CHAPTER 6

6 Accumulation and Localization of Antimony in *Trapa natans* and *Eichhornia crassipes* grown within a hydroponic system

6.1 Introduction

The contamination of water with toxic heavy metals and metalloids is a major environmental concern in many parts of the world. The main sources of heavy metals in aquatic ecosystems are of the anthropogenic type. Antimony (Sb) is a rare element in the Earth's crust, occurring at 0.2–0.3 mg/kg [1]. Sb is a toxic metalloid widely distributed in the environment and it has no known biological function and displays carcinogenic properties. Sb has no known biological function and can be toxic to plants. It is seen that its toxicity depends upon chemical form in which it exist. However, it has become widespread in the environment as a result of natural processes and human activities [2]. Sb (V) and As (V), which can coexist in the environment [3] present a serious threat to human and animal health because of their ability to enter food chains in large amounts. Sb is considered to be toxic in the environment at low level, and the World Health Organization has set the limit for Sb concentration in drinking water as 5μg/L.

Sb contamination is of great environmental concern with increasing knowledge of environmental fates and toxicological characteristics. Compared with Sb (+III), Sb (+V), which is thermodynamically of stable form, is reported to be the predominant species detected in oxic media [2].

Toxicity of antimony is not well known, but Sb (III) species are usually more toxic than Sb (V) species and is comparable in its biochemical behavior with arsenic and bismuth. Some algae and plants have high ability to accumulate As and Bi are also able to accumulate antimony also. Some works are interested in the influence of antimony to microorganisms. Interesting species is *Chlorella vulgaris* that demonstrates better growth parameters in medium supplemented with potassium tartrate in comparison with antimony-poor medium. Its ability to bioaccumulate antimony is also interesting—12 mg Sb to 1 g dry matter [9]. In living cells of these organisms toxic antimony(III) is converted to much less toxic antimony(V), bounded to low molecular-mass proteins and probably stored in vacuoles [10]. Bioavailability of Sb is very low because of very limited

bioavailability of this element [11]. There are no detailed studies interested in uptake, transport and mechanisms of toxic effect of antimony. We can suppose that mechanisms of antimony metabolism is similar to other heavy metals; after uptake, toxic Sb(III) form is converted to less toxic Sb(V) form, and consequently complexed with proteins (phytochelatins?) or carbohydrates and stored in vacuoles of plant cells. In addition to inorganic forms, organic, methylantimony compounds were also determined, in plant extracts from areas polluted by antimony through mining activities [12]. *Dittrichia viscosa* [13] *Digitalis purpurea*, *Erica umbellata*, *Calluna vulgaris* and *Cistus ladanifer* [14] were determined as potent Sb bioaccumulators, which are cyanobacteria and plants that bioaccumulate Sb from contaminated waters due to their ability to grow partially submerged, such as *Ceratophyllum* sp. [15].

Sb has no known biological functions and is toxic even at low concentration. Antimony is toxic to humans at chronic uptake rates exceeding 100 mg/d [16]; rats are susceptible to an intake of 11–75 mg/d. Due to its toxicity and potential carcinogenic nature, there are regulations regarding human exposure to Sb in the workplace [1].

The biogeochemical behaviour of Sb is in similar to the other group VI elements arsenic (As) and phosphorous (P). It is a metalloid that exists in four oxidation states: -III, 0, +III, and +V.

The metalloid Sb is an element of growing environmental concern. Its rapid growth in industrial use (e.g. flame retardants, alloys, pigments, semiconductors) is associated with an increased and uncontrolled release of Sb compounds into the environment, e.g. mining and smelting activities, incineration of waste, combustion of fossil fuels and spent ammunition [17]. Worldwide, Sb is the ninth most mined metal and its compounds are listed as priority pollutant by the US Environment Protection Agency [18]. Sb also used in the treatment of several tropical protozoan diseases, such as leishmaniasis [19] and in treating HIV [20].

It is debatable whether or not As is essential to human health, but there is no known human requirement for Sb [21]. Both metalloids are clastogenic in the trivalent state, and have carcinogenic potential [22]. Both have a strong affinity for thiol groups and may substitute for phosphorus (P) in biological reactions, which explains their inhibitory role in DNA replication and metabolic processes. There is

evidences that As is detoxified via methylation in biological systems, but less evidence for the same process occurring for Sb [22].

Considering their adverse effects on the health of humans and other organisms, the appropriate treatment of these metals is of great environmental importance. A variety of treatment methods have been developed for the elimination of these metals from water, including coagulation [23, 24] adsorption [25] ion exchange [26] and biological processes [27] however, these methods are expensive and require major investments in equipment and facilities. In contrast, phytoremediation is considered a cost effective and environmentally friendly technology for the treatment of waters contaminated by heavy metals [28, 29]. Therefore, under present investigation the Phytoremediation of antimony by aquatic macrophytes *Trapa natans* and *Eichhornia crassipes* is tested. Among various plant groups used for phytoremediation, aquatic macrophytes attain the most important position. Antimony content in plant species is generally between 0.1 and 200 μgm kg^{-1} [30] but it is easily absorbed when present in the environment in water-soluble form [31]. Traffic emissions were found to increase Sb content in the lichen *Xanthoria parietina* [32] and it was suggested that the presence of Sb in the environment may negatively influence lichen diversity, including the distribution of *X. parietina* [33].

Several species of aquatic macrophytes such as water hyacinth (*Eichhornia* sp.), Duck-weeds (*Lemna* sp., *Spirodella* sp.), small water fern (*Azolla* sp.) and water lettuce (*Pistia* sp.) have been used for the removal of heavy metals from waste water [34, 35, 36, 37]. The aquatic macrophytes are free-floating aquatic plants; entire root system of these plants is submerged in water. All of the above species take up metals from water producing an internal concentration several folds greater than surroundings. Water hyacinth (*E. crassipes*), a rooted macrophyte, known to grow profusely in polluted water bodies, eutrophic lakes and has great potential for the heavy metal accumulation. In spite of being noxious weed; this species has been an important choice for phytoremediation of heavy metals from waste water due to its several advantages over other species [36]. *Trapa natans* is also recently found to have phytoremediative potential and research works are going in the respective field. *T. natans* are manganese tolerant and is also characterized by manganese hyper accumulation properties [38].

Phytoremediation methods using aquatic plants to absorb metals/metalloids from their surrounding waters are highly efficient [39]. The bio removal process using aquatic plants contains two uptake processes; (i) biosorption which is an initial fast, reversible, and metal-binding process and (ii) bioaccumulation which is a slow, irreversible, and ion sequestration step [40, 41, 42, 43].

The ideal plant for remediation of a metal contaminated site would be a rapidly growing, high biomass plant with an extensive root system that can both tolerate and accumulate the contaminants in the polluted environment [44, 45, 46, 47]. Therefore, a careful selection of plant species to be used in remediation of metal contaminated environments must be made so that the effectiveness of phytoremediation can be evaluated [48]. Root systems are especially susceptible to metal stress, so that the root length of a plant can be used as an important tolerance index [49, 50, 51].

In order to survive and grow in metal contaminated environments plants must store toxic metal complexes in the compartments where metal cannot readily dissociate and where metal will not interrupt normal metabolic activities of the cell [52]. Localization study is therefore an essential part of understanding the mechanisms involved in the metal tolerance and metal bioaccumulation in hyperaccumulators. Scanning Electron Microscopy (SEM) with Energy Dispersive X-ray Analysis (EDX) technology offers a new approach to instantaneous analysis of elemental composition of various plant tissues and organs. Sahi et al. [53] has done their work on accumulation, speciation and cellular localization of Cu in *Sesbania drummondi* by SEM-EDX. They found that the distribution of Cu within the seedlings tissues predominantly accumulated in the corticular and vascular (xylem) regions of the root tissues. In the stem, most of the Cu was found within the xylem tissue. However, the deposition of Cu within the leaf tissues was in the parenchyma.

In the present research, application of TEM in plant physiology, such as identification of heavy metals and ion transport, heavy metal storage detoxification and employment in plant physiological experiments are summarized. TEM offers new areas of imaging and element mapping at high spatial resolution and yield information on element bonding and assignment of element accumulations to ultrastructural details in biological samples.

The aim of the present study is to assess the ability of *T. natans* and *E. crassipes* to accumulate Sb from contaminated water under laboratory conditions. The ultimate goal is to assess the application of these plants in the phytoremediation of metal/metalloid contaminated water.

6.2 Objectives of the study

The present study has been undertaken to meet the following objectives

1. To study the morphological effects of antimony exposure on the above two plants.
2. To examine the effect of antimony on chlorophyll content of these plants.
3. To determine the removal efficiency of antimony and their accumulation by the plants *Trapa natans* and *Eichhornia crassipes* grown hydroponically.
4. To confirm the internalization of antimony within and around the roots and shoot of the plants by SEM, with an energy dispersive X-rays (EDX).
5. To investigate the effect of Sb on ultrastructural changes in *T. natans* and *E. crassipes* using TEM.

6.3 Materials and Methods

6.3.1 Experimental set-up and design

E. crassipes and *T. natans* with approximately the same size and weight, 7–8 weeks old were used for the removal of Sb. The plants were washed thoroughly with tap water followed by de-ionized water prior to the experimentation. The washed plants were placed in plastic tubs with tap water under natural sunlight for ten days to let them adapt to the new environment (acclimatization). After acclimatization, the plants were taken out and washed again properly with extreme care such that the delicate roots, root hair and other parts remain intact. All the experimental sets were maintained in triplicate. The concentrations of metal ions in solution were determined before the commencement of the experiment. Solution samples were collected periodically from day 2^{nd} to day 10^{th} from experimental tub for the determination of metal concentrations with time span elapsed. Loss of water due to evaporation was made up daily by adding tap water to the mark in the experimental tank. All the plants were grown hydroponically for 10 days in modified Hoagland's nutrient solution. The modified Hoagland's solution contains (in M): KNO_3 $5X10^{-3}$, Ca $(NO_3).4H_2O$ $5X\ 10^{-3}$, $MgSO_4.7H_2O$

$2X10^{-3}$, KH_2PO_4 $1x10^{-3}$, FeSO4.7H2O $0.02x10^{-3}$, H_3BO_3 $0.045x10^{-3}$, $MnCl_2.4H_2O$ $0.01x10^{-3,}$ CuSO4.5H2O $0.3x10^{-6}$, $Na_2MoO_4.2H_2O$ $0.1x10^{-6}$, and $ZnSO_4.7H_2O$ $0.8x10^{-6}$ (pH) [54, 55].

Eichhornia crassipes

Trapa natans

Figure 6.1 *E. crassipes* and *T. natans* plants in control condition

6.3.2 Sb solution preparation

A stock solution (1000mg/L) was prepared in distilled water with analytical grade ($SbCl_3$). The reagents were dissolved in distilled water to get the desired concentration level as 0.18, 5.17 and 7.47 mg/L respectively. These concentrations were added to all the experimental sets. Experimental plants were grown in triplicate in each of the Sb treated solution.

6.3.3 Sb analysis in plants

On the 10^{th} day of the treatment, the plants were harvested from each tub. They were washed properly with tap water and then demonized water. Plants were then separated into leaves, shoots and roots. All plant parts were then oven dried at 65°C to a constant weight. After measuring dry weight, the plants were ground. Approximately 0.5g of each plant sample was placed in a beaker with 15ml high purity HNO_3-$HCLO_4$ (3:1) acid, mixed and allowed to stand at room temperature overnight and then heated to140-180°C for complete digestion (Reagent blanks were processed to ensure that Sb was not added during sample preparation.). The digested solution was analyzed for Sb using ICP-OES [56]. ANOVA was performed for all the data to confirm their validity using SPSS 18.0. A probability of 0.05 or lower was considered as significant (Statistical data in Annexure-II).

6.3.4 Phytoextraction ability

The phytoextraction ability of *T. natans* plants was assessed using both the bioaccumulation factor (BCF) and the translocation factor (TF).

6.3.4.1 Bioconcentration Factor (BCF)

The Bioconcentration factor (BCF) of metals was used to determine the quantity of heavy metals that is absorbed by the plant from the soil/water. This is an index of the ability of the plant to accumulate a particular metal with respect to its concentration in the substrate [57] and is calculated using the formula:

BCF = Metal concentration in plant tissue/Initial concentration of metal in substrate.

The higher the BCF value, the more suitable is the plant for phytoextraction [58].

6.3.4.2 Translocation Factor (TF)

To evaluate the potential of plants for phytoextraction, the translocation factor (TF) was used. This ratio is an indication of the ability of the plant to translocate metal from the roots to the aerial parts of the plant [44]. It is represented by the ratio

TF = Metal concentration (shoot +leaves)/Metal concentration (roots)

Metals that are accumulated by plants and largely stored in the roots of plants are indicated by TF values<1, with values>1indicating that metals are stored in the stem and leaves.

6.3.5 Scanning electron microscopy (SEM) and energy dispersion (EDS) studies

The SEM technique has been used in several studies to investigate the internal distribution of metals in plant tissues [59]. Root, petiole and leaf samples were washed in running tap water and carefully rinsed with distilled water before microscopy observations. For scanning electron microscopy analysis (SEM) with conventional preparation, small pieces of roots, petioles and leaves (3-4 mm) were immediately fixed in 3% glutaraldehyde in 0.05M phosphate buffer for 90 min, which was followed by secondary fixation in 2% osmium tetroxide in 0.01M sodium cacodylate buffer for 30 min [60]. The samples were dehydrated in an acetone series. SEM photograph were carried for the samples, using SEM model JEOL-JSM-6390 LV attach with energy dispersive X-ray unit, with an accelerating voltage of 20 kV.

6.3.6 Transmission Electron Microscopy (TEM) studies

Fresh root (about 1–3mm in length) and leaf sections (~1-2 mm) section of from top of both the control and Sb treated with different Sb concentrations were selected for TEM studies. Root and leaf sections were fixed in 4% glutaraldehyde

(v/v) in 0.2M sodium phosphate buffer (pH 7.2) for 6–8 h, post-fixed in 1% osmium tetroxide (OsO_4) for 1 h and washed in 0.2M sodium phosphate buffer (pH 7.2) for 1–2 h. Dehydration was carried out in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100%) followed by acetone (100%), then samples were infiltrated and embedded in Spurr's resin. Ultra-thin sections (80 nm) were prepared and mounted on copper grids and viewed under transmission electron micro scope (JEOL JEM-2100, Japan) at an accelerating voltage of 60.0 kV [61].

6.4 Results

6.4.1 Sb Toxicity

Accumulation of heavy metals/metalloids by the plant, not only cause structural and ultrastructural changes, but also result in toxicity symptoms and affect the metabolic processes within the plant cells. The mean metal concentration in the plants exposed to Sb is reported in mg/kg based on dry weight. In addition, symptoms of toxicity, such as fragility, chlorosis in leaf and browning and twisting to roots occurred only in 7.47mg/L Sb concentration in plants**.** Relative toxicity of Sb on *T. natans* was found greater than that in *E. crassipes.*

Table 6.1 Some of the morphological changes on 10^{th} day are discussed below

Species	Concentration(mg/L)	Morphological Changes (10^{th} day)
T. natans	0.18	Slight yellowing of leaf on leaf margins
	5.17	Yellowing spread to the leaf
	7.47	High chlorosis
E. crassipes	0.18	Marginal chlorosis
	5.17	Leaf curling and chlorosis increased.
	7.47	Leaf expansion retarded, no emergence of new roots, high chlorosis.

6.4.2 Sb disturbance on chlorophylls and photosynthetic activities

Table 6.2 Total chlorophyll content in the two species

Species	Concentration (mg/L) of stock solution	Chlorophyll content in mg/g fresh wt on 3^{rd} day	Chlorophyll content in mg/g fresh wt on 5^{th} day	Chlorophyll content in mg/g fresh wt on 10^{th} day
T.natans	Control	0.6 ± 0.01	0.6 ± 0.01	0.6 ± 0.01
	0.18	0.5 ± 0.01	0.4 ± 0.005	0.2 ± 0.01
	5.17	0.3 ± 0.01	0.2 ± 0.01	0.1 ± 0.01
	7.47	0.2 ± 0.01	0.2 ± 0.01	0.1 ± 0.01
E.crassipes	Control	0.18 ± 0.02	0.18 ± 0.02	0.18 ± 0.005
	0.18	0.16 ± 0.02	0.15 ± 0.02	0.09 ± 0.005
	5.17	0.13 ± 0.01	0.12 ± 0.01	0.06 ± 0.005
	7.47	0.11 ± 0.02	0.06 ± 0.005	0.04 ± 0.005

Data are mean of three replicates; ± is the standard deviation.

Sb accumulation results in a decrease in chlorophyll content in both plants *T. natans* and *E. crassipes.* Total chlorophyll content was found to be reduced as the concentration of Sb increases as shown in **Table 6.2** and **Table 6.3**. The reduction of chlorophyll concentrations with increasing Sb concentrations were statistically significant ($p<0.05$) (Annexure II, **Table 6.4.2.1-6.4.2.4**). However, chlorophyll concentration was remained unchanged in both the control plants. Sb concentrations at 0.18 mg/L, 5.17 mg/L and 7.47 mg/L after 10 days caused a significant reduction of total chlorophyll content like 0.2 mg chlorophyll/g fresh leaf weight, 0.1 mg chlorophyll/g fresh leaf weight and 0.1 mg chlorophyll/g fresh leaf weight in case of *T. natans* (**Table. 6.2**), and 0.09 mg chlorophyll/g fresh leaf weight, 0.06 mg chlorophyll/g fresh leaf weight and 0.04 mg chlorophyll/g fresh leaf weight (Table 6.2) in case of *E. crassipes* respectively.

The total chlorophyll content was found to be reduced as the concentration of Sb increases in the solution in *T. natans* and *E. crassipes* as shown in **Figure 6.2** and in **Figure 6.3** respectively. So, comparing the results of total chlorophyll content, leaf chlorosis in plants grown on metal-polluted water can be due to a low chloroplast density caused by a reduction in the number of chloroplasts per cell and a change in cell size, suggesting that the excess of metal interferes with chloroplast replication and cell division [62].

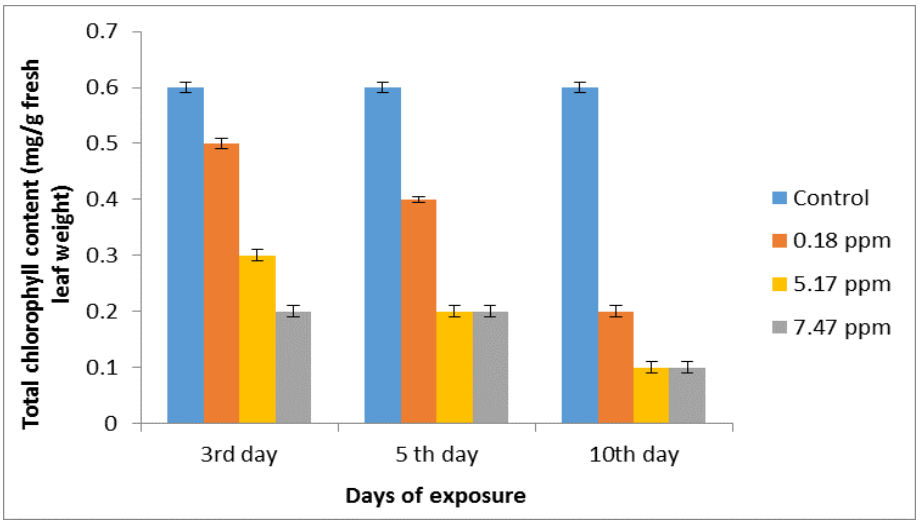


Figure 6.2 Graphical representation of total chlorophyll content in *T. natans* (Statistical data were given in Annexure II, Table 6.4.2.1-6.4.2.3)

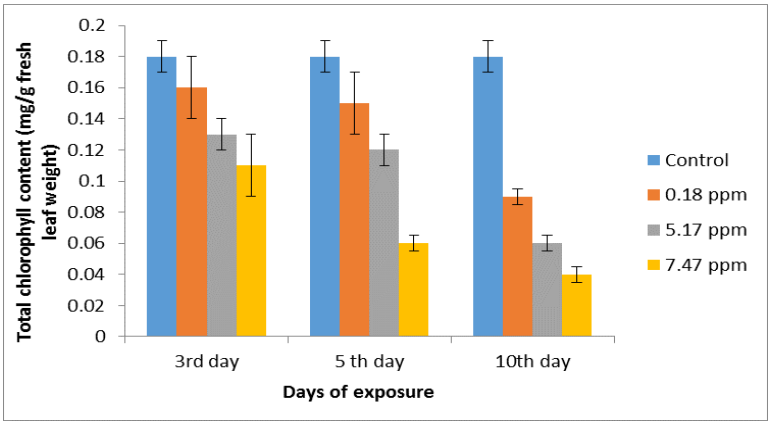


Figure 6.3 Graphical representation of total chlorophyll content in *E. crassipes* (Statistical data were given in Annexure II, Table 6.4.2.4)

This decline in chlorophyll content might be caused by an inhibition of an important enzyme of chlorophyll biosynthesis, δ-aminolevulinic acid dehydrogenase (δ-ALAD) and protochlorophyllide reductases [63]. Two main inhibitory mechanisms of metal action on enzymes can be distinguished-

(1) Binding of metals to functional groups such as SH-groups.

(2) Induction of metal deficiency in metalloenzymes and substitution of toxic for the essential metal in the enzyme complex [64].

δ –ALAD is a chloroplast enzyme. It catalyses the pathway of porphobilinogen (PGB) synthesis from δ- aminolevulinic acid (ALA) [65]. It is a metalloenzyme and its activity in plant is dependent on availability of Mg, as is an essential cofactor for δ-ALAD. Therefore Sb may also reduce δ-ALAD activity by exchanging the Mg at the active site [66]. Sb inhibited the activity of δ-ALAD activity by reducing tissue hydration, binding with –SH group of the enzyme [67]. Decreased chlorophyll content showed a positive correlation with reduced δ-ALAD activity as reported by Shibata and Ochiai [68]. The decrease in chlorophyll concentration may also be the result of an inhibited photosynthetic electron transport [69].

The potential metal sensitive sites which can be derived in the photosynthetic electron transport chain is the water splitting enzyme at the oxidizing site of PS I [70]. The primary site of Sb inhibition was on the oxidizing site of PS II, between the primary electron donor of PS II and the site of water oxidation [71]. Accordingly, the decreased chlorophyll content may also be attributed to the decomposition of the chloroplast membrane with excess Sb [72]

and through the induced Fe deficiency and nutrient imbalances, which in turn lead to interruption of chlorophyll biosynthesis [73].

6.4.3 Uptake of Sb from the solutions

Table 6.3 Phytoremediation potential of *T.natans* in removal of Sb

Initial conc. mg /L	Amount removed (%) 2^{nd} days	Amount removed (%) 4^{th} days	Amount removed (%) 6^{th} days	Amount removed (%) 8^{th} days	Amount removed (%) 10^{th} days
0.18	0.06±0.005(33.3)	0.07±0.005(38.8)	0.08±0.005(44.4)	0.08±0.005(44.4)	0.09±0.01(50)
5.17	2.07 ± 0.07(40.0)	2.18±0.01(42.16)	2.50±0.05(48.35)	3.21±0.11 (62)	4.08±0.005(78.91)
7.47	1.15±0.01(15.39)	1.84±0.05(24.63)	3.11±0.03(41.63)	3.42±0.24(45.7)	3.97±0.01(53.14)

Data are mean of three replicates; ± is the standard deviation.

The Phytoremediation potential of *T.natans* in terms of % removal of Sb was measured by determining concentration of Sb in solution with different concentration exposed to the plant as a function of contact time at 2^{nd} day's interval of time. The % removal was measured for 2^{nd}, 4^{th}, 6^{th}, 8^{th}, and 10^{th} days. It is evident from the table that % removal increases with the increases in exposure and found the maximum absorption on 10^{th} day. For 0.18 mg/L of initial concentration % removal was calculated as 33.3%, 38.8%, 44.4%, 44.4% and 50% at 2^{nd}, 4^{th}, 6^{th}, 8^{th} and 10^{th} days respectively. This removal % however increases with the increase in initial concentration upto 40.0%, 42.16%, 48.35%, 62%, and 78.91% at 5.17mg/L in 2^{nd}, 4^{th}, 6^{th}, 8^{th} and 10^{th} days respectively. However futher increase in initial concentration % removal somewhat decreases and found to be 15.39%, 24.63%, 41.63%, 45.7% and 53.14% in 7.47mg/L at 2^{nd}, 4^{th}, 6^{th}, 8^{th}, and 10^{th} days respectively. This decrease in % removal is not very clear and one explanation may be the concentration at 7.47mg/L could be toxic to plant and may cause some internal damage to the plant. However, it revealed from the data that biosorption of Sb by *T. natans* was very rapid on 2^{nd} day in comparison to other equal duration of time. The highest % of removal (78.91%) was observed at 5.17mg/L of initial concentration at 10^{th} day and it seems *T. natans* has the ability to remove very high concentration of Sb from solution and the plant may be potential for removal of Sb from aqueous solution and waste water (**Table 6.3**).

Table 6.4 Phytoremediation potential of *E. crassipes* in removal of Sb

Initial conc. mg /L	Amount removed (%) 2^{nd} days	Amount removed (%) 4^{th} days	Amount removed (%) 6^{th} days	Amount removed (%) 8^{th} days	Amount removed (%) 10^{th} days
0.18	0.05±0.005(27.7)	0.06±0.005(33.3)	0.07±0.005(38.8)	0.07±0.005(38.8)	0.09±0.005(50.0)
5.17	1.03±0.01(19.9)	1.24±0.01(23.98)	1.28 ± 0.03(24.7)	1.60±0.01(30.94)	2.03 ± 0.05(39.2)
7.47	0.92±0.03(12.31)	1.56±0.01(20.88)	1.60±0.03(21.41)	1.65±0.04(22.08)	1.99±0.005(26.6)

Data are mean of three replicates; ± is the standard deviation.

Similarly the Phytoremediation potential of *E. crassipes* interms of % removal of Sb was also measured by determining concentration of Sb in solution with different concentration exposed to the plant as a function of contact time at 48 hrs interval of time. The % removal was measured for 2^{nd}, 4^{th}, 6^{th}, 8^{th}, and 10^{th} days. It is evident from the table that % removal increases with the increase in exposure time and found that maximum absorption take place on 10^{th} day. For 0.18 mg/L of initial concentration % removal was calculated as 27.7%, 33.3%, 38.8%, 38.8%, and 50% at 2^{nd}, 4^{th}, 6^{th}, 8^{th}, and 10^{th} days respectively. This removal % however decreases as the with initial concentration increases from 5.15 mg/L to 7.47 mg/L as 19.9%, 23.98%, 24.7%, 30.94%, and 39.2% in 5.17 mg/L and 12.31%, 20.88%, 21.8%, 22.08% and 26.6 mg/L at 2^{nd} 4^{th}, 6^{th}, 8^{th}, and 10^{th} days respectively. It revealed from the data that biosorption of Sb by *E. crassipes* was very rapid on 2^{nd} day in comparison to other equal duration of time. The highest % of removal (50%) was observed at 0.18 mg/L of initial concentration and efficiency of removal of decreased thereafter with increase in metal concentration. However this plant could be used for removal of Sb at a lower initial concentration of Sb from aqueous solution and waste water. From this study it is evident that the amount Sb removal by *T. natans* and *E. crassipes* has been found to be influenced by initial concentration of the metal in the test solution. Moreover, *E. crassipies* is more effective in removing Sb at low initial concentration but *T. natans* can is more effective upto concentration of 5.17mg/L for removing Sb from solutions. However, there is a statistically significant ($p<0.05$) difference among the Sb concentrations in both plants with increase the number of days of exposure.

6.4.4 Removal efficiency of Sb from the solution

Table 6.5 Removal efficiency of Sb from the solution by *T. natans*

Parameters	Sb	Sb	Sb
Metal concentrations added to water (mg/L)	0.18	5.17	7.47
Remained metals in water after 10^{th} days experiment	0.09±0.01	4.08±0.005	3.97±0.01
Removal efficiency (%)	50	78.91	53.14

Data are mean of three replicates; ± is the standard deviation.

Table 6.6 Removal efficiency of Sb from hydroponic solution by *E. crassipes*

Metal concentrations added to water (mg/L)	0.18	5.17	7.47
Remained metals in water after 10^{th} days experiment	0.09±0.005	2.03 ± 0.05	1.99±0.005
Removal efficiency (%)	50	39.2	26.6

Data are mean of three replicates; ± is the standard deviation.

Sb removal efficiency of *T. natans* was highest (78.91%) at 5.17mg/L Sb, whereas for *E. crassipes* it was highest at 0.18 mg/L (50%) respectively.

6.4.5 Accumulation of Sb by the plants

A comparison of Sb concentrations in different morphological tissues of *T. natans* and *E. crassipes* suggested that the roots have significantly ($p<0.05$) higher concentrations of Sb in comparison to stem and leaves as shown in **Table 6.7** and **Table 6.8** respectively.

Table 6.7 Calculation of Translocation factor (TF) and Bioaccumulation factor (BCF) in *T.natans* after 10 days of harvesting

Initial conc. (mg/L)	Conc. in roots (mg/kg)	Conc. in shoot (mg/kg)	Conc. in leaf (mg/kg)	TF	BCF
0.18	45.94 ± 0.05	47.97 ± 0.1	39.78 ± 0.31	1.91± 0.01	742.69± 0.49
5.17	73.66 ±0.02	50.24 ± 0.16	58.60 ± 0.08	1.47 ±0.09	35.29±0.07
7.47	59.49 ±0.41	16.68 ± 0.01	43.36 ± 0.08	1 ± 0.1	16.01± 0.02

Data are mean of three replicates; ± is the standard deviation.

Table 6.8 Calculation of Translocation factor (TF) and Bioaccumulation factor (BCF) in *E. crassipes* after 10 days of harvesting

Initial conc. (mg/L)	Conc. in roots(mg/kg)	Conc. in shoot(mg/kg)	Conc. in leaf (mg/kg)	TF	BCF
0.18	12.64 ± 0.01	1.43 ± 0.1	1.60 ± 0.02	0.23 ± 0.005	87.05 ± 0.05
5.17	9.29 ± 0.34	0.90 ± 0.005	0.97 ± 0.005	0.20 ± 0.005	2.15 ± 0.03
7.47	3.52 ± 0.07	0.24 ± 0.03	0.25 ± 0.01	0.13 ± 0.01	0.53 ± 0.02

Data are mean of three replicates; ± is the standard deviation.

A comparison of Sb concentrations in different morphological tissues of *T. natans* after 10 days of harvesting suggested that the roots have significantly higher concentrations of Sb. In *T. natans*, Sb concentration in roots, shoots and leaves were 45.94 mg/kg, 47.97mg/kg and 39.78 mg/kg in 0.18 mg/L Sb concentrations, 73.66 mg/kg, 50.24mg/kg and 58.60 mg/kg in 5.17 mg/L Sb concentration and 59.49 mg/kg, 16.68 mg/kg and 43.36 mg/kg in 7.47 mg/kg Sb concentration respectively (**Table 6.7**) (Data in **Annexure-II**, **Table 6.4.5.1-6.4.5.3**).

In *E. crassipes*, Sb concentration in roots, shoots, leaf were 12.64 mg/kg, 1.43 mg/kg and 1.60 mg/kg dry wt. in 0.18 mg/L concentration; 9.29 mg/kg, 0.90 mg/kg and 0.97 mg/kg dry wt. in 5.17 mg/L concentration and 3.52 mg/kg, 0.24 mg/kg and 0.25 mg/kg dry wt. in 7.47 mg/L concentrations, respectively (**Table 6.8**) (Data in **Annexure-II**, **Table 6.4.5.4-6.4.5.6**).

There was significant difference in Sb uptake with increasing exposure time by the two species of plants ($p<0.05$) (**Table 6.3** and **Table 6.4**). The maximum Sb concentrations in roots, shoots and leaves of *T. natans* were 73.66 mg/kg, 50.24 mg/kg and 58.60 mg/kg and dry weight, respectively. And similarly the maximum Sb concentrations in roots, shoots and leaves of *E. crassipes* were 12.64 mg/kg, 1.43 mg/kg and 1.60 mg/kg dry weight, respectively. There was significant ($p<0.05$) difference of accumulation of Sb in different plant parts as seen from **Table 6.7** and **Table 6.8** between the two species. The highest bioaccumulation coefficients (the plant/solution concentration quotients) were 742.69 and 87.05 for *T. natans* and *E. crassipes*, respectively.

Hydroponic culture is an efficient method for screening of Sb tolerant for free floating plants of *T. natans*. The root sorption in *T. natans* is more because of

sorption of Sb ions by root surface area and also might be the direct contact of roots with the Sb solution.

The Sb levels of 5–10 mg/kg in plant tissue are reported as phytotoxic [74]. This indicate that the both plants possess a tolerance to Sb. Baroni et al. [75] found that the highest Sb concentrations in the roots of *Achillea ageratum*, *Plantago lanceolata* and *Silene vulgaris* were 338, 1150, and 249 mg/kg dry weight, respectively, while those in the leaves were 1367, 569, and 1163 mg/kg dry weight. Murciego et al. [76] also found high concentrations of Sb in *Dittrichia viscosa* (1136 mg/kg dry weight). These plant species appear to be good candidates for the phytoremediation of Sb contaminated soil; however, they are unsuitable for the cleanup of Sb contaminated water.

6.4.5.1 Bioconcentration Factor (BCF)

The BCF is a useful parameter for evaluating the ability of plants in accumulating heavy metals with respect to the initial concentration of heavy metals in the substrates [77]. According to Zayed et al. [77] BCF less than 300 represent a poor accumulator of heavy metals, whereas BCF values between 300 and 800 indicate moderate accumulator and BCFs greater than 800 represent a good accumulator.

The more the BCF, the more the plant is suitable for phytoextraction. The highest BCF for *T.natans* is 742.69 at 0.18 mg/L of Sb concentration, i.e. *T.natans* is a good bioaccumulator at the low concentration (**Table 6.7**). This results suggest that *T. natans* has the ability to remove Sb effectively from solution and plant may potentially be useful for removal of Sb from Sb containing wastewater. In the present study the calculated BCF values of the *T. natans* was more than 300 but below 800 and is considred as moderate accumulator of Sb according to classification made by Zayed et al. [77]. The highest BCF for *E. crassipes* is 87.05 at 0.18 mg/L Sb concentration, i.e. *E. crassipes* is a good bioaccumulator at the lowest concentration (**Table 6.8**). Therefore we can say that *E. crassipes* can be used Sb removal, though it is a poor accumulator of Sb in higher concentration.

Chandra et al. [78] suggested that the low concentration of metals in the shoot of aquatic macrophytes may be due to the slow mobility of metal transport from root to shoot and also the formation of complex compounds with COOH groups that may inhibit the translocation of metal to shoot.

6.4.5.2 Translocation Factor (TF)

Metals that are accumulated by plants and largely stored in the roots of plants are indicated by TF values <1. Values >1 indicate translocation to the aerial parts of the plant. In *T. natans*, TF values for all 3 concentrations of Sb was > 1, so we can say that Sb is stored in aerial parts. These values are comparable to those previously reported by others [79, 80].

Plant species were categorized according to their TF values into four groups. First group contain hyperaccumulator plants with TF values above ten. Second group contains hypertolerant plants with TF values above one but below ten. Third group contains tolerant plants with TF values less than one. This groups of plants have adopted an exclusion strategy. This strategy allows them to form metal stable complexes in their root cells, which results in a limited metal translocation to above-ground parts. The fourth group, categorized as excluders, as these plants can grow in heavy-metal polluted soils without accumulating significant quantities [81]. These plant species have TF values < 0.1. Possibly these plants use mechanism that avoids excessive uptake of metals and metal is absorbed and translocated only in nontoxic quantities. Since plants employed in phytoextraction treatments must accumulate more than 1000 mg/kg DW, should exhibit values of TF and BCF larger than unity, and should produce high quantities of biomass [82, 83, 84].

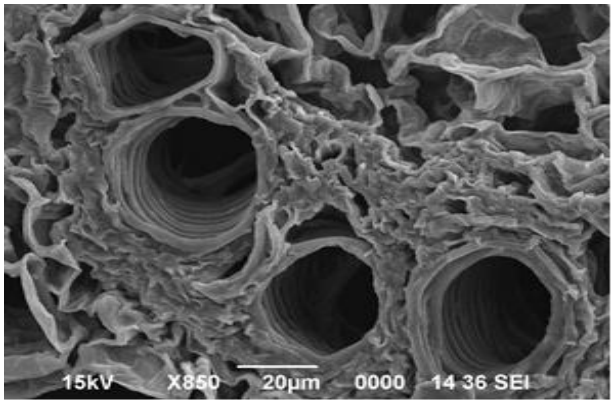
In *E.crassipes*, TF value for 0.18 mg/L concentration of Sb was 0.23, for 5.17mg/L it was 0.20 and for 7.47mg/L was 0.13 respectively. All the values are <1, so we can say that Sb is stored in root. In case of *E. crassipes*, the TF ranged from 0.13 to 0.23 in different concentration of Sb demonstrated their poor translocation of the metalloid from root to shoot.

Our data have shown that, after 10 days of treatment, the TF was >1 in *T.natans* plants exposed Sb solutions and the BCF was also >1 in all the treatments. These results suggest that *T. natans* can transport Sb from roots to shoot and that Sb could be accumulated by this plant, in accordance with the observations of Gupta and Devi [85] and Xiao et al. [86]. However, the recorded TF and BCF values are not high enough to define *T. natans* as a good accumulator of Sb from the solution. Same results for *E. crassipes* also. Comparing the above results we can say that among *T. natans* and *E. crassipes*, *T. natans* is more potential plant for Sb removal study.

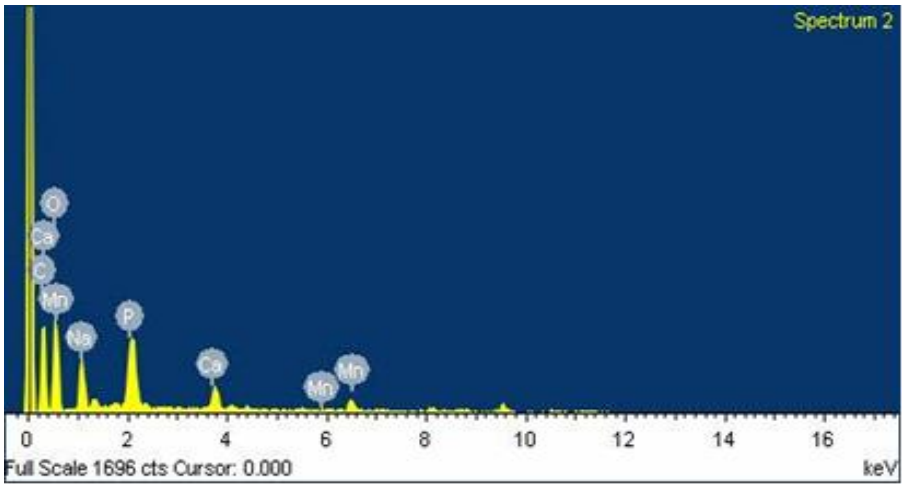
6.4.6 SEM X-ray microanalysis of root and shoot tissue for Sb distribution

To understand the Sb detoxification mechanism in *T. natans* and in *E. crassipes*, it is necessary to map the Sb transport pathway from roots to shoot as well as sites where Sb particles and high Sb percentages are localized. In order to investigate in more detail the internal distribution of metals within tissues, it is necessary to take the help of electron microscopes. The SEM technique has been used in several studies to investigate the internal distribution of metals in plant tissues [59].

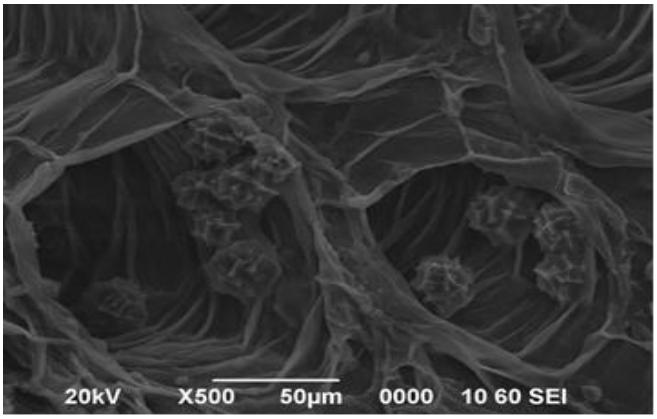
Thus, shoots and roots of Sb treated plant were examined by SEM equipped with energy dispersive X-ray spectrometer (EDX). The electron microscopy study also indicated different toxic effects of Sb on the cellular structure in shoot and root of *T. natans* and *E. crassipes.*



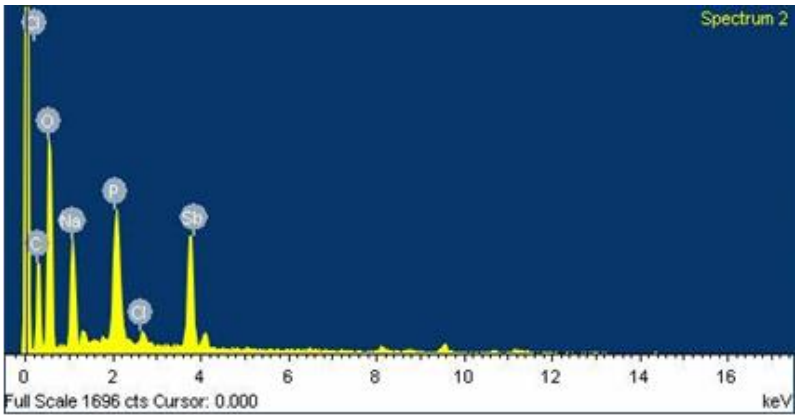
(**i**) Control shoots of *T. natans*



(**a**) EDX of Control shoot of *T. natans*



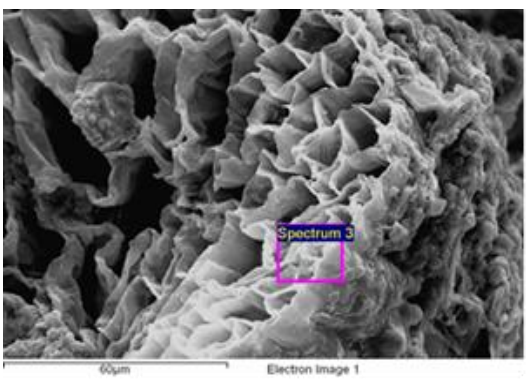
(**ii**) Sb treated shoot of *T. natans*



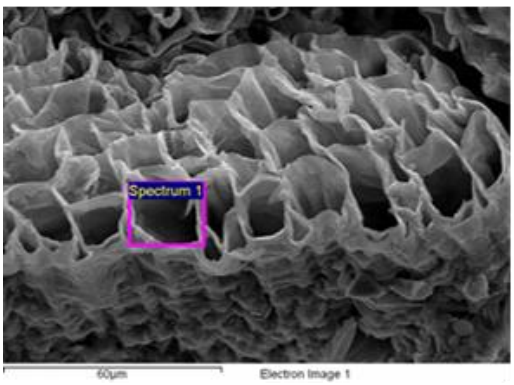
(**b**) EDX of Sb treated shoot of *T. natans*

Figure 6.4 SEM-EDX of fresh shoot of *T. natans* control (i, a); and exposed to Sb (ii, b) for 10th days

SEM micrographs of shoot of the control plant did not showed any abnormalities of the vascular bundles cells (**Figure 6.4i**). Sb treated shoot showed some anomalous growth on the xylem tissues (**Figure 6.4ii**). EDX of control shoot of *T.natans* showed the absence of Sb but the presence of other elements like O, Ca, C, Mn, Na, and P etc. (**Figure 6.4a**). EDX of Sb treated shoot of *T.natans* shows the Sb curve along with other elements such as C, Na, P, Cl etc. (**Figure 6.4b**).

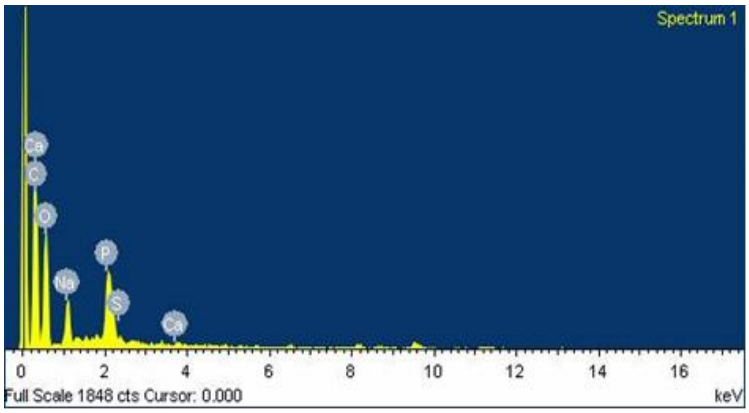


(**c**) Sb treated root of *T. natans*

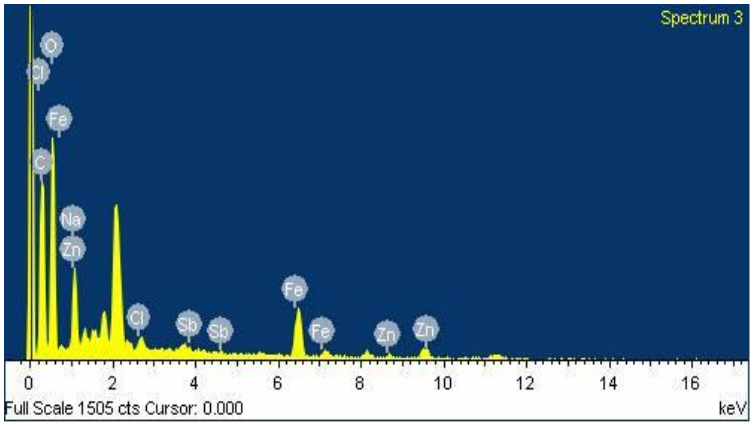


(**d**) Control root of *T. natans*

Plant exposed to 7.47 mg/L Sb solution showed some anomalous growth in thickness, narrow cortical area with large apertures in the central stele region. The roots of Sb treated plants also show a breakdown of spongy and palisade parenchyma cells (**Figure 6.4c**) followed by further loss of cell shape and decrease in intercellular spaces compared to the control group (**Figure 6.4d**). EDX spectra of the root of the control plant (**Figure 6.4iii**) showed presence of no Sb whereas Sb was confirmed in the treated plant besides other elements (**Figure 6. 4iv**).

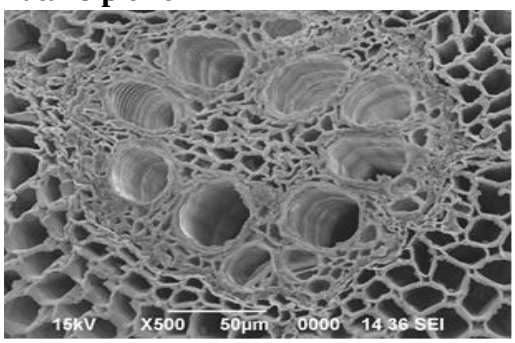


(**iii**) EDX of control plant of *T. natans*

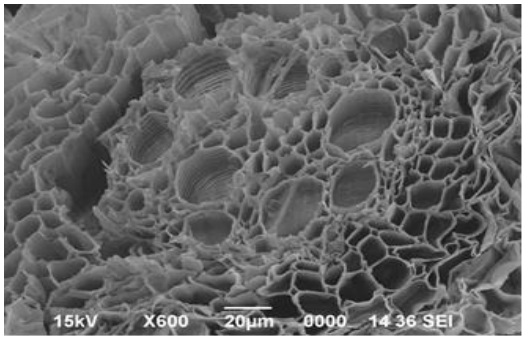


(**iv**) EDX of Sb treated *T. natans* plant

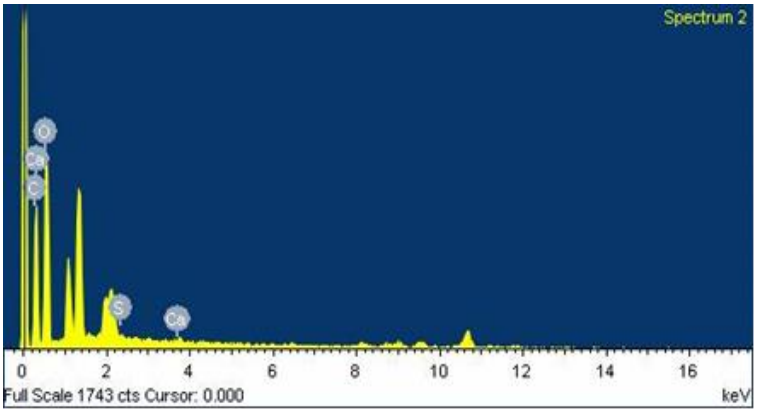
Figure 6.4 SEM- EDX of control (d, iii); and treated (Sb) (c, iv) root of *T. natans* plant



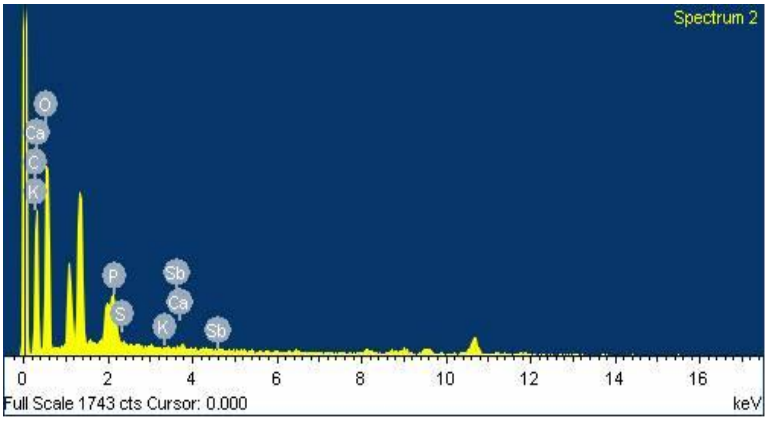
(**e**) Control shoots of *E. crassipes*



(**f**) Sb treated shoot of *E. crassipes*



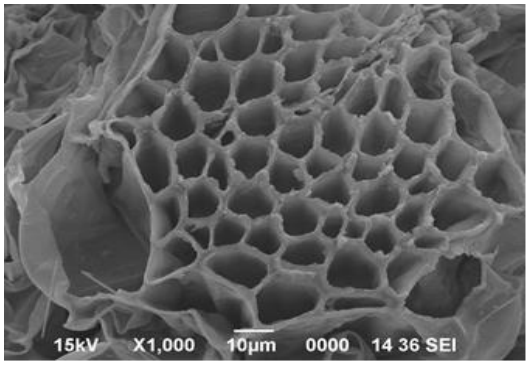
(**v**) EDX of control shoot of *E. crassipes*



(**vi**) EDX of the Sb treated shoot of *E. crassipes*

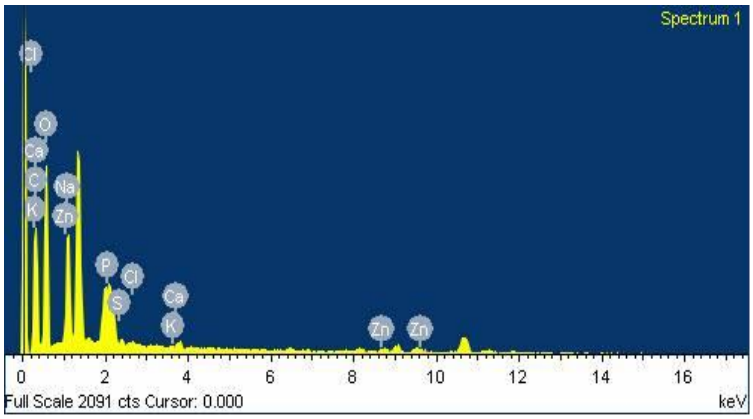
Figure 6.4 SEM- EDX of control (e, v); and treated (Sb) (f, vi) shoot of *E. crassipes* plant

From the SEM-EDX micrograph it has been seen that there was no abnormalities and no Sb curve was seen in the control plant of *E. crassipes* (**Figure 6.4e, v**). In the Sb treated shoot, the cortical region of the shoot is more or less intact, parenchymatous cells around the central stele and inner bundles shows damage and breakage also seen. Some Sb was also present in xylem tissue. Sb curve seen in treated shoot (**Figure 6.4f, vi**). The SEM micrographs of plant treated with Sb showed changes of the vascular cells of the shoot samples (**Figure 6.4f**). Exposure to 7.47mg/L Sb for 10 days resulted in a loss of cell shape, decrease in the intercellular spaces, and shrinkage of vascular bundle in *E. crassipes* (**Figure 6.4f**) as compared to the control (**Figure 6.4e**).

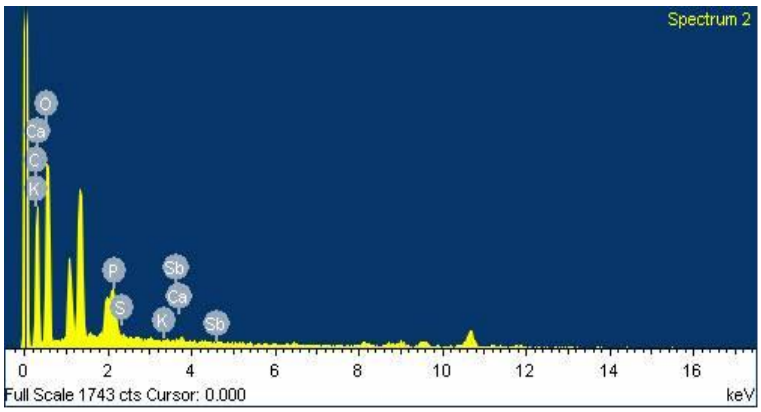


(**g**) Control root of *E. crassipes*

(**h**) Sb treated root of *E. crassipes*



(**vii**) EDX of control root of *E. crassipes*

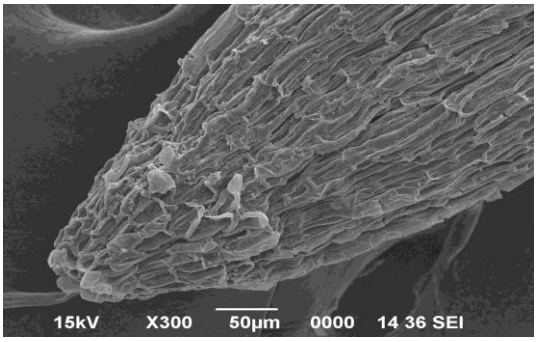


(**viii**) EDX of Sb treated root of *E. crassipes*

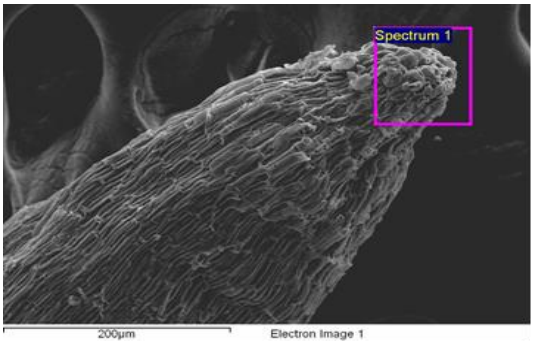
Figure 6.4 SEM- EDX of control (g, vi) and Sb treated root (h, viii) of *E. crassipes*

Sb localization within and around roots of *E. crassipes* growing in a hydroponic Sb solution was studied by energy dispersive X-ray microanalysis of cross section of roots. Root surface appears to be smoother in the control plant root than that the metal-loaded samples. No Sb curve seen in control root of *E. crassipes* but the presence of other elements seen such as Cl, O, Ca, C, K, Na and

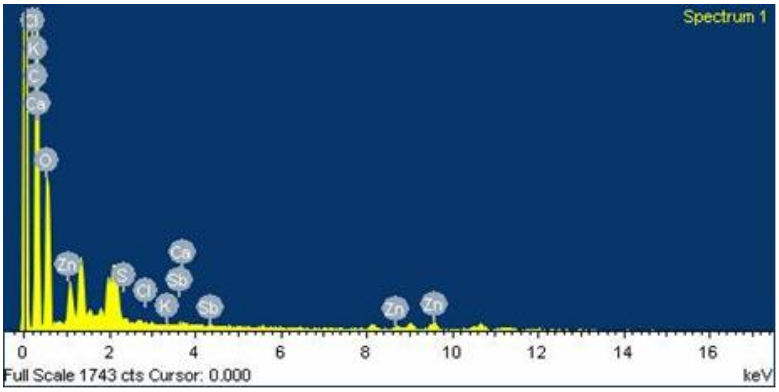
Zn etc.(**Figure 6.4g vii**). Cortical region of the treated root shows some anomalous growth and some cellular damage (**Figure 6.4h viii**). The examination of root cross-sections revealed that heavy deposition of Sb was in cortical region and electron-dense granules appeared bright when observed (**Figure 6.4g**).



(**h**) Root hair of control plant



(**i**) Root hair of Sb treated plant



(**ix**) EDX of Sb treated plant

Figure 6.4 Sb accumulation by the root hair of *E. crassipes*; (h) Root hair of control plant; (i) Root hair of Sb treated plant

In samples of *E. crassipes* treated with 7.47 mg/L Sb, the closer of root apex was seen, indicating an imbalance between the cell division and the start of cell elongation and differentiation (**Figure 6.4i**). This result is in accordance with the early development of vascular bundles observed in roots of the same plants and confirms the data of Dˇ urcˇekova et al. [87] who reported premature xylogenesis in barley roots exposed to Cd. In the tip of the root hair, showed a high atomic percentage of Sb accumulation (0.02) (**Figure 6.4ix**). It is assumed that the metal was more absorbed by the root hair cell wall. SEM combined with EDX analysis was used to identify the effects of metal sorption on surface morphology of the sorbent cells (*T. natans* and *E. crassipes* biomass). Scanning-electron microscopy analysis showed that Sb deposition was in vascular tissue in root as well in shoot.

In this study, examination of plant biomass before and after metal ion sorption by electron microscopy was carried out to locate the active sorptive areas

of these sorbents. EDX analysis was also used to confirm the identity of the metal ions on the plant roots, shoots and leaves cell surface. Prakasham et al. [88] found that the electron micrograph of *Chlorella* cells after Cu sorption was denser, and the surface had started to fracture in comparison with the smooth surface of cells (control) that were not in contact with metal. The change in structure of the cell material was probably influenced by the metal ions. Thus, it can be assumed that heavy metals attached to the functional groups such as carboxyl, amino, amide and hydroxyl that are found on algal cell surfaces lead to all these changes [88, 89]. When *Chlorella* biomass was introduced to higher Cu concentrations (20 mg/L), the shape of the cells became irregular and more folded, and they started to rupture and shrink. They also became attached to each other.

With the aim of obtaining Sb distribution patterns in *T. natans* and *E. crassipes* a study based on nuclear analytical techniques (scanning electron microscope (SEM) X-ray microanalysis) was carried out. X-ray microanalysis in leaves, shoots and roots of control plants did not show the studied metals. In Sb-treated plants, longitudinal analysis in roots demonstrated that Sb accumulated in both species, mainly in the root cap.

Transversal analysis of *T. natans* and *E. crassipes* demonstrated that Sb accumulated in a higher proportion on the root surface, decreasing in concentration towards the centre.

6.4.7 TEM Analysis

The ultrastructural investigation of the root and leaves cells of *T. natans* and *E. crassipes* exposed to high concentration of Sb (7.47 mg/L) for 10 days was carried out.

6.4.7.1 Chloroplasts

TEM investigations of the *T. natans* in a section of the leaf control, multiple chloroplasts were observed within the leaf cells (**Figure 6.5A**), however, they did not exhibit any abnormalities on the ultrastructural level of their organization (**Figure 6.5B**). Moreover, chloroplasts showed a disorganization of the inner membrane and relocation (dislodgment) of the stroma and grana when exposed to Sb solution (**Figure 6.5B**). Starch accumulation was evident within the chloroplast after application of both the plants into Sb solution which is found in the TEM observation (**Figure 6.5C**). In some cells, chloroplasts were totally disorganized

with a disruption of their envelope and a high swelling of thylakoids (**Figure 6.5D**).

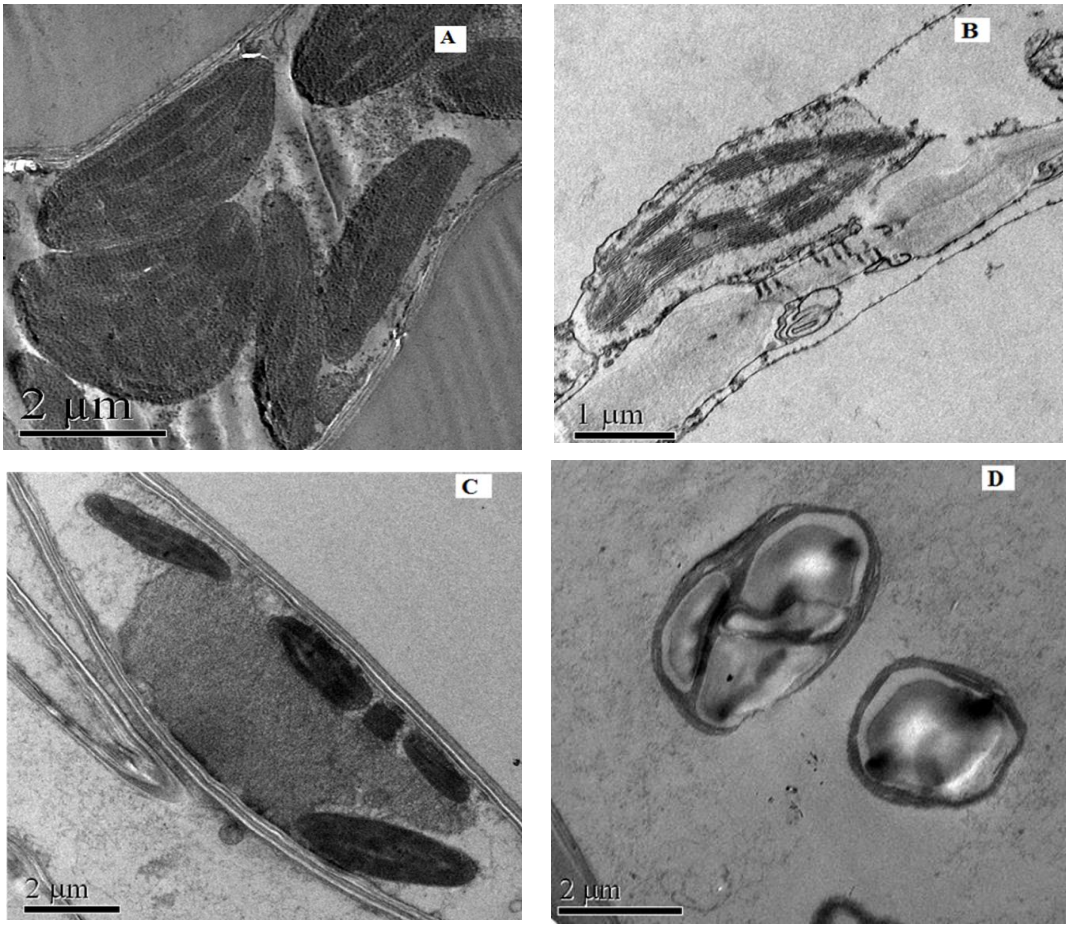


Figure 6.5 TEM micrograph shows a young mesophyll cell of control plant of *T. natans* showing numerous chloroplasts (A); A chloroplast with well-organized inner membranes with both grana and stroma in a cell of a young control leaf of *T. natans* (B); Chloroplast with a well-developed and organized thylakoid system in a cell of a mature control leaf (B); Damaged disintegrating chloroplasts showing the presence of irregular, rounded shape, numerous starch grains (C); disturbance of the orientation of the grana (D)

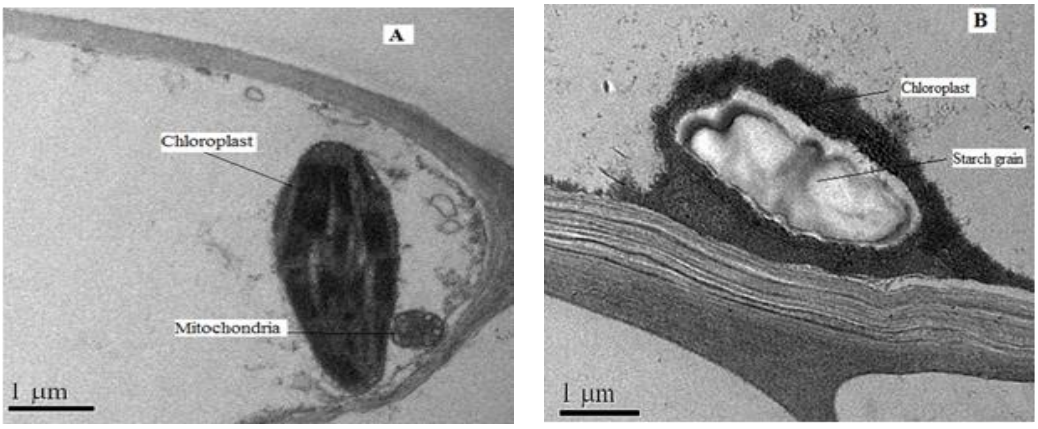


Figure 6.6 TEM micrograph shows a bean - shaped mesophyll chloroplast of control plant *E. crassipes*. The grana are orderly arranged and interconnected with stroma thylakoids (A); A mesophyll chloroplast of *E. crassipes* plant

treated with 7.47 mg/L Sb solution showing a large starch grain and disturbed grana structure (B)

In control leaf samples of *E. crassipes* chloroplasts with a well-developed lamellar system, some plastoglobuli were also seen (**Figure 6.6A**). In Sb treated samples the chloroplasts were generally swollen or irregularly bulged. The thylakoid system was lacking the typical arrangement (grana and intergrana thylakoids) (**Figure.6.6B**). Ultrastructural chloroplast alterations were related to impairing of photosynthetic activity shown in plants after heavy metal treatments [90, 91].

Ferro et al. [92] identified more than 100 proteins in the chloroplast envelope membranes. Among them, there is a metal transporter, a potential Cd/Zn transporting ATPase (AHM1), indicating the possibility for these metals to enter and accumulate with in the cell. Despite of severe destructions that toxic metals provoke in chloroplasts, they do not accumulate in these organelles [93, 94, 95, 96] they are rather found in abundance in the apoplast and the vacuole. This conclusion, valuable at least for higher plants, contrasts with the results of Nagel et al. [97] and Mendoza-Cózalt and Moreno-Sánchez [98] who found 50–60 % Cd in the chloroplast of a cell-wall deficient *Chlamydomonas reinhardtii* and the plant-like vacuole deficient *Euglena gracilis*, respectively. Both results indicate that even if chloroplasts have the capability to accumulate heavy metals, they do not represent the target organelle for these when the plant cell has a cell-wall and a vacuole. It also means that toxic metals are actively directed to the vacuole [94, 99].

6.4.7.2 Mitochondria

The mitochondria are important targets for toxic effects of heavy metals. In the control *E. crassipes*, multiple mitochondrion within the root cells were found (**Figure 6.7A**). At 7.47 mg/L Sb, some damages and ruptures were noted in the mitochondrion membranes (**Figure 6.7B**).

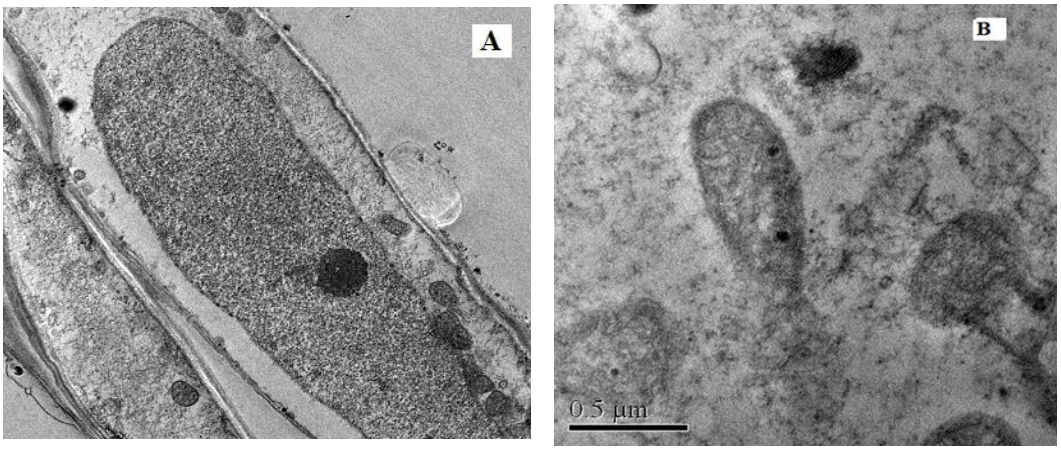


Figure 6.7 Transmission electron micrographs of root cells of *E. crassipes* showing numerous mitochondrion in the cortical cells of control plant (A); Ruptured mitochondria and reduced number of cristae of mitochondria in the cortical cells exposed at 7.47 mg/L $SbCl_3$ solution (B)

6.7.4.3 Root Vacuoles

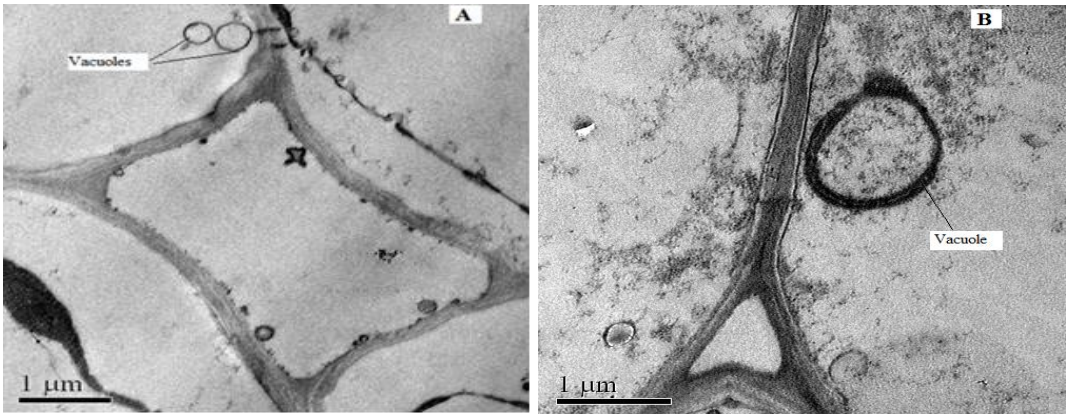


Figure 6.8 TEM micrographs of transverse section of roots of *T. natans*; (A) Control plant showing the vacuoles; (B) $SbCl_3$ treated plant root showing the deposition within the vacuole

Metal/metalloid deposition in vacuoles is a mechanism that may contribute to heavy metal/metalloid tolerance indeed, the accumulation of Sb in the vacuoles seems to be a most effective system for maintaining a very low cytoplasmic Sb concentration.

Several small vacuoles were also found in mature root cells of *T. natans* control plant (**Figure 6.8A**). Elemental distribution maps showed that Sb was predominantly localized in the vacuoles of root cells. However, due to significant metal accumulation in the Sb-treated plants, the cross sections of roots showed precipitation and an increase in the number of vacuoles in TEM micrographs of roots of *T. natans* (**Figure 6.8B**).

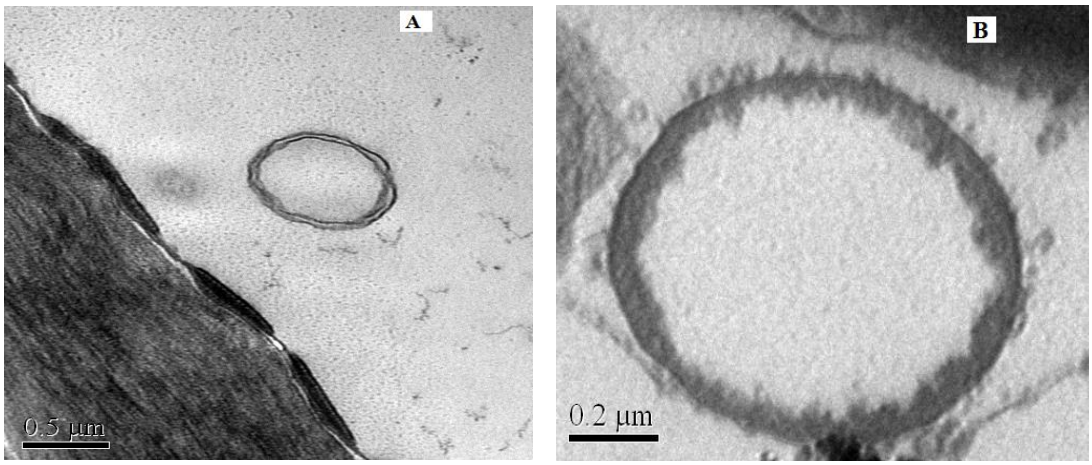


Figure 6.9 TEM micrographs of root cells of *E. crassipes*; in the control plant root cell no deposition within the vacuole (A); black deposits were found in the vacuole of $SbCl_3$ treated plant root cells (B)

No metal deposition was found in control root vacuole of *E. crassipes* (**Figure 6. 9A**). In the vacuoles of root cells of *E. crassipes*, electron dense granules were aggregated and formed into larger precipitates with circular or amorphic shape, which were encircled by the membrane (**Figure 6. 9B**). Root cells exposed to concentrations of 7.47 mg/L of Sb exhibited pronounced changes. In contrast to the control, the increased vacuolation was the first visible effect of metal toxicity on the meristematic cells treated with 7.47 mg/L Sb.

In the cross sections of roots showed precipitation and an increase in size of the vacuole in TEM micrographs of roots (**Figure 6.9B**).

6.4.7.4 Golgi body

The occurrence of cell plasmolysis and swelling organelles (like Golgi body) after heavy metal treatments could be explained by the loss of membrane selective permeability.

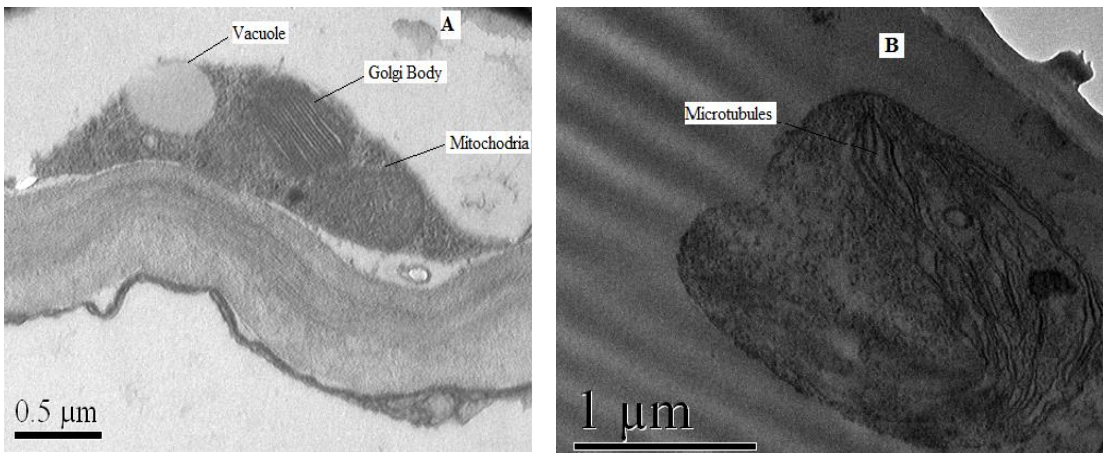


Figure 6.10 TEM micrograph of root cells of *T. natans* showing mitochondria, vacuole and Golgi appear well preserved (A); after application of Sb, the disorganization of microtubules in Golgi body appeared (B)

Cell and organelles develop swelling or shrinking as a consequence of ions moving across the membrane and shifting water [100]. The same authors referred the loss of membrane selective permeability to cellular energy depletion due to heavy metal toxic effects or a primary damage to membrane. Other studies confirmed our finding of cytoplasmic vesicles in heavy metal-treated plants [101, 102, 103].

6.4.7.5 Cell Wall

In our experiments the cell wall also played an important role in the storage of Sb. Cell walls accumulated more Sb than organelles and cytosol, which were considered as the first barrier against Sb entering into cells.

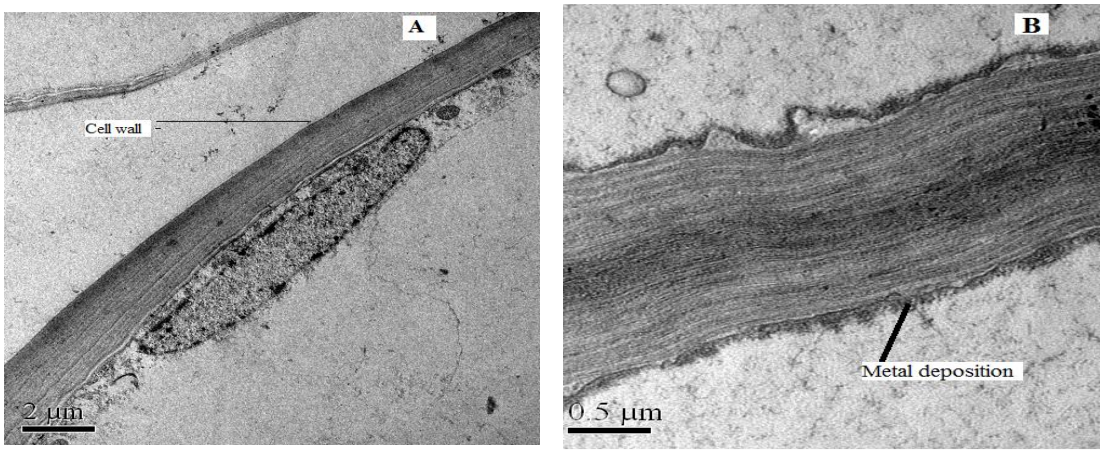


Figure 6.11 TEM micrographs of root cells of *T. natans* of control plant (A); The cell wall treated with 7.47mg L^{-1} Sb, showing numerous black deposits in the wall of the cell (B)

The cell wall of the root of control plant of *T. natans* did not exhibit any ultrastructural alterations (**Figure 6.11A**). When exposed to 7.47 mg/L of Sb solution, TEM examination showed ultrastructural changes in root tissues of *T. natans*. Numerous electron dense particles were observed in intercellular spaces, where they were often adsorbed on the surface of cell walls (**Figure 6.11B**).

Cell walls are another large buffer which can accumulate heavy metals and therefore prevent contact with the sensitive plasmalemma and cytoplasmic components [104, 105]. According to several localization studies, indicated that Pb, Al, Zn and Cd were accumulated in the cell walls of plants [106, 107, 108].

Sb concentrations analyzed in roots may reflect some proportion of metals that are merely adsorbed onto the root surface rather than within the root tissue. However, Vesk et al. [109] analyzed the localization of metals within roots of *E. crassipes* using energy dispersive X-ray microanalysis and found that Cu, Pb and Zn were not localized at the root surface, but were more highly concentrated in the inner root tissues. Levels were highest within the cells of the stele (the vascular bundles), in electron dense granules. Significant amounts were also seen in the cell walls.

6.5 Discussion

Evidence of chlorosis, leaf roll, leaf yellowing and failing of leaves were the main easily visible symptoms of Sb toxicity in *T. natans* and *E. crassipes* after 10 days of Sb application, especially at 7.47mg/L Sb level**.** Root et al. [110] and Chaffei et al. [111] recommended that chlorosis induced by Cd may be due to alterations in the Fe/Zn ratio, rather than to Fe deficiency, since plants treated with Cd showed a greater concentration of this micronutrient.

Decrease in chlorophyll content is one of most common responses to metal/metalloid stress in plants. Sb accumulation clearly showed the detrimental effects on chlorophyll content on both the aquatic plant, *T. natans* and *E. crassipes*. Consistently with our results, Pan et al. [112] investigated the chlorophyll content and the photosynthetic efficiency of plants of maize treated for 2 weeks with 10, 50, 100, 500 and 1000 mg kg^{-1} Sb in the soil and found that both parameters were negatively influenced only at the highest concentrations.

Analysis of Sb concentrations in plant organs showed that Sb concentrations in root were significantly higher than those in shoot in both the plants. Accumulation of Sb in the plant organs was highest in roots in all the sets of experiments. In *T. natans* Sb accumulation was more in leaves than shoots. It is thought that the direct sorption of Sb happens through the leaves; according to the morphology of this plant, leaves are in contact with the solution having large surface area. This result is comparable with Sune et al. [113]. They found that the Cr concentration found in the leaves of *S. herzogii*, which was higher in the abaxial than in the adaxial surface, detected by SEM X-ray microanalysis may suggest that direct sorption of the Cr in the solution happens through leaves, since due to the morphology of this plant, leaves are in contact with the solution. This does not

happen in *P. stratiotes*. Maine et al. [114] proposed that Cr uptake through direct contact between the leaves and the solution is the main cause of the increase of Cr in the aerial parts, being Cr poorly translocated from the roots to the aerial parts.

The concentration of Sb in the roots of *E. crassipes* being higher than in the aerial part but some amount of Sb was also found in leaves and petiole (shoot), demonstrating that this metalloid element was not totally immobilized in the plant. Some plants are potential to absorb Sb from environment but the amount absorb depends on both the activity of the root and its interaction with the surrounding solution.

With the aim of obtaining Sb distribution patterns in *T. natans* and *E. crassipes*, a study based on nuclear analytical techniques (scanning electron microscope (SEM) X-ray microanalysis) was carried out. X-ray microanalysis in leaves, shoots and roots of the both plants. Both the control plants did not show the studied metalloid. In Sb-treated plants, longitudinal analysis in roots demonstrated that Sb accumulated in both species, mainly in *E. crassipes* was in the root cap (**Figure 6.4**). This results was familiar with MacFarlane and Burchett [59] who reported by SEM X-ray microanalysis and revealed that accumulation of Cu predominantly in the cell walls. The SEM finding about Sb accumulation in roots is equally supported by the ICP-OES study for metal uptake is that roots have higher uptake of metal as compared to stem and leaves.

The chloroplasts from untreated plants were typical mesophyll chloroplasts, they show a well-organized internal membrane structure with normally developed grana and stroma thylakoids in *T. natans* (**Figure 6.5A**) and in *E. crassipes* (**Figure 6.6A**). Sb treated plastids show a disturbed shape (**Figure 6.5C**) in *T. natans*, with a wavy appearance of the grana and stroma thylakoids and the intrathylakoidal space swollen but the envelope intact (**Figure 6.6B**) in *E. crassipes*. Dilation of the thylakoid membranes was apparent in all chloroplasts.

TEM results for metal location revealed that most of the metal absorbed by plants was accumulated in roots and negligible amount of metals was found in leaves of these aquatic plants. Starch accumulation was evident within the chloroplast after application of the plant. *T. natans* into the Sb^{2+} solution which is found in the TEM observation (**Figure 6.5D**).

The changes in the shape of chloroplasts due to abiotic and biotic stresses are the results of an increase in the volume of the stroma and a disorganization of

the thylakoid membranes and have been previously reported in the literature [115]. Moreover, our results of injury to chloroplast associated with impairment of the photosynthetic function agree with similar results reported for Pb-treated lichens [116] and for Cd-treated *Elodea canadensis* and *Triticum aestivum* [117, 118].

In the plant cell, the whole process of photosynthesis is completed in the chloroplasts [119]. Therefore, the normal performing of plant photosynthesis depends on the integrity of chloroplast ultrastructure. Our experimental results demonstrated that after *T. natans* and *E. crassipes* treated with Sb^{2+} chloroplast was swollen, the membrane of chloroplast was damaged (**Figure 6.5D**). Lamellae structure of thylakoid was loose and disorderly, and the orderly arrangement of grana was also destroyed (**Figure 6.6B**) respectively.

The disorder and looseness of the lamellar structure could cause the decrease in the activity and function of PSII, and then the decrease in the photosynthetic function [120]. The destruction of the orderly arrangement of grana could cause the inhibition of the transfer and conversion of light energy [120].

Heavy metal-induced alterations of chloroplast arrangement were shown in different taxonomic groups of plants [118, 121, 122, 123]. Thus, the chloroplast seems to be a common target site of trace elements. The chloroplast alterations in leaves of plants exposed to heavy metals were related to an increase in the production of reactive oxygen species (ROS). These chemicals at high concentrations inside cells are able to cause oxidative damage to cellular structure and function [124]. Ultrastructural chloroplast alterations were related to impairing of photosynthetic activity shown in plants after heavy metal treatments [118, 125]. Our finding of photosynthetic impairment and the Sb inducted damage of enzymes involved in starch synthesis could be related to pyrenoid alteration, as already suggested by Demirevska-Kepova et al. [126].

Storage and localization of Sb as electron-dense precipitates in the vacuoles of root cells of *T. natans* and *E. crassipes* treated with Sb may play an important role for Sb detoxification by maintaining low levels of free Sb in cytosol [127, 128]. In fact, plants have a range of intracellular ligands potentially involved in metal accumulation and detoxification. Phytochelatins (PCs) and metallothioneins are two major sulfur-containing classes of metal chelating ligands that have been identified in plants and these may play a significant role in metal tolerance [128, 130, 131].

Our study shows that electron dense depositions were also observed all along the cell walls in TEM micrographs of their roots of *T. natans* (**Figure 6.11B**).

A number of studies indicate the importance of the cell wall in binding heavy metals in bryophytes as by many researchers [132, 133, 134, 135, 136].

Our study documented here some of the physiological implications of structural alterations caused by Sb due to metal uptake, translocation and accumulation. We believe that our results on the localization patterns and their effect on plant structural, morphological and physiological characteristics will contribute to understand and optimize the processes of phytoremediation of Sb by *T.natans* and *E. crassipes* plants.

6.6 Conclusions

Phytoremediation is emerging as a cost effective and environment friendly technology for the treatment of water and soil contaminated by metals and metalloids. In the present study, the aquatic macrophytes *E.crassipes* and *T.natans* which are easily cultivated and controlled, and well adapted to contaminated environments was tested for its ability to accumulate Sb from contaminated water in the laboratory experiments. In this study, the characteristics of accumulation and transportation of Sb in wetland plants are summarized as follows -

The results demonstrate that the plants, *T. natans* and *E. crassipes* stand out as good candidates for phytoremediation of Sb grown hydroponically. Sb concentration showed a constant decrease in all the concentrations with increasing days of exposure. Morphological symptoms like unhealthy flowering, chlorosis and necrosis indicates Sb toxicity. Chlorophyll content is greatly affected by Sb toxicity in both the plants.

The distribution of Sb in the root is greater than in shoot; after uptake of Sb by roots, most of the Sb will be stored in root tissues, and less transported in to the shoots, 2) Plaque can act as a buffer area for the uptake of toxic metals (Sb) into root tissues, but it does not block the Sb transport into root tissues.

Removal efficiency of *T.natans* was found to be highest in 5.17mg/L concentration (78.91%). In *E. crassipes*, it was highest at 0.18mg/L concentration (50%) respectively. *E. crassipes* was found to store the Sb in roots, *T. natans* was found to store Sb in aerial parts as BCF for *T.natans* is positively correlated with

concentration, thus it increase with increase in solution. BCF for *E. crassipes* is negatively correlated with concentration, thus it decreases with increase in solution.

Comparing both BCF and TF was shown for both the plants, which indicated that these species are good candidate for the remediation of Sb pollution. For BCF, harvesting of above ground part of plants with high BCF and TF is very important in the view of toxicology. Harvesting will prevent the accumulated heavy metals/metalloid get into food chain and thus avoid negative potential risk to environment.

The Sb treated roots and shoots showed internal tissue damage in the SEM-EDX studies. Appearance of new leaves and roots, though unhealthy indicates that the plant has the ability to survive under stress to a great extent. TEM microanalysis showed that Cd is accumulated in the vacuoles and cell wall and thereby prevent it entering plant cells. Metal deposition in vacuoles is a mechanism that may contribute to heavy metal tolerance: indeed, the accumulation of Sb in the vacuoles seems to be a most effective system for maintaining a very low cytoplasmic Sb concentration. In our experiments the cell wall also played an important role in the storage of Sb. X-ray TEM microanalysis highlights the accumulation of Sb in cell walls and vacuoles. It is necessary to elucidate what internal/external factors play important roles in metal and metalloid (e.g. Sb) uptake and tolerance in order to select suitable wetland plants with high levels of tolerance.

However, *T. natans* and *E. crassipes* seem to be unable to survive for a longer period at a very high concentration of Sb (e.g.7.47 mg/L) because, in such conditions, we observed extreme morpho-anatomical, cytological and ultrastructural changes at the root and leaf level unavoidably limit and alter the root function. Although the results of the present study suggest that *T. natans*, a herbaceous free floating plant growing in Sb contaminated solution have greater phytoaccumulation potential than *E. crassipes*.

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