006	0.050±0.001
20	0.05±0.001	0.054±0.001	0.057±0.001	0.034±0.001
25	0.04±0.001	0.049±0.001	0.044±0.001	0.030±0.001
50	0.027±0.001	0.044±0.001	0.034±0.002	0.022±0.001
F-ratio				
Time period(3, 56)	9.58*			
Concentration(6, 56)	787.0*			
Time period × Conc. (18, 56)	17.16*			
HSD value	0.075143			

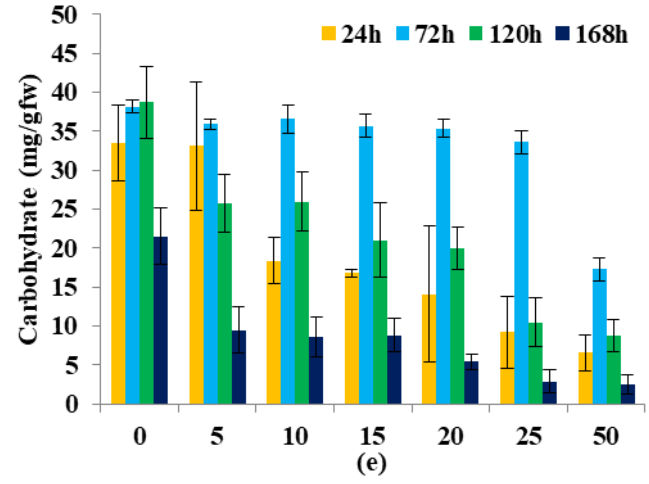
***significant at p≤0.05.** Results are presented in Mean ± S.E

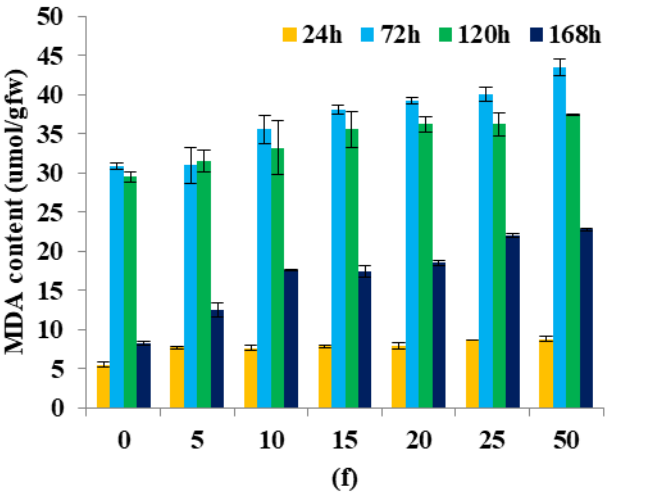
Effect on carbohydrate content

Carbohydrate metabolism showed a decreasing trend with increasing fluoride concentration in nutrient medium. Results revealed that carbohydrate content was significantly ($p<0.05$) lower than controls at all the concentrations **(Table 1)**. In the present investigation, maximum decrease of 88.4% in carbohydrate content was recorded at the highest concentration of fluoride (50 ppm) at time period of 144 h whereas the minimum decrease of 1.15% observed at lowest concentration of fluoride (5 ppm) at 24 h **(Fig. 2 e)**. Asthir and Singh (1995) reported decrease in carbohydrates content of caryopsis of Sorghum plant due to accumulation of fluoride which inhibited the conversion of sugars to carbohydrates [26]. Formation of reducing sugar such as glucose, fructose and mannose exposed to fluoride decreased their concentration which may be due to the conversion of these reducing sugars into non- reducing sugars. Hence, increased level of non-reducing sugars in the plant tissues might be the strategy adopted to overcome fluoride toxicity [27].

Effect on MDA content

Malondialdehyde (MDA) content showed statistically significant ($p<0.05$) increase in fronds of fluoride treated *Spirodela polyrhiza* as compared to control **(Table 1)**. Maximum percent increase of 175% at 50 ppm fluoride concentration at an exposure period of 144 h while minimum increase of 0.2% was recorded at low concentration of fluoride (5 ppm) at the time interval of 72 h **(Fig. 2 f)**. Elevation in MDA levels in fronds of duckweed as an assay of fluoride induced oxidative stress. High levels of MDA content was reported in leaves of *Camellia sinensis* under fluoride stress [28]. Plant cells generate ROS under stressed conditions resulting in oxidative damage and the accumulation of malondialdehyde (MDA) [29]. It indicates oxidative damage and cell membrane integrity. Concomitant increase in lipid peroxidation occurred during stressed conditions due to elevated levels of hydrogen peroxide or generation of reactive oxygen species (ROS) in plant cells [30].





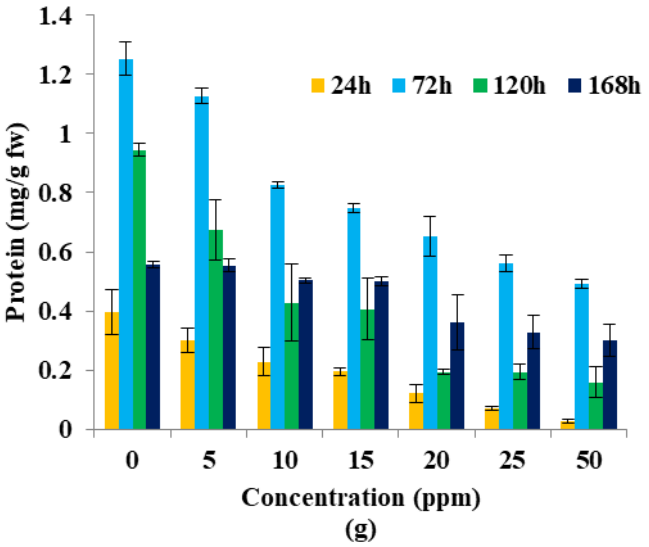


Fig 2. Effect of fluoride on (e) Carbohydrate content (f) MDA content) (g) Protein content c in *Spirodela polyrhiza* during different time intervals. Values are mean of triplicates ± SE, n=3.

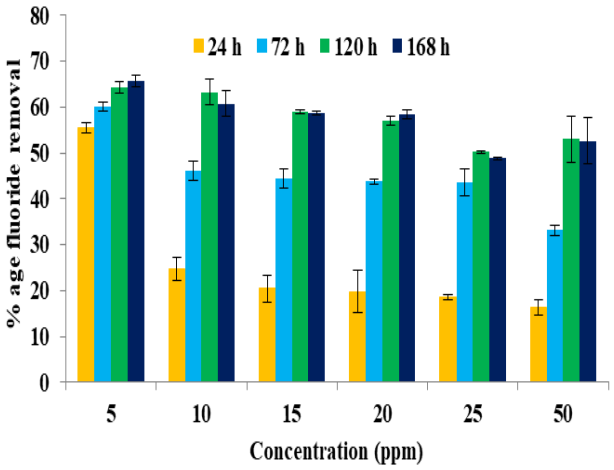
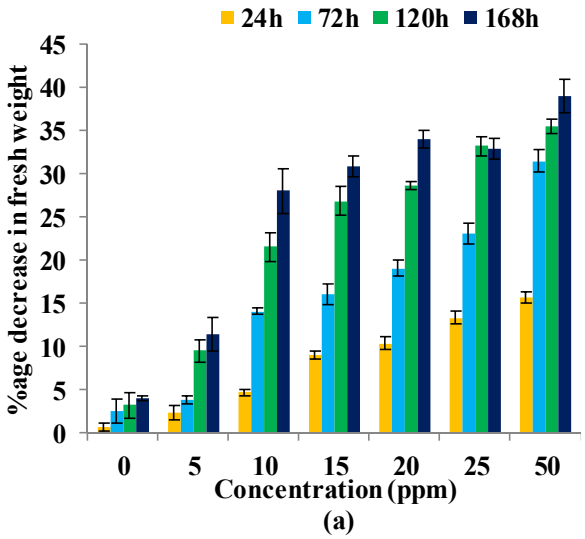


Fig 3. Percent fluoride removal by *Spirodela polyrhiza* during different time interval.



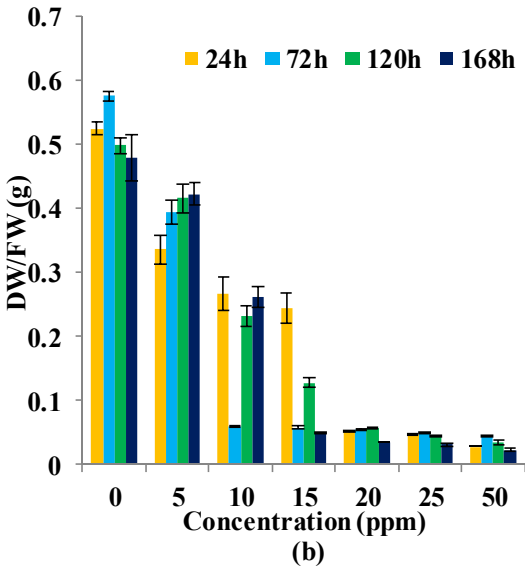


Fig. 4. Effect of fluoride on growth parameters of *Spirodela polyrhiza* (a) Percentage decrease in fresh weight (b) Dry to fresh weight ratio.

Percentage removal of fluoride by experimental plant

Fluoride toxicity induced various visible injury signs like chlorosis, necrosis, tip burn or scorching which initiated on the margins of leaf and finally caused internal cellular injuries [31]. From the entire investigation it was observed that the experimental plant *Spirodela polyrhiza* **(Fig. 3)** showed significant ($p<0.05$) potentiality with respect to removal of fluoride from water. The removal of fluoride was recorded after 24 h, 72 h, 120 h and 168 h with respect to final concentration. It was observed that percentage removal of fluoride from the solution increased with increasing time period and decreased at elevated concentrations. The fluoride solutions induced different toxic effects on *Spirodela polyrhiza* when exposed for longer periods but at its higher concentrations. The maximum percentage removal of 55.5%, 60.08%, 64.2% and 65.6% fluoride was observed at low concentration of 5 ppm at time interval of 24, 72, 120 and 168 h respectively, while at high concentration of 50 ppm the maximum percentage removal of fluoride analysed was 16.2%, 33.2%, 52.9% and 52.5% **(Table 2)**. The removal was found to be almost constant after 120 h and 168 h at same concentration. It means removal efficiency of this macrophyte slowed down during longer exposure period at high concentration. However, the plant is efficient in the removal of fluoride indicating that higher fluoride removal could be possible at longer exposure period.

Effect on plant growth parameters

Growth rate of *Spirodela polyrhiza* in the present investigation is adversely affected under fluoride stress. The percentage decrease in fresh weight was significantly increased and dry to fresh weight ratio was significantly reduced ($p\leq0.05$) at higher concentrations. The maximum percentage decrease in fresh weight was observed at 50 ppm fluoride solution at the time interval of 168 h **(Table 3) (Fig. 4 a)**. This may be due to the accumulation of fluoride by *Spirodela polyrhiza* [32]. Moreover, high dry weight to fresh weight ratio (DW/FW) was observed at lower concentration of 5 ppm at the time interval of 120 h and 168 h. Lower dry weight to fresh weight ratio was observed at high concentration of 50 ppm during all the intervals. **(Table 4) (Fig. 4 b)**.

CONCLUSION:

From the above study it was elucidated that *Spirodela polyrhiza* possess a high level of tolerance to fluoride induced stress based upon its fluoride removal potential. However, further studies are required to understand the specific fluoride tolerance mechanisms and various strategies adopted by *Spirodela polyrhiza* during stressed conditions.

CONFLICT OF INTERESTS:

The authors indicate no conflict of interests.

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