



BABEŞ-BOLYAI UNIVERSITY  
FACULTY OF CHEMISTRY AND CHEMICAL  
ENGINEERING



# Phytoextraction studies using aquatic plants

**PhD Thesis**

**Abstract**

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Cluj-Napoca

2015

## **Acknowledgment**

*This work was possible due to the financial support of the Sectorial Operational Program for Human Resources Development 2007-2013, co-financed by the European Social Fund, under the project number POSDRU/159/1.5/S/132400 with the title „Young successful researchers – professional development in an international and interdisciplinary environment”.*

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## 1. General introduction

### 2. Introduction

One of the most considerable global problems is the removal of the organic and inorganic pollutants from urban, industrial and household activities. From the beginning of the 20<sup>th</sup> century, paralelly with the increase of the population and the anthropogenic activities, the discharge of the solid and liquid waste pollutants into the environment has also notably increased [1]. Most of the contaminants entering the ecosystems are chemicals. In everyday life 60,000 chemicals are used by humans in form of fuels, consumer products, industrial solvents, drugs, pesticides, fertilizers or as food additives [2].

Nowadays, the development of new, suitable and highly efficient remediation technologies for removing the different types of pollutants has gained the attention of the researchers. Bioremediation is an alternative method designed for the removal, uptake and annihilation of a wide variety of pollutants using different kinds of biomaterials and live cells in controlled conditions. Compared to other removal techniques, bioremediation has evolved into a promising, economical and environmentally friendly strategy [3].

Bioremediation techniques can be utilized in-situ or ex-situ in the polluted environments. The process contains several sub-processes like biosorption, bioaccumulation, bioprecipitation, bioreduction, biodegradation, phytoextraction, phytostabiliation, phytofiltration, phytovolatilization, phytodegradation or rhyzodegradation [4].

**Phytoremediation** is part of bioremediation, which is an alternative method based on the use of green plant systems to remove hazardous pollutants from the environment [5, 6]. The main advantage of the phytoremediation method is that this eco-friendly technology can be carried out *in-situ* to accumulate, degrade, remove or minimize the impact of the different kinds of pollutants from the environment.

**Phytoextraction** is the uptake of contaminants from soils or aqueous solutions by plants root, accumulated and translocated them to their above tissues. Two kind of phytoextraction are known; continuous (natural) and chelat-assiseted (induced) phytoextraction. The continuous phytoextarction describes the use of “natural” plants, while the induced phytoextarction is based on the use of the plants in cooperation with an chelating agent to help the mobility for the less available metals [7]. This technique generally is applied to remove heavy metals, however it can be also use to annihilate organic compounds [8].

The **major limiting factors** of this promising remediation technique are the nature of **plant species** that is how they tolerate toxic effects, and their ability to remove the pollutants. The ideal species for the phytoremediation methods have to perform some principal criteria such as having high biomass, deep root system, faster growth rate, being easy to harvest, having the ability to adapt and tolerate a wide range of environmental conditions with a potential to tolerate different kinds of pollutants [9, 10].

### 3. General objectives and goals

Based on the literature overview, it can be observed that there is an increased interest in promoting new, efficient, ecofriendly methods for the removal of different contaminants from wastewaters and in determining the accumulation and transport processes.

The **aim** of this thesis is to develop an alternative biological method for the removal of inorganic and organic pollutants using various aquatic plants and to determine the biochemical responses to the induced stress.

In order to achieve these goals, the following objectives are to be taken into consideration:

- The selection of the aquatic plants as well as the organic and inorganic pollutants for the phytoextraction studies.
- The optimization of the effect of operational parameters such as aquatic plant quantity, initial concentration of the pollutants, initial pH and temperature of the medium in order to achieve the highest phytoextraction efficiency and capacity.
- The study of the phytoextraction mechanisms using Fourier transformation infrared spectroscopy (FTIR), ultraviolet-visible spectroscopy (UV-Vis), thin layer chromatography (TLC), scanning electron microscopy (SEM) and energy dispersive spectrometer (EDS).
- The characterization of the responses of the plants to the induced abiotic stress through the qualitative and quantitative analysis of various biochemical markers (photosynthetic pigments: chlorophyll *a*, *b* and carotenoids *x+c*, total proteins, antioxidants: ascorbic acid and glutathione, phytochelatin precursors, as well as phytochelatin synthesis and levels).
- The analyses of the experimental data using equilibrium isotherms and kinetic models for the design and for a better understanding of the complex phytoextraction processes.
- The development of an artificial neural network (ANN) model to predict and to offer quantitative estimations about the phytoextraction capacity and removal efficiency.

## **4. Experimental part**

### **4.1. Selection of the inorganic pollutants and aquatic plants for the phytoextraction studies**

#### **4.1.1. Heavy metals**

In this thesis, three heavy metals were chosen as inorganic pollutants, due to their hazardous characteristics and their negative effect on the live-organisms. The selected heavy metals for the phytoextraction studies are the  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$ .

#### **4.1.2. Aquatic plants**

Five hydrophytes were selected for the phytoextraction studies due to their physiological properties. The chosen aquatic plants are floating and submerged species: *Elodea canadensis* Mich. (*Elodea canadensis*), *Eichhornia crassipes* Mart (*Eichhornia crassipes*), *Salvinia natans* Kunth (*Salvinia natans*), *Lemna minor* L. (*Lemna minor*), and *Pistia stratiotes* L. (*Pistia stratiotes*).

#### **4.2.1. Experimental conditions, materials and methods**

The phytoextraction experiments were performed in hydroponic systems to eliminate all mass transfer limitations, to produce more surfaces for the preferential uptake of pollutants, and for a better screening of the potential crops in controlled conditions.

Samples were withdrawn from the aqueous solutions every day to monitor the decrease of heavy metals concentration in time. The hydrophytes after the experiments were used further to determinate the accumulated heavy metals quantities and to analyse the plant biochemical responses to the induced stress.

##### ***Plant material and growing conditions***

*Elodea canadensis* Mich. (*Elodea canadensis*), *Eichhornia crassipes* Mart (*Eichhornia crassipes*), *Salvinia natans* Kunth (*Salvinia natans*), *Lemna minor* L. (*Lemna minor*), *Pistia stratiotes* L. (*Pistia stratiotes*) submerged and floating aquatic species were grown in a greenhouse (University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca, Romania) with addition of fertilizer (Complex 3, 0.5 %). Plant in the age of 40-60 day was selected for the phytoremediation experiments. The plants were kept under



controlled conditions for an acclimatization period of 3 days in modified Hoagland nutrient solution at room temperature with a 14/10 h (light/dark) photoperiod.

The modified Hoagland nutrient solution contains as follows: 1.25 mM KNO<sub>3</sub>, 1.25 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.5 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.25 mM KH<sub>2</sub>PO<sub>4</sub>, 11.6 μM H<sub>3</sub>BO<sub>3</sub>, 4.5 μM MnCl<sub>2</sub>·4H<sub>2</sub>O, 10 μM Fe(III)EDTA, 0.19 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.12 μM Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, and 0.08 μM CuSO<sub>4</sub>·5H<sub>2</sub>O (the chemicals were purchased from Merck Germany) [11]. All chemicals used were of analytical grade.

### ***Phytoremediation of heavy metals***

After the 3 day acclimatization period, the plants were exposed to Cu<sup>2+</sup>, Zn<sup>2+</sup>, and Cd<sup>2+</sup> alone (monometallic system) or in a combination (multimetallic system – a model system which was more similar to the natural conditions), in concentrations of 5 mg/L - 10 mg/L. The control plants were left without heavy metal treatment.

The working/ stock solutions were prepared from the salts such as Cd(NO<sub>3</sub>)<sub>2</sub>, ZnSO<sub>4</sub> ×7H<sub>2</sub>O and CuSO<sub>4</sub> ×5H<sub>2</sub>O, the initial concentrations were checked by atomic absorbance spectrophotometer (GBC SensAA Dual Australia) with flame atomization systems using Photron P410 HCL-D2 lamp at the following wavelengths: 228.8 nm (Cu), 213.9 nm (Zn) and 324.7nm (Cd).The calibration solutions were made in the range of 1 - 5 μg/ml for Cu<sup>2+</sup>, 0.2 – 2 μg/ml for Zn<sup>2+</sup>, and 0.2 – 1.8 μg/ml for Cd<sup>2+</sup>.

### ***Sampling and sample analysis***

In order to determine the Cu<sup>2+</sup>, Zn<sup>2+</sup> and Cd<sup>2+</sup> contents in the plants, the samples were oven-dried at 70 °C for 24 h. Thereafter, the tissues were ground and digested with nitric acid and hydrogen peroxide (8:3, v: v). The resulted samples were filtered and stored at 4 °C, until further analysis was made. The samples heavy metals concentration was determined by atomic absorption spectrophotometer (GBC SensAA Dual, Australia).

### ***Statistical analysis***

Statistical analysis including calculation of average values, standard errors was performed by Microsoft Package. The significant differences was observed between the exposed plants and their control at P<0.05 values.

#### 4.2.2. Results of monometallic systems

The phytoextraction (phytoaccumulation) abilities of five different aquatic plants were studied separately exposed to monometallic solutions containing  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  or  $\text{Cd}^{2+}$  in a concentration of 10 mg/L. Taking into consideration the physical characteristics of the plants, the obtained results were divided into two groups:

1.) Morphologically big plants such as *Eichhornia crassipes* and *Pistia stratiotes*; in this case the accumulation and distribution of heavy metals were analysed in the root and the leaves of the plants;

2.) Morphologically small hydrophytes such as *Elodea canadensis*, *Salvinia natans* and *Lemna minor*; in this case the accumulation of heavy metals was determined by analysing the whole plant.

The obtained results in the case of morphologically big plants after the phytoextraction experiments of heavy metals using monometallic systems are shown in the Table 1 and in the case of morphological small plants are presented in Table 2.

The obtained results in Table 1 presented that the distribution of the three heavy metal ions was unequal in the different parts of the plants; the heavy metals were accumulated in higher concentration in the roots of the plants. In opposition with the result obtained in the case of  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  metals, the *Pistia stratiotes* species was able to accumulate with 1.87-fold more Cu ion in the leaves than in the roots. These results can be explained by the nature of the plant, which has different metal accumulation and defence mechanism Literature reports showed that various species have unique ecophysiological behaviour and capacity to accumulate heavy metals which are compartmentalize efficiently in the cell wall, vacuoles or in other specific subcompartments of the cytosol in order to keep them away from active metabolic sites in plant cells [12].

The heavy metal accumulation in case of the morphologically small plants was different in the three species (Table 2). It can be noticed that the *Salvinia natans* showed a greater ability to accumulate  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$ ; by 3.5– fold 3.8-fold as well as 4.2- fold greater concentrations than in the case of *Elodea canadensis*, and by 3-fold, 5-fold as well as 3-fold higher concentration than in the case of *Lemna minor*. These results are in good agreement with the findings of Dhir. et al., who reported that *Salvinia natans* is a metal hyperaccumulator plant, which was able to tolerate and bioconcentrate high quantities high quantities of  $\text{Fe}^{2+}$ ,  $\text{Cr}^{6+}$  and  $\text{Ni}^{2+}$  metals [13].

**Table 1.** Accumulation and distribution of heavy metals (mg/g DW) in the *Eichhornia crassipes*. and *Pistia stratiotes* hydrophytes exposed to monometallic system ( $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$  initial concentration 10 mg/L,  $t = 24 - 28$  °C, pH = 5.6, photoperiod 14/10 h artificial light). Values represent  $\pm$  SD (n=3), small letters represent the statistical significant difference at  $P < 0.05$

Species	Heavy metals	Concentration	Concentration	Accumulated	Accumulated
		in control sample leaves (mg/g)	in control sample roots (mg/g)	concentration in root (mg/g)	concentration in leaves (mg/g)
Eichhornia crassipes	$\text{Cu}^{2+}$	0.005	0.003	$1.468 \pm 0.3^b$	$0.365 \pm 0.02^d$
	$\text{Zn}^{2+}$	0.075	0.041	$0.817 \pm 0.07^c$	$0.322 \pm 0.03^d$
	$\text{Cd}^{2+}$	0.0	0.0	$1.728 \pm 0.2^a$	$0.139 \pm 0.01^d$
Pistia. stratiotes	$\text{Cu}^{2+}$	0.009	0.004	$1.334 \pm 0.2^b$	$2.496 \pm 0.6^a$
	$\text{Zn}^{2+}$	0.033	0.013	$1.118 \pm 0.4^b$	$0.951 \pm 0.01^b$
	$\text{Cd}^{2+}$	0.0	0.0	$1.428 \pm 0.3^b$	$0.276 \pm 0.04^c$

**Table 2.** Accumulation of the heavy metals in *Elodea canadensis*, *Lemna minor* and *Salvinia natans* after the monometallic solutions exposure ( $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$  initial concentration 10 mg/L,  $t = 24 - 28$  °C, pH = 5.6, photoperiod 14/10 h artificial light). Values represent  $\pm$  SD (n=3), small letters represent the statistical significant difference at  $P < 0.05$

Species	Heavy metals	Concentration	Accumulated
		in control sample leaves (mg/g)	concentration in leaves (mg/g)
Elodea canadensis	$\text{Cu}^{2+}$	0.044	$1.340 \pm 0.1^a$
	$\text{Zn}^{2+}$	0.086	$0.583 \pm 0.02^b$
	$\text{Cd}^{2+}$	0	$0.444 \pm 0.01^b$
Lemna minor	$\text{Cu}^{2+}$	0.0034	$1.558 \pm 0.6^c$
	$\text{Zn}^{2+}$	0.044	$0.447 \pm 0.01^c$
	$\text{Cd}^{2+}$	0	$0.62 \pm 0.06^{\text{NS}}$
Salvinia natans	$\text{Cu}^{2+}$	0.0074	$4.719 \pm 0.5^a$
	$\text{Zn}^{2+}$	0.003	$2.236 \pm 0.6^c$
	$\text{Cd}^{2+}$	0	$1.901 \pm 0.2^c$

### 4.2.3. Result of multimetallic systems

The phytoextraction (phytoaccumulation) ability of the aquatic plants was chosen to be studied exposed to multimetallic systems, in order to simulate wastewater conditions containing multiple inorganic contaminants. Similarly as in the case of monometallic systems, the multimetallic system experiments were studied and presented, divided into two groups by taking into consideration the morphological characteristics of the plants. The obtained results are presented in the Table 3 as well in Table 4.

The presence of three heavy metal ions in the solutions has a major influence on the phytoextraction of the plants, and it was noticed that the phytoaccumulation ability of the hydrophytes increased after exposure of the multimetallic solution (Table 3). The *Eichhornia crassipes* was able to accumulate 4.1-fold higher concentration of  $\text{Cu}^{2+}$  as well as  $\text{Zn}^{2+}$  than in the case of monometallic system. In the case of the *Pistia stratiotes*, a better performance was observed for the phytoaccumulation of the  $\text{Cd}^{2+}$ , which was three, time higher than in case of the phytoextraction of monometallic solution (Table 3).

In the case of morphologically small plants, the hydrophytes had diverse facilities to accumulate heavy metals from the multimetallic solutions (Table 4). The phytoextraction (phytoaccumulation) ability of *Lemna minor* increased vigorously. The presence of the three metal ions had a positive effect on the uptake and accumulation of  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  metals, which was 6.9-fold, 10.9-fold and 4.4-fold higher than in the case of monometallic systems. In the case of the other two species the phytoaccumulation ability increased or decreased depending on metals (Table 4).

Plants can uptake heavy metals via their roots and in certain cases such as in the case of submerged plants via their leaves. The metal uptake occurs in two pathways: extracellular (apoplastically), which is a fast process followed by intracellular (symplastically), which is a slow one. The uptake of heavy metals in the fast stage takes places by physical and chemical sorption (adsorption) as well as by ion exchange processes. In the slower stage takes place the intracellular uptake and the transport of the metals into the cells [14].

Controversial interactions take place when the plants are exposed to more than one metals: synergetic or antagonistic effect, which can be explained by the competition or association of the heavy metals for the binding sites at membrane transporters, at metallo-enzymes, at metallothioneins or at other target molecules with metal sensitivity [15].

The obtained results demonstrate that the accumulation capacity of the plants increased during the phytoremediation of multimetallic solutions, showing a synergetic effect on the uptake capacity.

**Table 3.** Accumulation and distribution of heavy metals (mg/g DW) in *Eichhornia crassipes* and *Pistia stratiotes* exposed to multimetallic solutions ( $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$  initial concentration 10 mg/L,  $t = 24 - 28^\circ\text{C}$ ,  $\text{pH} = 5.6$ , photoperiod 14/10 h artificial light) Values represent  $\pm$  SD (n=3), small letters represent the statistical significant difference at  $P < 0.05$

Species	Heavy metals	Concentration	Concentration	Accumulated	Accumulated
		in control sample leaves (mg/g)	in control sample roots (mg/g)	concentration in root (mg/g)	concentration in leaves (mg/g)
Eichhornia crassipes	$\text{Cu}^{2+}$	0.004	0.005	$5.841 \pm 0.4^a$	$1.769 \pm 0.6^c$
	$\text{Zn}^{2+}$	0.029	0.0063	$3.032 \pm 0.2^b$	$1.736 \pm 0.8^c$
	$\text{Cd}^{2+}$	0	0	$3.585 \pm 0.5^b$	$0.391 \pm 0.3^d$
Pistia stratiotes	$\text{Cu}^{2+}$	0.0033	0.0079	$2.989 \pm 0.7^b$	$3.646 \pm 0.06^b$
	$\text{Zn}^{2+}$	0.013	0.047	$4.899 \pm 1.1^a$	$3.178 \pm 0.3^b$
	$\text{Cd}^{2+}$	0	0	$3.290 \pm 0.4^b$	$0.889 \pm 0.02^c$

**Table 4.** The accumulated heavy metal concentrations in *Elodea canadensis*, *Lemna minor* and *Salvinia natans*, exposed to multimetallic solutions ( $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$  initial concentration 10 mg/L,  $t = 24 - 28^\circ\text{C}$ ,  $\text{pH} = 5.6$ , photoperiod 14/10 h artificial light). Values represent  $\pm$  SD (n=3), small letters represent the statistical significant difference at  $P < 0.05$

Species	Heavy metals	Concentration	Accumulated
		in control sample leaves (mg/g)	concentration in leaves (mg/g)
Elodea canadensis	$\text{Cu}^{2+}$	0.036	$0.482 \pm 0.01^b$
	$\text{Zn}^{2+}$	0.089	$0.594 \pm 0.03^b$
	$\text{Cd}^{2+}$	0	$1.516 \pm 0.2^a$
Lemna minor	$\text{Cu}^{2+}$	0.003	$10.810 \pm 1.3^a$
	$\text{Zn}^{2+}$	0.046	$4.891 \pm 1.2^b$
	$\text{Cd}^{2+}$	0	$2.784 \pm 0.9^c$
Salvinia natans	$\text{Cu}^{2+}$	0.0067	$3.564 \pm 0.1^b$
	$\text{Zn}^{2+}$	0.0026	$4.472 \pm 0.3^b$
	$\text{Cd}^{2+}$	0	$1.094 \pm 0.4^d$

### 4.3. The biochemical responses of the plants to the induced abiotic stress

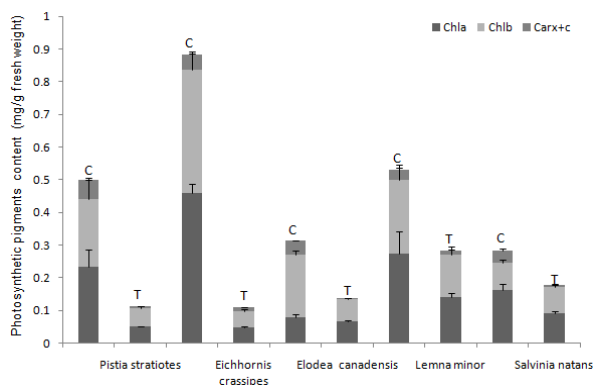
Adverse environmental conditions change and affect the plant metabolism, resulting in biochemical responses in the evolution and adaptation of the exposed species. Plants have developed evolving defense systems in order to tolerate the abiotic stress effects, however these complex systems are not completely clarified and more research is needed in this area

. In order to analyze the responses of the aquatic plants to the induced stress effect and to understand the phytoremediation mechanism, the changes of several **biochemical “markers”** of stress were studied such as photosynthetic pigments, total protein, antioxidant (ascorbic acid and glutathione), and phytochelatin levels and phytochelatin synthesis.

#### 4.3.1. Analysis of the photosynthetic pigments

The *Elodea canadensis*, *Eichhornia crassipes*, *Lemna minor*, *Pistia stratiotes* and *Salvinia natans* species were exposed to multimetallic system ( $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  in an initial concentration of 10 mg/L). After the phytoextraction experiments, in order to investigate the biochemical responses and to determinate the resistance of the plant to the induced abiotic stress, the photosynthetic pigments content of the plants (chlorophyll *a*, chlorophyll *b* and carotenoids  $x+c$ ) was analysed.

The aquatic plants photosynthetic pigments content was determinate quantitatively from the control and treated plants using absorption coefficients at specific wavelengths given by Lichtenthaler[16, 17]. The obtained results are presented in the Fig. 3.



**Fig. 3.** The photosynthetic pigments content ( $\text{Chl}_a$  and  $\text{Chl}_b$ , as well the  $\text{Car}_{x+c}$ ) of control plants (C) and the aquatic plants exposed to heavy metals (T) in multimetallic systems.

The results confirmed that the three heavy metal ions had a negative effect on the photosynthetic activity of the hydrophytes during the experiments (Fig. 3). A high decrease in the content of the photosynthetic pigments was shown at *Eichhornia crassipes* (Chl<sub>a</sub> 89%, Chl<sub>b</sub> 86%, Car<sub>x+c</sub> 72%) and at *Pistia stratiotes* (Chl<sub>a</sub> 77%, Chl<sub>b</sub> 73%, Car<sub>x+c</sub> 89%) species. Moreover, in the case of the other three species a smaller decline was observed: at *Elodea canadensis* Chl<sub>a</sub> 16%, Chl<sub>b</sub> 63%, Car<sub>x+c</sub> 96%, at *Salvinia natans* Chl<sub>a</sub> 43%, Chl<sub>b</sub> 4.6%, Car<sub>x+c</sub> 83%, and at *Lemna minor* Chl<sub>a</sub>, 48%, Chl<sub>b</sub> 42% Car<sub>x+c</sub> 56%.

The obtained results are in agreement with previous literature reports such as Naumann et al. [18], who showed that Cu<sup>2+</sup> monometallic treatments may inhibit the chlorophyll and carotenoids biosynthesis in the case of aquatic plants.

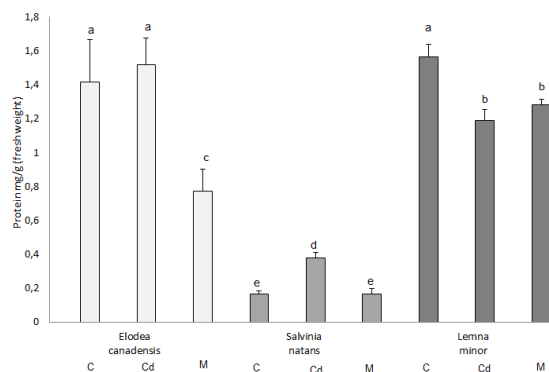
The *Elodea canadensis*, *Salvinia natans* and *Lemna minor* were chosen for **further biochemical analysis** to determine the responses of the plant to the induced heavy metal stress.

#### 4.3.2. Analysis of total protein content

The total protein content of three aquatic plants, was determined after 6 day exposure of Cd<sup>2+</sup> mono- and multimetallic solutions (heavy metals initial concentrations was 65 µM/L, as 4.10 mg/L Cu<sup>2+</sup>, 4.30 mg/L Zn<sup>2+</sup> and 7.30 mg/L Cd<sup>2+</sup>).

The hydrophytes total protein content was extracted following the method of Wang et al. [19, 20]. The total protein content was estimated according to the method described by Bradford [21], using bovine serum albumin as standard.

Comparing the results it can be noticed that the *Salvinia natans* protein level was increased (2.27-fold greater concentration of protein compared to the control) in the case of experiment using monometallic system. However, the *Elodea canadensis* and *Lemna minor*'s protein content decreased (1.83- fold and 1.22-fold smaller than the controls) after the phytoextraction of heavy metals using monometallic system. These results can be explained by two processes such as the synthesis of new proteins or by the misregulation as well the degradation of the existent proteins. These observation were also presented in our previous work [22] and also are in good agreement with other literature reports [23], [24].



**Fig. 4.** The aquatic plants total protein content in control plants and after the phytoextraction of mono- and multimetallic solutions (C- Control, Cd- monometallic solution, M –multimetallic solution containing  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  in  $65\mu\text{m/L}$  initial concentration). Values indicated by letters are significantly different from the corresponding control of each species at  $P \leq 0.05$  level.

#### 4.3.2. Antioxidants as biochemical “markers” of stress

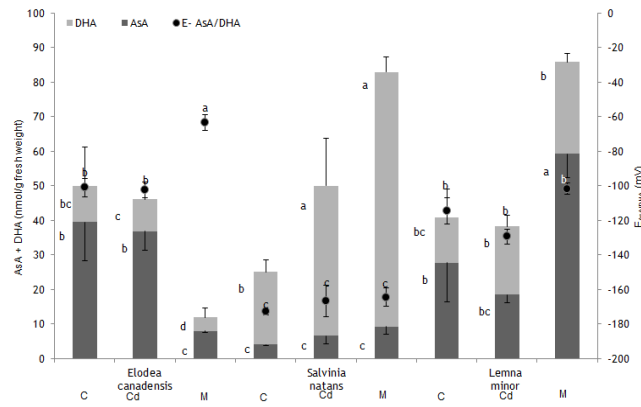
The variation of concentrations and synthesis of several antioxidants (non-enzymatic and enzymatic antioxidants) may be accomplished as general responses during the abiotic stress [25]. Two major water-soluble antioxidants are known to play a key role in the plants defense mechanism which is the sulfur-containing glutathione and ascorbate. Ascorbate (AsA) and glutathione (GSH) non-enzymatic antioxidants are important component of the ascorbate-glutathione cycle controlling the level of hydrogen peroxide. GSH also participates in the detoxification of heavy metals and the maintenance of metal homeostasis [26]. It can remove toxic molecules (peroxides, xenobiotics) through conjugation and it controls cellular redox state through degradation of  $\text{H}_2\text{O}_2$ . Moreover the glutathione oligochelatins ( $[\gamma\text{-Glu-Cys}]_n\text{Gly}$ ), the phytochelatins, are able to bind the heavy metals [27]. The glutathione (GSH) or phytochelatins (PCs) can form complexes with heavy metals which are transported to vacuoles of the cells as a final step of detoxification [28].



### 4.3.3. Analysis of ascorbic acid

To evaluate the aquatic plants responses to the metallic stress, quantitative analysis was performed for the ascorbic acid (AsA). The plants (control as well the treated hydrophytes) total ascorbic acid content were extracted and analyzed by HPLC (Waters, Milford, MA, USA) method using a diode array (W996) detector as described previously by Szalai et al.[29].

The comparing results of ascorbic and dehydroascorbic acid (AsA/DHA) content in the three aquatic species after the phytoextraction experiments is presented in the Fig.5. The results of total AsA are shown by the oxidized and reduced form as well the calculated redox potentials (using the Nernst equation).



**Fig. 5.** Effect of heavy metals on ascorbate concentration (AsA), dehydroascorbate (DHA) and on their half-cell reduction potential ( $E_{DHA/AsA}$ ). The plants were exposed to no metals (C: control), to  $Cd^{2+}$  or to  $Cu^{2+}+Zn^{2+}+Cd^{2+}$  (M: multimetallic system) for 6 days. Values indicated by letters are significantly different from the corresponding control of each species at  $P \leq 0.05$  level

It can be observed that the phytoextraction of mono- and multimetallic solution had different effect on the aquatic plants AsA and DHA content (Fig. 3). The *Salvinia natans* and *Lemna minor* produced 3.31-fold and 2.10-fold higher concentrations of total ascorbate (AsA+DHA) after exposure of the multimetallic system. These two species AsA/DHA ratio was also increased, especially in the case of *Salvinia natans* hydrophyte (Fig. 5)[30]. Plants can face the heavy metal stress through their protective mechanisms by mitigating and repairing ROS damages in the cells. The overexpression of antioxidants such as AsA and DHA might be a

powerful tool for the survival of plants with the highest metal accumulation according to literature reports [31, 32]

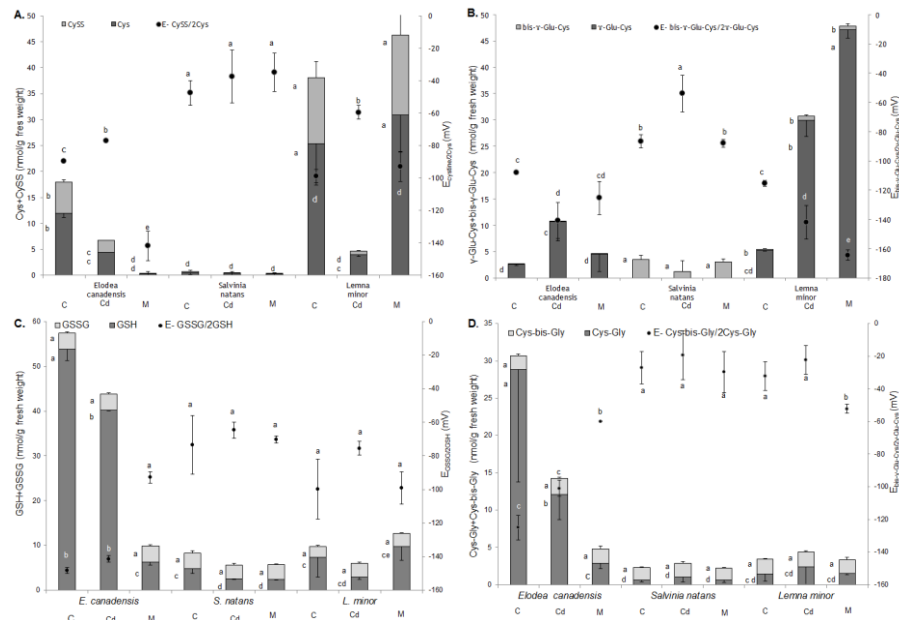
Previous literature report presented that the terrestrial plants AsA and DHA levels depends upon the plant species and some environmental factors (such as light, temperature, etc.), biotic or abiotic stressor (presence of organic or inorganic pollutants) [33]. These explanations are in good agreements with the results obtained in the present research and also explain the variation of the AsA and DHA contents after the experiments.

The participation of AsA in the reduction of heavy metal stress-induced damages and in phytoremediation can be considered to be involved in the ascorbate-GSH cycle maintaining the reduction of GSH, which is a precursor of the heavy metal-complexing phytochelatins.

#### 4.3.4. Analysis of thiol compounds

Another important aspect to determine the effect of heavy metals ( $\text{Cd}^{2+}$  mono - and  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  multimetallic solution phytoextraction, initial concentration of heavy metals  $65\mu\text{M/L}$ ) was the analysis of **thiol compounds**: cysteine (Cys), cystine (CySS),  $\gamma$ -glutamyl-cysteine ( $\gamma$ -Glu-Cys), bis- $\gamma$ -glutamyl-cystine (bis- $\gamma$ -Glu-Cys), glutathione (GSH), glutathione disulphide (GSSG), cysteinyl-glycine (Cys-Gly) and cystinyl-bis-glycine (Cys-bis-Gly). The thiol compounds were extracted and analysed according to methods described previously [34, 35]. [36, 37] [38].

The obtained results for the thiol compounds are presented in the Fig. 6. It can be observed from the results that the *Lemna minor* was show a greater **cysteine and cystine (Cys+cystine)** and  **$\gamma$ -glutamylcysteine** ( $\gamma$ -Glu-Cys) concentrations after the phytoextraction of multimetallic system. Also a great induction of GSH synthesis was only observed exposed to  $\text{Cd}^{2+}$  or multimetallic solution in the case of *Lemna minor*. It can be mentioned that the  $\gamma$ -Glu-Cys synthesis is the rate limiting step in GSH formation, which was assimilated by the greater GSH concentration in *Lemna minor*. hydrophyte These results are in good agreements with other literatures reports using terrestrial plant [34]. The low GSH content in Cd-treated *Lemna minor* can be explained by the immediate use of GSH for PC synthesis, since the amount of its degradation product, Cys-Gly remained low after the phytoextraction experiments of heavy metals.



**Fig. 6.** Effect of heavy metals on the concentration of thiols and thiol disulphides and on their half-cell reduction potential (E). The plants were treated with no metals (C: control),  $Cd^{2+}$  or  $Cu^{2+}+Zn^{2+}+Cd^{2+}$  (M: multimetallic solution) for 6 days. Values indicated by letters are significantly different from the corresponding control of each species at  $P \leq 0.05$  level **A.** Cys/CySS, **B.**  $\gamma$ -Glu-Cys /bis- $\gamma$ -Glu-Cys, **C.** GSH/GSSG, **D.** Cys-Gly/Cys-bis-Gly.

The thiol groups from the glutathione molecules acts as a reducing agent, nucleophilic agent and may undergo oxidation and forms the glutathione disulfides (GSSG), this reaction is a reversible process. The thiol (-SH) groups are reactive towards electrophilic compounds and mend cellular detoxification. During the heavy metal stress the glutathione is able to form chelates with metals in the plants and this process is catalyzed by the glutathione S-transferase [39]. The GSH represent a dual role in the plants growth as a basic component of phytochelatinns and as an antioxidant metabolite during the abiotic stresses.

In order to analyze further plant adaptation to the heavy metal stress, the phytochelatin activity and levels were determinate of these three aquatic species. **The phytochelatinns (PCs)** are synthesized from GSH and have different degree of polymerization regulated by the number of the incorporated  $\gamma$ -Glu-Cys dipeptides. The synthesis of PCs is catalyzed by  $\gamma$ -Glu-Cys dipeptidyl transpeptidase (EC 2.3.3.15) called **phytochelatin synthase (PCS)**. PCS activity in

the plants can be studied by determination of increasing phytochelatin concentrations [40]. The obtained result for the aquatic plant's PCS activity and PCs levels after the phytoremediation experiments are presented in the Figure 7 and Table 6.

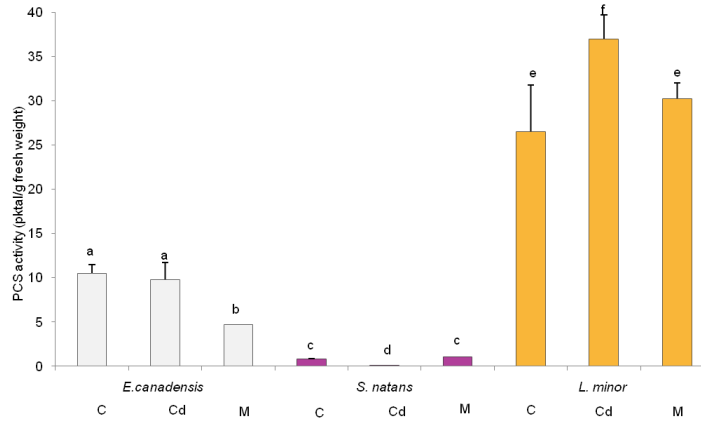


Fig.7. Effect of heavy metals on the phytochelatin synthase activity. The plants were treated with no metals (C: control), Cd or Cu+Zn+Cd (M: multimetallic system) for 6 days. Values indicated by asterisks are significantly different from the corresponding control of each species at the  $p \leq 0.05$  level

**Table 6.** Phytochelatin (PC) content of the plants exposed to no metals (C: control), Cd or Cu+Zn+Cd (M: multimetallic system) for 6 days. Values indicated by asterisks are significantly different from the corresponding control of each species at the  $p \leq 0.05$ (\*).

Species	PCs	PCs (nmol/g fresh weight)		
		C	Cd	M
<i>Elodea canadensis</i>	PC <sub>2</sub>	1.88 ±0.42	1.34* ±0.43	1.36* ±0.2
	PC <sub>3</sub>	15.96 ±1.7	13.38 ±2.1	15.94 ±1
	Total	17.84±1.0	14.72±1.3	17.32±0.9
<i>Salvinia natans</i>	PC <sub>2</sub>	2.19 ±0.073	1.51* ±0.5	0.70* ±0.05
	PC <sub>3</sub>	16.30 ±1.3	16.42*±0.3	12.16*±1.4

<i>Lemna minor</i>	Total	18.49±1.1	17.93±0.4	12.86*±0.8
	PC <sub>2</sub>	0.96 ±0.13	1.32* ±0.46	1.23 ±0.26
	PC <sub>3</sub>	9.87 ±0.7	12.61* ±1	14.36* ±1.1
	PC <sub>4</sub>	0	4.07±0.7	8.86 ±0.9
	PC <sub>6</sub>	0	42.22±0.57	51.30 ±0.4
	PC <sub>7</sub>	0	44.31±0.5	33.89 ±0.7
	Total	10.83±0.8	119.25*±1	109.64*±0.7

It can be observed from the obtained results that the PCS activity of *L. minor* was about 3-fold greater compared to *Elodea canadensis* (Fig.7). The highest PCS activity was observed in the case of monometallic solution in the *Lemna minor* plant.

The most interesting finding of this part of the research was that the highest phytoremediation capacity in *Lemna minor* was associated with the synthesis of PCs with high degree of polymerization (PC<sub>4</sub>, PC<sub>6</sub> and PC<sub>7</sub>). It should be noted, that in the other two species only PC<sub>2</sub> and PC<sub>3</sub> were detected.

It can be concluded that the *Lemna minor* produce preminent results during the heavy metals uptake and accumulation which was accomplished by the activity of plants defense mechanism to the produced abiotic stress. The PCs are known generally as metal chelators, they could have important role in the transport and storage of the metals in the plant cells. In this study the *L. minor* presented an adaption to the Cd toxicity through the synthesis of polymerization of longer chain-lengths PCs, which might have a major role in metal homeostasis.

Previous literature reports described that the PCs with higher degree of polymerization were more efficient in the complexation of Cd [41] and the highest heavy metal accumulation and the synthesis of the longest PC chains (PC<sub>4</sub>) was observed in *Fucus spp.* deriving from contaminated sites [39].

#### 4.4. Selection of the organic pollutants for the phytoextraction studies

Triphenylmethane dyes are synthetic colorants with aromatic structure, extensively used by different industries [42]. Their persistence in the environment leads to serious ecological and

health problems [43]. The triphenylmethane dyes are known to be highly toxic to mammalian cells and mutagenic and carcinogenic to humans [42, 44].

### ***Plant material and growing conditions***

The free-floating aquatic plants (*Lemna minor*, *Salvinia natans*) and submerged aquatic plant (*Elodea canadensis*) were chosen for phytoremediation experiments due to their unique physical and biological properties and its high tolerance for abiotic stresses. The plants were grown in the greenhouse of the University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca, Romania, with the addition of Complex III fertilizer (0.5%). Following the 30 day growing period, they were used for the phytoremediation experiments.

### ***Experimental conditions***

The phytoremediation experiments were carried out in controlled conditions (at room temperature  $23 \pm 2$  °C, illuminated with a lamp with the 14/10 h light/dark photoperiod), in 250 ml Beaker glass containing 200 ml synthetic wastewater and the aquatic plants along with the macro- and micronutrients in batch mode (from the modified Hoagland nutrient solution).

### ***Methods for dye determinations***

The stock solutions of CV and MG were obtained by dissolving 1 g of dye in 1 L distilled water. The working solutions were prepared by diluting the stock solutions with a Hoagland nutrient solution.

In order to determinate the plant's phytoremediation efficiency and capacity, the systems concentrations changes were followed in time by withdrawn samples every day from the synthetic dye solutions. The dyes concentrations were determined spectrophotometrically (CV  $\lambda_{\max} = 590$  nm and MG  $\lambda_{\max} = 618$  nm) using a double beam UV-visible spectrophotometer (UV-Vis: GBC Cintra 202).

The percentage of removal efficiency of the *aquatic plants* were calculated by equation (1) below, where E (%) is the dye removal efficiency,  $C_i$  is the initial dye concentration and  $C_f$  is the final dye concentration measured from the aqueous solutions in mg/L.

$$E(\%) = \frac{C_i - C_f}{C_i} \cdot 100 \quad (1)$$

The plants phytoremediation capacity was calculated by the formula (2), where  $q_{max}$  is the plant's uptake capacity (mg/g),  $C_i$  is the initial concentration (mg/L),  $C_f$  is the final concentration (mg/L) measured from the aqueous solutions,  $V$  is the volume of the solution (L), and  $m$  is the plant quantity (g).

$$q_{max} = (C_i - C_f) \cdot \frac{V}{m} \quad (2)$$

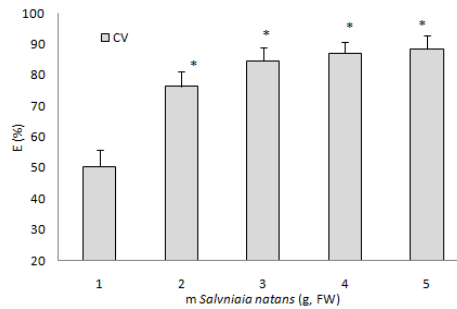
#### Operational parameters and the phytoremediation process characterisation

The following operational parameters were analysed: Effect of plant quantity (between 1 and 5 g); Effect of initial dye concentrations (between 10 to 300 mg/L initial dye concentrations); Effect of initial pH (values of 2-10 initial pH); Effect of temperatures (5 to 40 °C). The obtained results are presented only in the case of one selected plant in this short version of thesis; however the extended PhD thesis contains the results for all the three aquatic plants.

#### 4.4.3. Optimization of the operational parametrs

##### *The effect of plant quantity*

In order to study the effect of plant weight over the phytoremediation efficiency of triphenylmethane dyes, various plant quantities (1 - 5 g) were tested. The obtained results are presented in the Fig.8 for the *Salvinia natans* specie.



**Fig. 9. Fig. 9.** The effect of *Salvinia natans* plant quantity on the removal efficiency (E, %) of the CV dye; effect of plant quantity:  $m_{plant} = 1-5$  g (FW),  $C_i = 50$  mg/L,  $V = 0.2$  L,  $t = 23 \pm 2$  °C, 10 day phytoextraction period; values mean  $\pm$  standard deviations (n = 3); \*significant difference at  $P < 0.05$ .

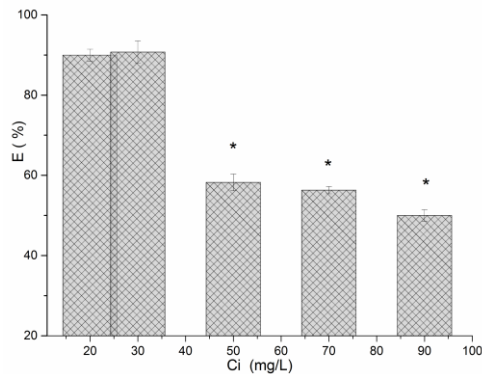
A beneficial effect on the removal efficiency was observed when a plant quantity was increased. This can be explain with the fact that the larger amount of biomass provides to be more surface area (roots and leaves), which include the availability of more active adsorption sites and active phytoextraction through the plant's roots.

### ***The effect of initial dye concentrations***

The initial dye concentration is an important factor in the phytoremediation process. To evaluate the effect of CV and MG dyes initial concentrations' on the removal efficiency, the aquatic plants were exposed to different initial dye concentrations in the range of 20 - 90 mg/L at *Elodea canadensis*, in the range of 10 – 120 mg/L at *Salvinia natans* and in the range of 40 - 300 mg/L initial dye concentrations at *Lemna minor* specie.

According to the obtained results it can be observe that the aquatic plants highest removal efficiency was attained at the smallest initial concentration and it could be noticed that the removal capacity of aquatic plants is depended on the initial dye concentrations.

The hydrophytes were able to tolerate higher concentrations of dyes, however the removal efficiency decreased. The plant surface's active sites and uptake capacity depend on the plant's saturation and at higher concentrations the process' efficiency is starting to decrease. The initial dye concentration provided an important driving force to overcome all mass transfer resistances between the dye and the aqueous solid phases [45].



**Fig. 13.** Effect of initial dye concentrations: CV on the removal efficiency (E, %) using *Elodea canadensis*. Values mean  $\pm$  standard deviations (n = 3); \*significant difference at  $P < 0.05$



### *The effect of the initial pH*

The pH of the aqueous medium is an important factor which affects directly the living system's biological and biochemical functions. Generally the pH of medium has a major influent in the plants growth regulation and could also affect the mobility and availability of ions and in the uptake of some nutrients [46, 47].

The highest removal efficiency was measured at the initial pH value of 7 in the case of CV dye using *Lemna minor*, *Salvinia natans* or *Elodea canadensis*, although in the initial pH range from slightly acidic (pH 5) to alkaline (pH 9), the hydrophytes shown high removal efficiency with no considerable differences (Table 9). These results are in good agreement with the results of Reema et al. who found that the *Lemna minor* activity in the removal of Methylene blue is highest in the pH range of 6-7.5 [48].

**Table 9.** The aquatic plants phytoextraction efficiency at various initial pH: *Salvinia natans*  $m_{\text{plant}} = 4$  g (FW),  $C_i = 50$  mg/L CV and MG dyes,  $V = 0.2$  L,  $t = 23 \pm 2$  °C.

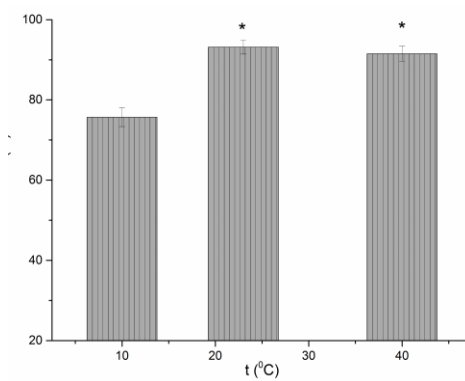
Dye	The initial pH of the dye solutions	The final pH of the dye solutions	Dye removal efficiency (E = %)
CV	3.	6.52	68.054
	5	7.1	88.432
	7	7.33	88.316
	8.5	7.49	88.4737
	10	8.33	88.155
MG	3	3.8	28.84±5.6
	4	5.98	45.99 ±4.76
	5	6.63	69.03 ±3.69
	6	6.84	81.85±3.70
	7	7.26	85.28±2.61

The aquatic plants can tolerate a wide range of initial pH from acidic to alkaline. Their dye removal efficiency was notable in various cases and the hydrophytes had characteristic properties to equilibrate the pH which may present the plants' responses to the induced water

stress. These results can be explained by the plants' metabolic reactions involving consumption, production or transfer of protons during the processes [49].

### *The effect of temperature*

The temperature has a major effect during the phytoremediation process on the plants' biochemical processes, such as enzyme activity, translocation of nutrients and photosynthesis of plants [46]. Generally, the aquatic plant's optimal growth temperature is between 10 and 30 °C. Lower or higher temperatures can be considered stress factors for the plant and can influence the phytoremediation efficiency.



**Fig.15.** Effect of temperature on the phytoremediation efficiency of CV dye using *Elodea canadensis*:  $m_{\text{plant}} = 4$  g (FW),  $C_i = 30$  mg/L CV dye,  $V = 0.2$  L. \*significant difference at  $P < 0.05$

The results indicate that generally the phytoremediation experiments of triphenylmethane dyes using aquatic hydrophytes were performed at room temperature. However, *Lemna minor*'s the phytoremediation efficiency of MG dyes at 23 and 40 °C temperatures was high in both cases and there was no significant differences between the two values. The obtained results are in good agreements with the findings of Khataee et al. as well Torok et al, demonstrating that the various aquatic species' dye removal occurs with high efficiency at 25 °C [50].

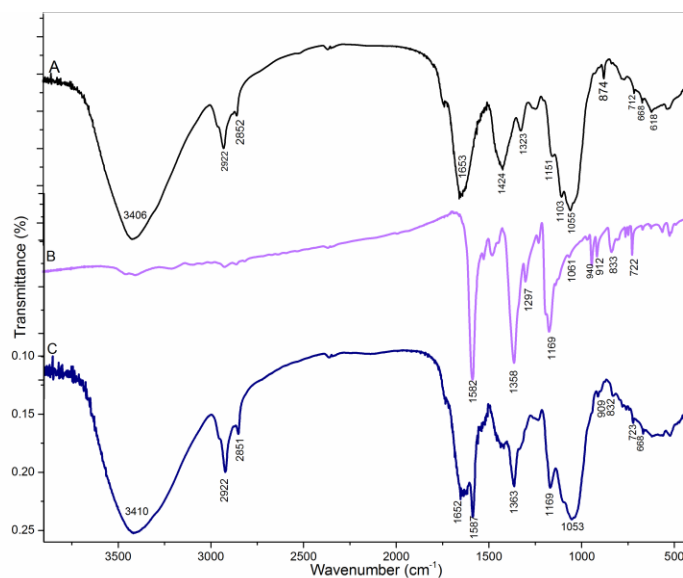
In the case of submerged macrophytes, at low temperature the composition of plasma membrane lipids is changing, which alters the plant membrane fluidity, resulting in lower membrane permeability and lower metal uptake.[51] Therefore, this hypothesis is in good agreement with our results, whereat at 10 °C the *Elodea canadensis* phytoremediation efficiency

are found to be lower. Our observations are compatible with the results of other literature reports with different kind of plants [52, 53].

## 4.5. Spectral analysis

### 4.5.1. Fourier transformation infrared spectroscopy (FTIR)

Fourier transformation infrared spectroscopy (FTIR) was used to further characterise the aquatic plants after the phytoremediation experiments. CV and MG dyes, aquatic plants before (control) and after phytoremediation were subjected to Fourier transform infrared spectroscopy (FTIR) analysis. The plant samples were prepared encapsulating 1.2 mg of finely grounded plant particles in 300 mg of KBr. Infrared spectra were obtained using a JASCO 615 FTIR spectrometer  $4000\text{-}500\text{ cm}^{-1}$  (resolution,  $2\text{ cm}^{-1}$ ) and data were processed with ORIGIN PRO 8.5 software



**Fig. 18.** FTIR spectra analysis of *Elodea canadensis* control plant (A), CV dye (B) and after the phytoextraction experiments of CV dye (C).

The  $2000\text{ to }500\text{ cm}^{-1}$  contain a large number of vibrations attributed to the plant and specific fingerprint peaks of the dyes [54]. It can be mentioned that the triphenylmethane dyes spectra contain three specify intense peaks that spectra contains at the range of  $2000\text{ - }1000\text{ cm}^{-1}$

<sup>1</sup>which can be considered as the dyes' fingerprints zone. Generally the hydrophytes treated with triphenylmethane dyes have shown major changes in this aforementioned spectra region.

In the case of *Elodea canadensis*, (Fig.18) reveal the appearance of three new peaks at  $1587\text{ cm}^{-1}$ ,  $1363\text{ cm}^{-1}$  and  $1169\text{ cm}^{-1}$ . It can be mentioned that the appearance of new peaks in the treated plant spectra clearly indicates the CV and MG dye uptake by the aquatic plants. The peaks at  $1587 \pm 2\text{ cm}^{-1}$  and at  $1370 \pm 7\text{ cm}^{-1}$  can be attributed to the benzene rings' C=C stretching vibrations and C-C aromatic stretching vibrations. The new peak in the FTIR spectra found at  $1168 \pm 3\text{ cm}^{-1}$  can be associated with the secondary amine stretching vibrations and the asymmetric stretches of Ar-NR<sub>2</sub> peaks from the dyes. These observations are in good agreement with the results reported for the removal of malachite green using *Limonia acidissima* and with the FTIR analysis of the treated *Staphylococcus epidermidis* [55, 56]

#### **4.5.2. Energy-dispersive X-ray spectroscopy (EDS)**

The elemental composition of control aquatic plants and plant exposed to phytoremediation were analysed with a Scanning Jeol JEM 5510LV (Japan) coupled with Oxford Instruments EDS Analysis System Inca 300 (UK). To determine the elemental composition of the aquatic plants: *Lemna minor* and *Salvinia natans* samples were washed with distilled water and dried then were analysed.

The results obtained from the EDS spectra of *Salvinia natans* plant are presented in Table 11. It was observed a variation in the elemental content after the exposure of triphenylmethane dyes. The hydrophyte Na, P, S, Cl, K, Fe and Cu contents were decreased representatively exposed to MG dye, however in the case of phytoextraction experiment using CV dye only the Si and P decreased significantly. These simultaneous decreases of elements could suggest the stress effect produced by triphenylmethane dyes on the plants metabolisms. The P, S and K elements are known to play a particularly critical role in plants' growth, metabolism (such as enzyme activation, protein synthesis, photosynthesis or energy transfer) and also contributes to plant survival under various biotic and abiotic stresses [57-60]. The deficiency of the mentioned elements can be regarded as a growth-limiting factor which also enhanced the decrease of the photosynthetic activity in plants [61].

Conform Marschner's report the high concentrations of potentially toxic ions and nutrient imbalance of the solutions produce a depressed uptake, impaired internal distribution and transport of minerals which are in concordance with our results [62].

**Table 11.** EDS analysis of the *Salvinia natans* control and the aquatic plant after the phytoremediation experiments with triphenylmethane dyes;  $C_1 = 100 \text{ mg/L}$ ,  $V = 0.2 \text{ L}$ , initial  $\text{pH} = 3.5 \text{ (MG)}/3.8 \text{ (CV)}$ ,  $t = 23 \pm 2 \text{ }^\circ\text{C}$ , until 12 days; values mean  $\pm$  standard deviations ( $n = 3$ ), \*significant difference at  $P < 0.05$ .

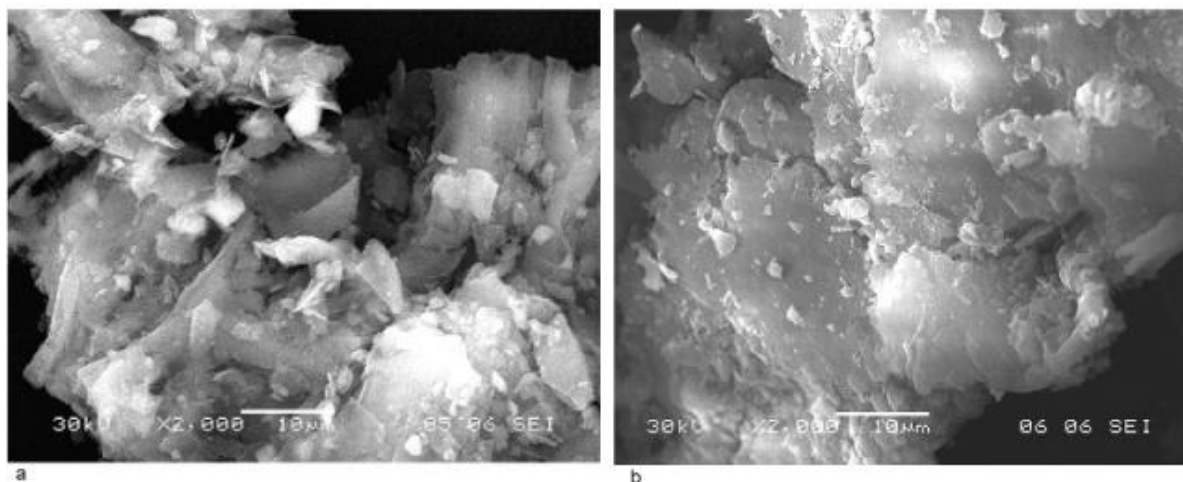
Nr.	Elements	Wt (%) Content of the control plant	Wt (%) Content of the plant after the phytoextraction of MG	Wt (%) Content of the plant after the phytoextraction of CV
1	C	52.98 $\pm$ 1.42	46.63 $\pm$ 3.66	53.25 $\pm$ 5.73
2	N	4.33 $\pm$ 0.41	4.72 $\pm$ 0.73	5.92 $\pm$ 0.55*
3	O	28.31 $\pm$ 1.79	32.23 $\pm$ 1.85	25.21 $\pm$ 2.04
4	Na	0.83 $\pm$ 0.15	0.08 $\pm$ 0.01*	0.37 $\pm$ 0.07
5	Al	0.16 $\pm$ 0.02	0.94 $\pm$ 0.27	0.13 $\pm$ 0.03
6	Si	2.44 $\pm$ 0.67	7.17 $\pm$ 0.65*	0.18 $\pm$ 0.13*
7	P	1.90 $\pm$ 0.17	0.45 $\pm$ 0.11*	0.74 $\pm$ 0.25*
8	S	1.40 $\pm$ 0.26	0.24 $\pm$ 0.11*	0.92 $\pm$ 0.27
9	Cl	1.19 $\pm$ 0.11	0.08 $\pm$ 0.14*	4.5 $\pm$ 2.16
10	K	3.07 $\pm$ 0.52	0.23 $\pm$ 0.07*	6.61 $\pm$ 3.17
11	Ca	2.75 $\pm$ 0.8	5.73 $\pm$ 0.9	1.50 $\pm$ 0.67
12	Fe	0.41 $\pm$ 0.21	1.23 $\pm$ 0.35*	0.43 $\pm$ 0.31
13	Cu	0.26 $\pm$ 0.11	0*	0.16 $\pm$ 0.04

#### 4.4.3. Scanning electron microscopy (SEM)

The surface of *Salvinia natans* was evaluated using a JEOL (USA) JSM 5510 LV SEM microscope. Prior to the analysis, the samples were dried and mounted on a stainless stab with a double stick tape. Afterwards they were coated with a thin layer of gold (10 nm) under vacuum ( $1.33 \times 10^{-6} \text{ mBar}$ ) to improve electron conductivity and image quality.

In order to characterize the surface morphology of *Salvinia natans*, SEM studies were conducted. It is possible to observe in Fig. 19 (a) that the aquatic plant has a rough and porous

surface with a fibrous and heterogeneous structure. The morphological changes and the surface properties of *Salvinia natans* involve the increase of the surface area, increasing thus the uptake capacity of MG dye. After the phytoremediation process, due to the interaction between the aquatic plant surface and dye molecules, the surfaces become saturated forming a dense structure (Fig. 19. b).

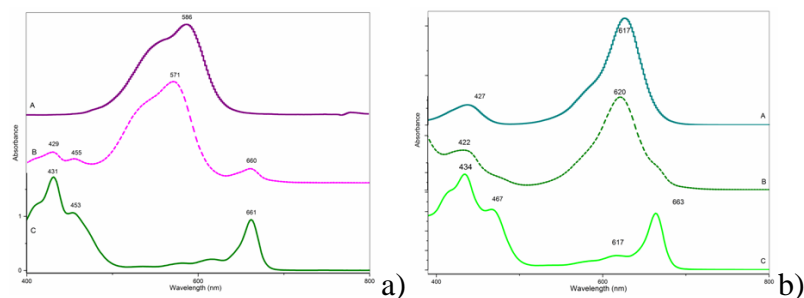


**Fig. 19.** The SEM micrographs of *Salvinia natans* control plant (a) and *Salvinia natans* after the phytoremediation of MG (b).

#### 4.5.4. Phytoaccumulated dyes analysis using UV-Vis spectroscopy

The content of the from control *Lemna minor* plant's pigments was extracted from the control plant as well as from hydrophytes exposed to phytoremediation experiments and were registered using UV-Vis spectroscopy.

The results are presented in Fig. 20. The results demonstrate that the plant's cells contain the accumulated dyes in an intact form without degradation. The wavelength bands of the dyes are similar to the control dyes' wavelength bands, except that the peak maximums are shifted from  $\lambda_{\max}$  586 to 571 nm by CV and from  $\lambda_{\max}$  617 to 620 nm in case of MG.



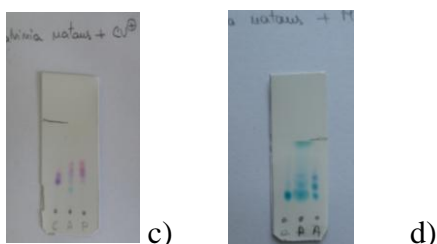
**Fig. 20.** UV-Vis spectra of the CV and MG diluted in EtOH (A), the phytoaccumulated CV and MG content extracted with EtOH from *Lemna minor* (B), and the photosynthetic pigments extracted with EtOH from control *Lemna minor* (C) [The a) for the CV and b) for the MG dye].

#### 4.5. 5. Characterisation of the parallel phytodegradation process

The triphenylmethane dyes such as CV are considered to be resistant to degradation (in natural conditions) and they persist in the environment for longer period [63, 64]. It was previously reported that several bacteria and fungus have the ability to decolorize and degrade triphenylmethane dyes [65].

In order to investigate the hydrophytes phytodegradation capacity, a parallel process of phytoremediation, dye molecules were analysed from the solutions. Thin layer chromatography was performed for the detection of degradation products, using a mobile phase: methanol, ethyl-acetate, n-butanol, water and acetic acid in 1:2:3:1:0.2 (v/v). The degradation compounds from the dye solutions after phytoremediation were extracted with ethyl acetate.

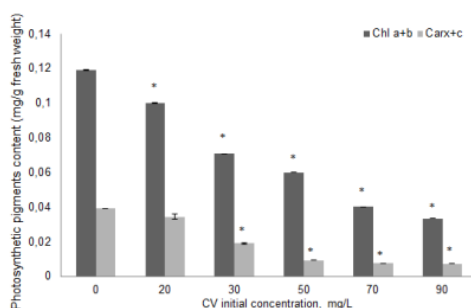
It was noticed that phytoremediation methods can be explained by parallel sub-processes, such as phytoextraction/phytoaccumulation in the plants and phytodegradation in the aqueous solutions. The obtained results from the TLC analysis are presented in the Figure xxxx. Compared to the control dye new spots can be observed, which indicate the dyes' phytodegradation by *Salvinia natans* and *Lemna minor*.



**Fig. 22.** TLC analysis of control dyes (CV and MG) and the degraded metabolites after the phytoremediation experiments with CV (a) and MG (b) dyes using *Lemna minor* or in the case of *Salvinia natans*: CV (c) and MG (d).

#### 4.5.6. Photosynthetic pigments determination

The CV and MG dyes such as the Cu, Zn and Cd heavy metals can cause several changes in the plants physiological and biochemical processes. The photosynthetic pigments (chlorophyll *a*, *b* and carotenoid *x+c*: xanthophylls and carotenes) of the control and treated *aquatic* plants (*Elodea canadensis*, *Salvinia natns* and *Lemna minor*) were analysed in order to determine the biochemical responses produced by the CV and MG dyes.



**Fig. 24.** The photosynthetic pigments content of: b) *Elodea canadensis* in control and exposed to various initial concentration of CV dye (mean  $\pm$  SD,  $n = 3$ , \* indicates the significantly difference at  $P < 0.05$ )

The total chlorophyll ( $Chl_{a+b}$ ) content is significantly decreased after the CV phytoremediation (exposure at initial concentration in range of 20 - 90 mg/L) in *Elodea canadensis* (Fig. 24.b). The highest decrease was observed in the case of 70 and 90 mg/L initial concentration experiments. The  $Chl_{a+b}$  content decline was by 66 % and 72 % compared to the control plant. The *Elodea canadensis*  $Car_{x+c}$  content were also affected after the phytoremediation of CV dye at initial concentration higher than 30 mg/L. Therefore, it can be noticed that the exposure in high concentration of CV dye, induce biosynthesis deregulation, wherein the plants cannot resist to the induced abiotic stresses.



## 5. General conclusions

### Phytoremediation of heavy metals

- ❖ Five hydrophytes, *Elodea canadensis*, *Eichhornia crassipes*, *Salvinia natans*, *Lemna minor*, and *Pistia stratiotes* were selected due to their physiological properties for the phytoextraction studies to remove **Cu<sup>2+</sup>, Zn<sup>2+</sup> and Cd<sup>2+</sup> heavy metals** from mono- and multimetallic aqueous solutions.
  - -In the **monometallic systems** the highest heavy metal accumulation was achieved by *Salvinia natans* (4.719 mg/g DW Cu, 2.236 mg/g DW Zn<sup>2+</sup>, and 1.901 mg/g DW Cd<sup>2+</sup>) and by *Pistia stratiotes* (3.83 mg/g DW Cu<sup>2+</sup>, 2.069 mg/g DW Zn, and 1.704 mg/g DW Cd<sup>2+</sup>). The results demonstrated that the nature of the aquatic plants plays an important role in the phytoaccumulation potential of the heavy metals. *Salvinia natans* was able to accumulate 3.5-fold more Cu<sup>2+</sup> than *Elodea canadensis* and 3-fold more than *Lemna minor*.
  - In the **multimetallic systems** different interactions take place when the plants are exposed to more than one metal (synergetic or antagonistic effects) what can be explained by the association of the binding sites on the membrane transporters, on the metallo-enzymes, on the metallothioneins or on other target molecules with metal sensitivity. In the case of Cd<sup>2+</sup> accumulation, *Eichhornia crassipes* and *Pistia stratiotes* species phytoextraction (phytoaccumulation) facility became 2.14-fold and 2.466-fold (3.97 mg/g DW, 4.17 mg/g DW Cd<sup>2+</sup>) bigger than in the case of monometallic systems. *Lemna minor* accumulated 6.9 fold more Cu<sup>2+</sup>, 11 fold more Zn and 4.48 fold more Cd<sup>2+</sup> ions (10.81 mg/g DW at Cu<sup>2+</sup>, 4.89 mg /g DW at Zn<sup>2+</sup>, 2.784 mg/g DW at Cd<sup>2+</sup>) compared to the results obtained at monometallic systems. However, in the case of *Salvinia natans* only the Zn<sup>2+</sup> accumulation while in the case of *Elodea canadensis* only the Cd<sup>2+</sup> accumulation increased 2-fold actually 3.4-fold.
  - The obtained results demonstrated that the heavy metal bioaccumulation was not **proportional in the parts of the plants**; the heavy metals were phytoaccumulated in higher concentrations in the roots (70%). The Cu<sup>2+</sup> was the only exception, because it was accumulated in higher concentrations in the leaves of *Pistia stratiotes* (46.55 % higher than the concentrations in roots).

- ❖ The accumulation of the toxic heavy metals activates various **protective mechanisms** in the aquatic plants. The plants react to the effect of the induced stress through several biochemical processes, which are considered to be “markers” of the abiotic stress.
  - The results showed that the heavy metals had an influence on the **photosynthetic pigments** (chlorophyll *a*, *b* and carotenoids) content of the hydrophytes. The highest decrease of photosynthetic pigments was present in *Pistia stratiotes* (84.30 % in Chl<sub>a</sub>, 75.16 % in Chl<sub>b</sub>, 92 % in Car<sub>x+c</sub>) and in *Eichhornia crasipes* species (89.57 % in Chl<sub>a</sub>, 87.27 % in Chl<sub>b</sub>, 39.89 % in Car<sub>x+c</sub>).
  - The toxic effect of the heavy metals has a major impact on the **total protein** content of the plants. The plants can respond to the induced stress by two pathways: by generating new proteins, by increasing the level of the proteins or by the degradation of several proteins. An increase of the total protein content was observed in the case of *Salvinia natans* (55%) and *Elodea canadensis* (6.8%) species exposed to Cd<sup>2+</sup> monometallic systems. However the phytoremediation of Cd<sup>2+</sup> from multimetallic solutions results in the decrease of the total protein content in the case of *Elodea canadensis* (45%) and *Lemna minor* (18%).
  - Ascorbate is an important component of the ascorbate-glutathione cycle, which plays key role in the plant defence mechanisms. The **total ascorbate** (AsA+DHA) concentration, ratio and reduction potential results showed that the *Salvinia natans* and *Lemna minor* were able to activate their defence mechanisms to the induced stress after Cd<sup>2+</sup> exposure in mono- and multimetallic systems. It can also be mentioned that these results harmonize with the phytoremediation capacity of the plant.
  - The **glutathione** content of the plants was investigated together with their precursors, **cysteine (Cys)** and **γ-glutamyl-cysteine (γ-Glu-Cys)**, the degradation product, **cysteinyl-glycine (Cys-Gly)**, and the thiols' half-cell reduction potential was also determined. The *Lemna minor* **cystine (CySS)**; **Cys**; **GSSG**; **γ-Glu-Cys** content increased during the Cd<sup>2+</sup> phytoextraction experiments using multimetallic systems. These results can be connected to the quantity of the accumulated heavy metals and demonstrate that the *Lemna minor* presents preeminent results by activating its defense mechanisms during the abiotic stress.

- Remarkable results were obtained at the quantitative analysis of **phytochelatins (PCs)**. It was observed that the phytochelatin synthase (PCS) activity and the PCs levels of the aquatic plants were in accordance with the phyto remediation capacity of the plant.
  - According to the literature, this is the first time when PCs with higher degree were identified from *Lemna minor*. During the phytoextraction experiments, the *Lemna minor* produced PCs with high degree of polymerization such as **PC<sub>4</sub>**, **PC<sub>6</sub>** and **PC<sub>7</sub>**.
  - The *Elodea canadensis* and *Salvinia natans* produced only two kinds of groups of PCs: **PC<sub>2</sub>** and **PC<sub>3</sub>**.

### **Phytoextraction of triphenylmethane dyes**

- The *Elodea canadensis*, *Salvinia natans* and *Lemna minor* phytoextraction efficiency and capacity were determined to remove **crystal violet (CV)** and **malachite green (MG)** **triphenylmethane dyes** from aqueous solutions.
- The **optimised phytoextraction parameters** were determined. The plant quantity, the initial concentration of the dyes, the initial pH and the temperature can affect the phyto remediation efficiency and capacity.
  - A beneficial effect on the removal efficiency was observed by increasing the **plant quantity**: the highest removal efficiency was achieved at 3 and 4 g plant (FW) quantities (using  $C_i = 100$  mg/L at *Lemna minor*;  $C_i = 50$  mg/L in the case of *Salvinia natans*;  $C_i = 30$  mg/L at *Elodea canadensis*). The obtained results demonstrate that the phyto remediation process mechanism was influenced by the active binding sites on the surfaces of the plants.
  - The highest removal capacity of the hydrophytes was attained at the highest **initial concentration** ( $C_i = 90$  mg/L using *Elodea canadensis*,  $C_i = 120$  mg/L using *Salvinia natans*, and at  $C_i = 300$  mg/L using *Lemna minor*.) It can be mentioned that the capacities of these aquatic plants are highly influenced by the active uptake facilities and the saturation of the plants.
  - The results clearly showed that the nature of the three aquatic plants was different. The highest removal efficiency and capacity were obtained in the case of *Lemna*

*minor*, which underlines that this hydrophyte is more suitable for the removal of the triphenylmethane dyes from aqueous solutions.

- The three hydrophytes were able to remove the triphenylmethane dyes at various range of **initial pH**. The plant had the facilities to reconstruct the medium pH to 7 during the experiments, which can be explained by several interactions such as H<sup>+</sup> buffering, H<sup>+</sup> consuming and producing in equilibrium reactions and transport processes during the experiments. The highest removal efficiency was achieved in the case of initial pH 7 for the removal of CV dye using the three hydrophytes, in case of initial pH 5 for the phytoextraction MG dye using *Lemna minor* and in case of initial pH 7 for the removal of MG dye using *Salvinia natans*.
- Analysing the phytoextraction efficiency of the aquatic plants at various temperatures, it was found that the highest removal efficiency of triphenylmethane dyes was performed at room temperatures (23 ±3°C) in all cases.

### **Spectral characterization of phytoaccumulation**

- ❖ The results obtained by **FTIR analysis** clearly indicate that the CV and MG dyes were bioaccumulated by the aquatic plants. In the FTIR spectra the appearance of new peaks was noticed, which can be attributed to the specific fingerprint peaks of the CV and MG dyes in the range of 2000 - 1000 cm<sup>-1</sup>.
- ❖ **UV-Vis** and FTIR analysis showed that the phytoaccumulated dyes presented in the plant are in an intact form, however **TLC analysis** showed the possibility of phytodegradation of the dye molecules (in the aqueous solutions).
- ❖ **UV-Vis spectroscopy and EDS** analysis was used to study the abiotic stress effect induced by the triphenylmethane dyes on the photosynthetic pigments and elemental composition of the plants. The major changes in elemental composition and in photosynthetic pigments during the phytoextraction experiments predict that the impact of stress on plants is highly negative. These results can be associated with the plant's removal capacity, with the metabolic deregulation and with the limited growth of the plants exposed to triphenylmethane dyes.

- ❖ In order to characterize the surface morphology of *Salvinia natans*, **scanning electron microscope studies** were conducted. The results showed that the surface of the hydrophyte suffered morphological changes during the phytoremediation experiments.

### **The isotherms and the kinetics models**

The equilibrium established during the phytoextraction process can be well characterized by the **isotherms and the kinetics data**.

- The obtained adsorption equilibrium data in the case of *Salvinia natans* and *Elodea canadensis* were found to fit successfully to both of the **Langmuir** and **Freundlich isotherms**, which defines that the removal of CV and MG dyes is carried out on monolayer as well as on multilayer surface adsorption. The **Dubinin-Radushkevich isotherm** model predicts that the phytoremediation process has chemisorption nature in the case of *Elodea canadensis* plant.
- The phytoextraction process was found to correspond to the **pseudo-second-order kinetics** for both triphenylmethane dyes using *Salvinia natans* and *Elodea canadensis*. The results of the kinetics studies confirm that the adsorption of CV and MG dyes takes places *via* surface exchange reactions, like chemisorptions.
- An **artificial neural network (ANN) model** was developed to predict the phytoremediation capacity and removal efficiency. The use of *Salvinia natans* in phytoremediation process of MG dye solution was successfully predicted by applying a three layered feed-forward back-propagation network with four neurons in hidden layer.

The obtained results offer an alternative method for the removal of heavy metals ( $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$ ) and triphenylmethane dyes (CV and MG) from aqueous solutions using several aquatic plants, which prove to be promising biofilters in future wastewater treatment applications. The induced abiotic stress effect was analysed through the determination of biochemical responses of the plants, which offers important information about the nature of the plant and of its capacities.

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