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Phytoremediation of heavy metals with cucumber, white poplar and aquatic plants

SUMMARY

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1 Introduction

Human activities have contaminated large areas irreversibly. Pollution of air, soil, and water with heavy metals is a major environmental problem. There is a need to search for alternative methods to solve the problem. Metals cannot be easily degraded and the cleanup usually requires their removal. The European Environment Agency has estimated that it would be between EUR 59 and 109 billion the total costs of the clean-up of contaminated sites. At present not even highly industrial countries can afford to clean up contaminated sites. In Germany, for instance, only 30% of the soils from contaminated areas are cleaned up in soil remediation facilities, while the rest is stored untreated in waste disposal facilities. With the increasing extent of extraction and processing of heavy metals from their ores, the subsequent soil contamination occurring especially in mining areas and nearby waste stockpiles affects the surrounding biota. As a consequence, heavy metal pollution may result in (non)occupational exposure of humans to heavy metals like cadmium, lead and nickel in toxic concentrations.

The widespread contamination of soils with heavy metals presents currently one of the most serious environmental concerns. Although a small portion of heavy metals in soils is derived from natural processes (e.g. bedrock weathering), a much higher amount originates from anthropogenic sources such as the mining and smelting industry, use of mineral fertilizers and pesticides, sewage sludge application and in the case of Pb, from the former use of leaded gasoline. Since none of the heavy metals is biodegradable, bioaccumulation from air, water, soil or animal feed is observed with latency periods that can last decades. The metal content of shoot tissues depends on the uptake and translocation ability of root and vascular tissues. The metal distribution inside the plant represents *per se* an indicator of the tolerance and/or accumulation mechanisms. Plants producing a high amount of biomass in a short time and accumulating heavy metals in their aerial parts are ideal candidates for phytoremediation. Therefore tree species, besides supplying an important biomass, are promising plant materials to be used as phytoremediators also because little dispersal from the accumulated heavy metal is likely to occur from wood.

Hydroponic culture is a very useful tool for selecting from a considerable number of plant specimens. It reduces not only the period of growth and treatment of the plants but also the space required carrying out the experiment. In general, data obtained by a hydroponic screening need to be confirmed by field performance trials, even though some results obtained in hydroponics and in field experiments are similar. Soil-grown specimens certainly reflect

real-world conditions much better than hydroponics. Nevertheless, in terms of metal accumulation and tolerance, the results may be soil-specific and therefore difficult to compare. Unfortunately, due to the high variability of parameters like type of hydroponics, heavy metal concentration, iron(III) source of the hydroponics, treatment time, etc., the few reports on poplar grown in nutrient solutions containing Cd, Pb or Zn cannot be really compared.

Aquatic macrophytes have great potential for the phytoremediation of water contaminated with heavy metals. They are taxonomically closely related to terrestrial plants, but are aquatic phanerogams, which live in a completely different environment. Their characteristics to accumulate metals make them an interesting research objects for testing and modeling ecological theories on evolution and plant succession, as well as on nutrient and metal cycling. They are easy to culture in laboratory and are thus a convenient plant material for ecotoxicological investigations. Elevated metal concentrations induce several defense strategies in plants. Chelation of metals/ metalloids is part of the first response in protection.

Trying to find responses for these questions the goals I formulated, for the experiments that I was going to do, are the following:

1. Ensure the iron supply with a thorough selection of complexing ligands in cucumber, as a model plant, to follow the phytoextraction and accumulation of heavy metals in typical Strategy I. type plants.
2. By using hydroponics determine the distribution of iron and heavy metals in the plants, to determine inhibitory effects and favored mediums for phytoextraction.
3. Using the same parameters as for the model plant determine the translocation factor for poplar in case of heavy metals.
4. Because phytoextraction efficiency is determined by the biomass/ heavy metal ratio determine the Poplars tolerance for heavy metals measuring and comparing the affected biomass with that in the control group.
5. For phytoextraction the mobility and accumulation of heavy metals is key that is why I need to determine the ability to take up and transport heavy metals toward the shoot.
6. Because the whole biomass of an aquatic plant is in the contaminated medium I need to determine the aquatic plants ability to accumulate heavy metals and its effects on the biomass development.
7. For the use of phytoremediation in practice I need to find species of interest for use in phytoremediating water.

2 Literature review

Over the centuries, human activities have contaminated large areas in both developed and developing countries. The European Environment Agency has estimated the total costs for the clean-up of contaminated sites in Europe to be between EUR 59 and 109 billion (1). At present not even highly industrial countries can afford to clean up contaminated sites. In Germany, for instance, only 30% of the soils from contaminated areas are cleaned up in soil remediation facilities, while the rest is stored untreated in waste disposal facilities. Heavy metals are a major factor of this pollution due to their atmospheric deposition, (2) (3) (4) (5), their leaching tendency, and the fact that they are undegradable. Plants have a natural propensity to take up metals. Some metals, such as Cu, Co, Fe, Mo, Mn, Ni, and Zn, are essential mineral nutrients. Others, however, such as Cd and Pb, have no known physiological activity (6). On the one hand, this propensity is a drawback for human health when contamination of food crops is too high. It does, on the other hand, serve as a tool for phytoremediation.

Phytoremediation, the use of plants to remediate contaminated soil, is an emerging technology (7) (8) (9) (10) that will require greater understanding of the underlying mechanisms for its optimisation. One clear distinction in the use of this technology relates to whether inorganic or organic compounds are the primary targets of remediation, although mixed pollution situations do of course exist. Phytoremediation of inorganic substances has increasingly split into various subtypes, the main division being between phytostabilization and phytoextraction.

3 Experimental part

3.1 Methods for plant growth and analysis

3.1.1 The use of hydroponics as a method for plant growth

In static solution culture, plants are grown in containers of nutrient solution, such as glass Mason jars (typically, in-home applications), plastic buckets, tubs, or tanks. The solution is usually gently aerated but may be unaerated. If unaerated, the solution level is kept low enough that enough roots are above the solution so they get adequate oxygen. A hole is cut in the lid of the reservoir for each plant. There can be one too many plants per reservoir.

Reservoir size can be increased as plant size increases. A homemade system can be constructed from plastic food containers or glass canning jars with aeration provided by an aquarium pump, aquarium airline tubing and aquarium valves. Clear containers are covered with aluminium foil, butcher paper, black plastic, or other material to exclude light, thus helping to eliminate the formation of algae. The nutrient solution is changed either on a schedule, such as once per week, or when the concentration drops below a certain level as determined with an electrical conductivity meter. Whenever the solution is depleted below a certain level, either water or fresh nutrient solution is added, A Mariotte's bottle, or a float valve, can be used to automatically maintain the solution level. In raft solution culture, plants are placed in a sheet of buoyant plastic that is floated on the surface of the nutrient solution. That way, the solution level never drops below the roots. (11)

Plant nutrients used in hydroponics are dissolved in the water and are mostly in inorganic and ionic form. (12) (13) Numerous 'recipes' for hydroponic solutions are available. Many use different combinations of chemicals to reach similar total final compositions. Commonly used chemicals for the macronutrients include potassium nitrate, calcium nitrate, potassium phosphate, and magnesium sulfate. Various micronutrients are typically added to hydroponic solutions to supply essential elements; among them are Fe, Mn, Cu, Zn, B, Cl, and Ni. Chelating agents are sometimes used to keep Fe soluble. Many variations of the nutrient solutions used by Arnon and Hoagland have been styled 'modified Hoagland solutions' and are widely used. (14) Scientists are researching how different amounts of light, temperature and carbon dioxide, along with plant species can be grown and cultivated on planets like Mars. (15)

Some of the reasons why we chose hydroponics are the following:

- No soil is needed, which eliminates factors that we cannot regulate or duplicate
- The water stays in the system for further measurements
- It is possible to control the nutrition levels
- No risk of pollution because of the controlled system
- Stable and high yields
- Pests and diseases are easier to get rid of than in soil because of the container's mobility

3.1.2 Analytical instrumentation

The digestion of the dried plant material was performed in a pressure-controlled microwave digestion system (MDS-2100 CEM Corporation, Matthews, USA). The analyses of the digested samples were carried out by an EXTRA IIA total-reflection X-ray fluorescence spectrometer manufactured by Atomika Instruments GmbH (Oberschleissheim, Germany).

3.2 The study of the accumulation and distribution of iron and heavy metals in cucumber plants

3.2.1 Materials and Methods for cucumber growth in hydroponics

Cucumber seeds (*Cucumis sativus* L. cv. joker) were germinated in Petri dishes on wet filter paper for one day in darkness at 26 °C. The seeds with radicles were planted on plastic net placed between polystyrene disks which were put into PVC cups each containing 200 ml 0.5 mM CaSO₄ solution for an additional day in order to achieve a slightly faster radicle elongation under isoosmotic conditions ensured by the salt solution. Then they were covered with wet filter paper to keep the plants in a dark, moisture-filled atmosphere at 26 °C for one more day. After this step, the plants were transferred into modified Hoagland nutrient solutions with the following chemical composition: 1.25 mM KNO₃, 1.25 mM Ca(NO₃)₂, 0.5 mM MgSO₄, 0.25 mM KH₂PO₄, 11.6 μM H₃BO₃, 4.5 μM MnCl₂ *4H₂O, 10 μM Fe(III) citrate or Fe(III) EDTA, 0.19 μM ZnSO₄ *7H₂O, 0.12 μM Na₂MoO₄ *2H₂O, 0.08 μM CuSO₄ *5H₂O. The nutrient solutions were replaced three times per week. For each change, they were freshly prepared from stock solutions. (16) (17)



Picture 1 Cucumber plant in Hydroponics

The plants were grown in a controlled environment at 22–26 °C and illuminated with metal halogen lamps and fluorescent tubes with light intensity of 75 W/m² and 14/10 hours light/dark photoperiod.

At the development of the second leaf, 3 plants (1 plant per pot) were transferred separately into each heavy metal contaminated nutrient solution containing Pb(NO₃)₂, NiSO₄, or Cd(NO₃)₂ in a concentration of 10 µM and iron was added as Fe(III) citrate or Fe(III) EDTA in the same concentration. Fe(III) citrate and Fe(III) EDTA, both having an Fe : ligand molar ratio 1:1, were purchased from Merck. The control plants (3 for each iron supply) remained in the „heavy metal free“ solutions. The Cd, Ni and Pb content of the control nutrient solutions were checked by graphite furnace atomic absorption spectrometric measurements. The plants were harvested after a growth period of 32 days. Then, the plants bearing 5-10 leaves were decapitated 5 mm above the root collar; the roots were rolled into filter paper and spin-dried for 1 minute in order to remove the remnants of the nutrient solution. The leaves were detached from the stems by using a silicon knife. The plants were divided into eight groups as follows: control, lead-, nickel- and cadmium-contaminated groups supplied either with Fe(III)citrate or Fe(III)EDTA. The leaves belonging to the same leaf-storey as well as cotyledons were stored together in filter paper prior to their subsequent sample preparation. Root and leaf samples were dried in an oven at 80 °C until constant weight was achieved.

3.2.2 Sampling and sample preparation of plant material

Both the dried leaf and root samples were dissolved using a microwave-assisted digestion procedure. The mass of the leaves was approximately 40 - 50 mg, meanwhile that of the roots samples was 0.4 - 0.8 g. If the mass of the root samples exceeded these values, the sample was divided into two parts and subjected to microwave-assisted digestion separately. Depending on the sample mass, 3 or 8 mL concentrated nitric acid of Suprapur® grade, supplied by Merck, were added to the samples. In both cases, eight vessels were subjected to microwave-assisted digestion simultaneously. The nominal power value of the microwave-assisted digestion system was 950 W ± 50W. Samples were digested by applying a three-step digestion procedure as follows: 1. Power: 25%, pressure: 20 psi, time: 3 min; 2. Power: 30%, pressure: 80 psi, time: 10 min; 3. Power: 30% for leaf, stems and cotyledons and 50% for root, pressure: 100 psi, time: 20 min. After digestion, the solutions resulted were filled up with deionized water to 5 or 25 mL, depending on the sample mass digested.

For the elemental analysis of the digested cotyledon, leaf, stem and root samples, each sample was spiked with a standard solution of Ga, used as an internal standard, to give a final

concentration of 1 mg L⁻¹. From these spiked and mixed solutions, 25 µL were dropped onto a quartz carrier plate and dried on a ceramic-coated hot plate (Cole Palmer, Chicago, IL, USA) at 80 °C for 30 min in a clean box. From each solution, three parallel samples were prepared and analysed. BCR 414, trace elements in plankton was used for validation of results with recoveries ranging between 84% and 105%. Alternatively, representative samples were analyzed by graphite furnace atomic absorption spectrometry (GF-AAS) which was also used for the determination of heavy metal blank values of the control nutrient solutions.

Plants	Dry weight (g) ±SD	Fe (umol)±SD		Heavy metals(umol)±SD
		Total	Shoot	Total
Control	2.54 ± 0.15 a	31.4±1.8a	3.7±0.4a	–
Cd-treated	0.44± 0.07 b	27.2±1.8b	0.2±0.03b	Cd:5.5±0.3
Ni-treated	2.25±0.01 c	27.1±1.4b	2.1±0.1c	Ni:17.4±1.1
Pb-treated	2.24±0.08 c	28.2±1.1b	2.9±0.2d	Pb:38.9±3.4

Table 1 Dry weight, total and shoot Fe and heavy metal content of 32 day-old cucumber plants (*Fe(III) citrate*)

Grown in nutrient solution containing 0 or 10 µM Cd(II), Ni(II) and Pb(II), respectively, and Fe(III) citrate as the iron supply. (See also Section 2.1; SD, data standard deviation; n = 3.) The values indicated by different letters in a column are significantly different at P < 0.05 (ANOVA with Tukey–Kramer post test).

Plants	Dry weight (g) ±SD	Fe (umol)±SD		Heavy metals(umol)±SD
		Total	Shoot	Total
Control	2.74 ± 0.13 a	7.4 ± 0.5 a	3.7 ± 0.3 a	–
Cd-treated	0.57 ± 0.14 b	5.3 ± 0.7 b	0.4 ± 0.06 b	Cd: 6.2±0.5
Ni-treated	2.12 ± 0.15 c	14.9 ± 1.8 c	3.0 ± 0.2 c	Ni: 14.4±0.7
Pb-treated	2.78 ± 0.14 a	12.6 ± 0.7 c	3.8 ± 0.4 a	Pb: 18.7 ±1.2

Table 2 Dry weight, total and shoot Fe and heavy metal content of 32 day-old cucumber plants (*Fe(III) EDTA*)

Grown in nutrient solution containing 0 or 10 μM Cd(II), Ni(II) and Pb(II), respectively, and Fe(III) EDTA as the iron supply. (See also Section 2.1; SD, data standard deviation; n = 3.) The values indicated by different letters in a column are significantly different at $P < 0.05$ (ANOVA with Tukey–Kramer post test).

3.2.3 Results and Discussion

Cucumber plants grown in nutrient solutions with 10 μM Pb(II) or Ni(II) ions developed similar number of leaves and root mass as the control plants independently of the applied Fe(III) supply (Table 1 and 2). In the case of the Cd(II)-treated plants, a decrease of about 80% in the dry weight was observed independently of the iron(III) form used in the nutrient solutions indicating a severe growth inhibition by Cd in these plants. The decrease in the weight of the plants was accompanied with severe leaf chlorosis and necrosis in the leaf edges. Moreover, in the case of Cd(II)-treated plants, only 5-6 leaves developed during the total growth period. When Fe(III) citrate was used as iron source of the hydroponics, the lead and nickel contamination decreased only by about 12% the dry weight of the plants compared to the control plants. Nickel contamination in hydroponics having Fe(III) EDTA as iron form decreased about 23% the total plant weight, meanwhile when lead was added to the hydroponics, practically, the plant weight was identical to that of the control plants.

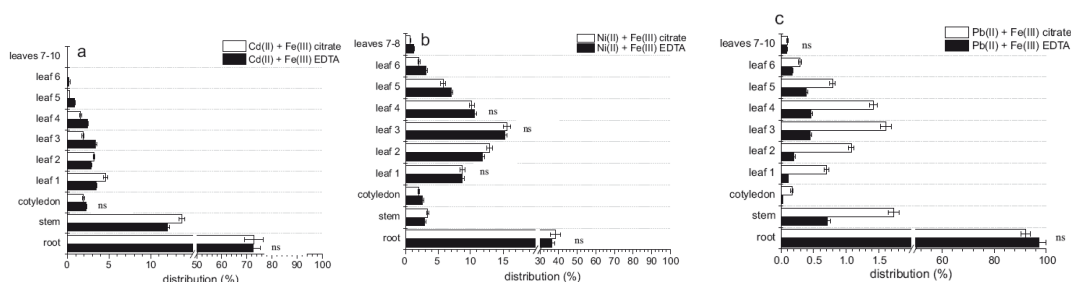


Figure 1. Cadmium Nickel and Lead distribution in 32 day-old cucumber plants Grown in nutrient solution containing 10 μM Cd(II) and Fe(III) citrate or Fe(III) EDTA as iron supply Differences between the Fe(III) EDTA and Fe(III) citrate treatment were analyzed by t-test. All data pairs are significantly different at $P < 0.05$ except for those indicated with “ns” for non significant.

The distribution pattern of the heavy metals investigated within the different plant compartments (root, stem, cotyledon, leaves) in the case of Fe(III) citrate and Fe(III) EDTA as iron sources was very similar as it can be seen in Figures 8-10. Independently of the iron(III) supply of the hydroponics, the extent of heavy metal accumulation varied as follows: Cd < Ni < Pb. The mobility of these heavy metal ions within the plants from the root towards the shoots was the following: Pb < Cd < Ni. In the case of both iron(III) supplies, the majority

of Pb accumulated in the root system; a moderate accumulation of Cd was observed in the stem of the plants, while Ni could be determined in significant amounts in the leaves.

Iron accumulation was higher when Fe(III) citrate was used as iron form in the hydroponics than when the very stable Fe(III) EDTA was applied in all treated plants and even for the control plants. When Ni(II) and Pb(II) ions, which also form stable complexes with EDTA, were added to the nutrient solutions containing Fe(III) EDTA in identical concentration, the Fe accumulation was roughly doubled compared to that of the control plants. In spite of the increased uptake, it should be emphasized that most of iron remained in the roots (Figure 11-14). However, except for Cd contamination, the amounts of transported Fe were similar independently of the Fe(III) chelate used for the hydroponic experiments (Table 1 and 2). The pH of the freshly prepared nutrient solutions (measured with a Radelkis OP equipment) were acidic: that of Fe(III) citrate containing hydroponics ranged between 4.7 and 4.8 independently of the heavy metal ion treatment, meanwhile that of the Fe(III) EDTA was slightly less acidic as the pH range varied between 4.8 and 5.1. The pH of the nutrients solution after two days increased with about 0.5 units.

Competition of 10 μM Cd(II), Ni(II) and Pb(II) with 10 μM Fe(II) or Fe(III) for the complexing agents could be simulated (Figure 15-18). The speciation study was performed by taking into consideration the pH and chemical composition of the nutrient solutions in addition to EDTA and citrate complexes. Formation of mixed hydroxo complexes for Fe(III) was also considered. The complex stability as well as acid protonation constants were taken from Martell AE and Smith RM (18) (19). The results obtained for the iron accumulation as a function of the applied Fe(III) complex compound (Fe(III) EDTA vs Fe(III) citrate) suggest that the iron accumulation in the test plants is determined by the stability of complexes. This phenomenon is in very good agreement with the recent results of Lucena and Chaney (2006) who demonstrated, by ferric chelate reductase activity and Fe concentration measurements, that both reduction of Fe(III) to Fe(II) and Fe concentration in the xylem sap in green-stressed cucumber plants were higher for the less-stable Fe chelates, except for Fe(III) EDTA. According to the speciation studies, Fe(III) EDTA is the predominant complex at the pH of the nutrient solutions and formation of other EDTA complexes is unlikely (Figure 15-18). As Fe(III) is reduced to Fe(II) on the root cell plasma membranes of plants that is also accompanied by acidification, competition between Fe(II) and the heavy metal ions employed in this study for the chelating agents was also investigated. In the lack of the quantifiable extent of this reduction for the whole period, the extreme case of the complete reduction was used for this type of complex species simulation. If Fe(II) was the predominant iron source in

the nutrient solution, due its lower stability constant with EDTA by about 10 orders of magnitude compared to that of Fe(III) EDTA, formation of Cd(II) EDTA, Ni(II) EDTA and Pb(II) EDTA would be more likely. Since, in previous studies conducted by our group, EDTA could not be detected by HPLC (20), a possible explanation for the lower amounts of Pb and Ni taken up when the iron chelate employed was Fe(III) EDTA compared to the amounts of metals taken up when Fe(III) citrate was used can be supported by the results of these simulations. In the case of citrate as ligand, except for Fe(III), there are no significant differences between the complex stability values of the heavy metals (M) investigated with citrate in the form of ML, MHL, ML₂, MLOH⁻ or M₂L₂(OH)₂²⁻ complexes. Thus, the formation of any citrate complex with the heavy metals investigated - cannot be expected at the acidic pH of the nutrient solutions. This finding may support the results obtained for the heavy metal uptake which is higher for Ni and Pb when Fe(III) citrate was the iron source of the nutrient solutions. A clear relationship between the complex species simulation and heavy metal uptake could not be established for Cd, because the amounts of Cd taken up were similar, independently of the Fe(III)chelating agent used, pointing to the fact that the extent of Fe(III) reduction is not uniform for each heavy metal contaminant.

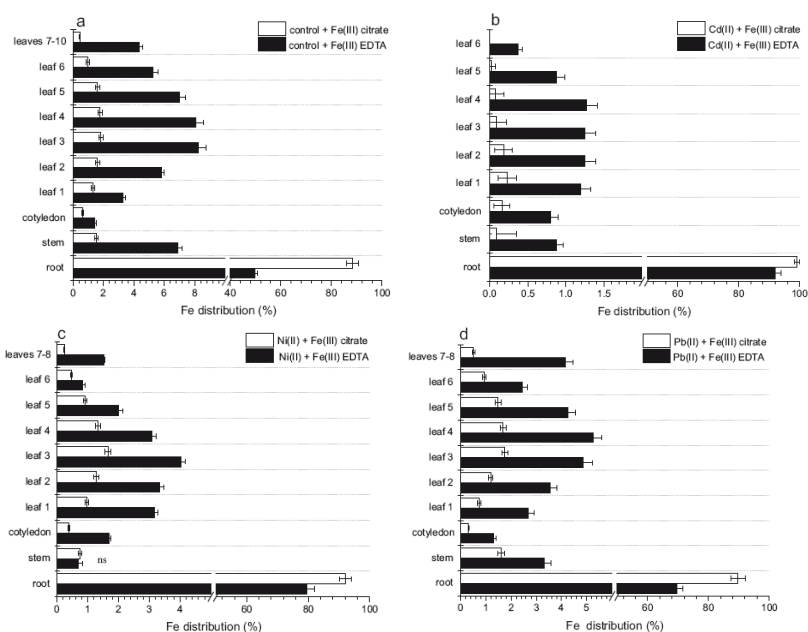


Figure 2 Iron distribution in 32 day-old control cucumber plants control and nickel lead cadmium treated Grown in nutrient solution containing Fe(III) citrate or Fe(III) EDTA as iron supply. Differences between the Fe(III) EDTA and Fe(III) citrate treatment were analyzed by t-test. All data pairs are significantly different at $P < 0.05$ except for those indicated with “ns” for non significant.

Our results suggest that citrate ions do not interfere with the uptake of essential or toxic elements compared to EDTA that forms very stable complexes with Fe(II) and Fe(III) as

well as with the heavy metals investigated. It also turned out that the total Fe content ($\mu\text{mol}/\text{plant}$) of plants supplied with Fe(III) citrate were similar in the control and heavymetal contaminated plants even in the case of the Cd(II)-contaminated plants (Table 1).

This is a very important outcome of the performed experiments, which, to our knowledge, until now has not been emphasized by any research group. It seems that, when a 1 : 1 mol ratio of divalent heavy metal ion and Fe(III) (which is reduced to Fe(II) prior to uptake) supply is ensured in the hydroponics, there is no interference or any competition in the Fe accumulation – expressed as μmoles - even in the case of Cd(II)-contaminated plants where the growth was severely affected by cadmium contamination. Generally, regarding iron transport, it could be observed that even if higher amounts of iron had been taken up, similar amounts of iron, expressed as micromoles, were transported from the root towards the shoot except for Cd. This is an evidence for the fact that iron uptake, storage and transport are carefully regulated processes in cucumber. However, the distribution of Fe depends on physiological factors, too. With this respect, the effect of the applied heavy metals should be taken into account. None of them is known to affect the root iron chelate reductase in Fe sufficient Strategy I plants. The transporter proteins responsible for FeII uptake in these plants may carry Cd (21) but not Pb and Ni. These previous findings can further explain that in the case of Fe-EDTA supply, the total amount of Fe accumulated by the plants can be significantly different while in case of Fe-citrate the much higher Fe content due to poor chelation may obscure the differences (Table 1 and 2). After the FeII is released at *FRO2* it may compete with Cd for uptake explaining the smaller accumulation of Fe in these plants (Table 2). Nickel is a micronutrient with a transport system different from that of Fe, although its uptake mechanism has not been described, yet (22). Lead is not essential and is unlikely to compete for transporters due to its size but both Pb and Ni form more stable complex with EDTA than FeII which may enhance FeII uptake or immobilization in the apoplast leading to the formation of iron plaque after reoxidation. This latter has not been demonstrated in our previous studies with Mössbauer spectroscopy (23). The precipitation of FeIII as hydroxides in the root apoplast may be favoured in iron deficient plants with high iron reducing rates leading to the accumulation and reoxidation of FeII at increasing pH values. Furthermore, the interaction with Fe translocation was shown only for Cd as the inhibition of *FRD3*, the citrate effluxer to xylem (24). This is the reason of the low Fe content in the Cd-treated plants supplied either with Fe-EDTA or Fe-citrate (Tables 2,3; Figures 8-14). The order established for the accumulation of the heavy metals investigated is in accordance with our previous results (25). Regarding the lead mobility within the plants, though its retention in iron

precipitates cannot be excluded, it could also be accumulated in the root cell walls in form of lead pyro- or orthophosphate (14) or depending on the C.E.C. may have replaced other divalent metals forming carboxylates in case of the Fe citrate supply (26). The results concerning the mobility of Ni was in good agreement with our previous results when the high nickel transporting potential of citric acid in the xylem sap of nickel-contaminated cucumber plants was demonstrated by two independent hyphenated techniques (27) (28). Again, citrate ions proved to exert less influence on the uptake of nickel and lead ions, which is in accordance with the fact the EDTA forms stable complexes with these ions, meanwhile citrate ions, not.

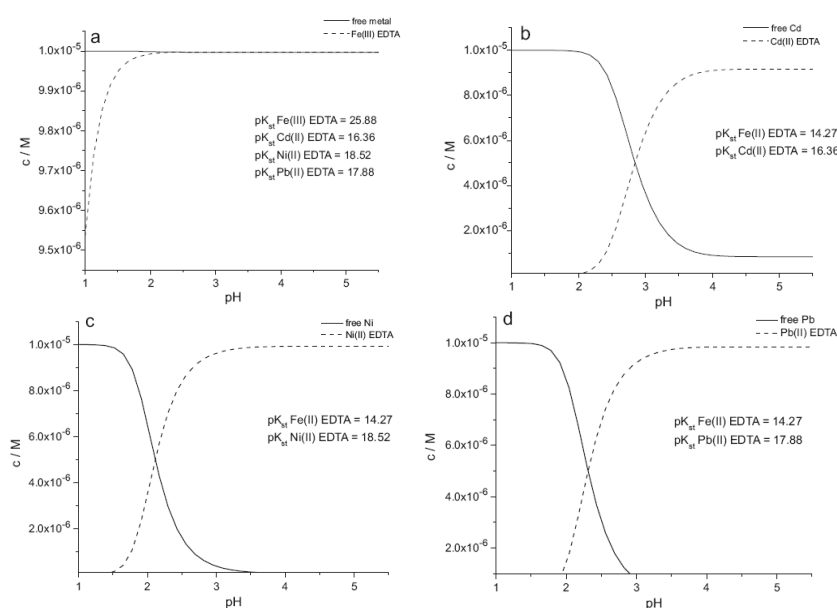


Figure 3 *Speciation curves for Fe(III) EDTA and Fe(II) EDTA and metal ions (Cd, Ni or Pb) The investigated heavy metal ions in an acidic pH range did not exceed that of the nutrient solutions.*

The discussion based on the relationship of critical stability constants for complex compounds and metal ion uptake cannot be strictly applied for cadmium, as it is well-known that higher plants grown on Cd-containing substrates show disturbed water balance. Adverse effects of Cd on stomatal function, water transport and cell wall elasticity have been reported, among others, by Poschenrieder et al. (29).

3.2.4 Conclusion and Original Contribution

Our results have some important implications in the understanding of iron uptake and distribution within cucumber plants under heavy metal stress. For example, a higher iron supply can be ensured with a thorough selection of complexing ligands – based mainly, but not exclusively, on the stability of complexes formed during the iron uptake – when the

growing conditions, e.g. heavy metal contamination, may limit iron uptake. This could be achieved in the case of cucumber plants grown in cadmium-, lead- and nickel-containing nutrient solutions supplied with iron(III) citrate in identical concentration with that of the heavy metals. We suggest that the accumulation of iron is determined mainly by complexation/chelation processes in the root/medium interface, while its distribution is affected by the internal competition with heavy metals and their inhibitory effects on the transport systems involved. However, as the Fe transport is highly regulated, yet affected by various heavy metals, synergistic or antagonistic effects caused by binary or ternary mixtures of these heavy metals should be investigated further.

3.3 Study of iron, cadmium, lead and nickel accumulation in poplar grown in hydroponics

Poplar (*Populus jacquemontiana* var. *glauca* cv. Kopeczkii) was grown in hydroponics containing 10 μ M Cd(II), Ni(II) or Pb(II), and iron as Fe(III) EDTA or Fe(III) citrate in identical concentrations. The present study was designed to compare the accumulation and distribution of Fe, Cd, Ni and Pb within the different plant compartments. Generally, iron and heavy-metal accumulation were higher by factor 2–7 and 1.6–3.3, respectively, when Fe(III) citrate was used. Iron transport towards the shoot depended on the Fe(III) chelate and, generally, on the heavy metal used. Lead was practically accumulated only in the root. The amounts of Fe and heavy metals accumulated by poplar were very similar to those of cucumber grown in an identical way indicating a strong Fe uptake regulation of these two Strategy I plants: a cultivar and a woody one. The Strategy I Fe uptake mechanism together with the Fe(III) chelate form in the nutrient solution had significant implications in the iron and heavy metal uptake. Poplar seems to have a phytoremediation potential for Cd and Ni as their transport towards the shoot was characterized by 51–54% and 26%–48% depending on the iron(III) supply in the nutrient solution.

Hydroponic culture is a very useful tool for selecting from a considerable number of plant specimens. It reduces not only the period of growth and treatment of the plants but also the space required carrying out the experiment. In general, data obtained by a hydroponic screening need to be confirmed by field performance trials, even though Watson et al. (30) have pointed out that results obtained in hydroponics and in field experiments are similar. Soil-grown specimens certainly reflect real-world conditions much better than hydroponics.

Nevertheless, in terms of metal accumulation and tolerance, the results may be soil-specific and therefore difficult to compare (31). Unfortunately, due to the high variability of parameters like type of hydroponics, heavy metal concentration, iron(III) source of the hydroponics, treatment time, etc., the few reports on poplar grown in nutrient solutions containing Cd, Pb or Zn cannot be really compared.

Up to our knowledge, the uptake and accumulation capability of Ni by poplar grown in hydroponics supplied with different iron(III) chelators has not been explored yet. Moreover, the choice of iron(III) source in order to ensure adequate iron supply to the test plants is generally overlooked. Therefore, the present study aimed at investigating Cd, Fe, Ni and Pb accumulation and distribution in poplar plants grown in hydroponics supplied with two different iron(III) chelators: Fe(III) citrate and Fe(III) EDTA by a fast and easy-handling multielemental technique: total-reflection X-ray fluorescence spectrometry suitable for analyses of plant materials (32).

3.3.1 Materials and Methods

Plant growth experiments using hydroponics (33) were conducted on poplar, *Populus jacquemontiana* var. *glauca* (Haines) Kimura, 1982, cv. Kopeczkii. Briefly, micropropagated plants were transferred to nutrient solution and were further grown in chambers equipped with metal halide lamps and fluorescent tubes ensuring controlled environment of 14/10 h light ($120 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) / dark photoperiods, 24/20 °C and 70/75% relative humidity. The plants were growing in modified, quarter strength nutrient solution (basic solution; Csog et al., (33)) containing either 10 μM of Fe(III) citrate or 10 μM Fe(III) EDTA as iron source. Under these conditions, they developed several minor leaves (called later on throughout the text old leaves). At the appearance of the fourth normal-sized leaf, plants were marked. Then, groups consisting each of 5 plants were further grown in basic nutrient solution containing 10 μM of $\text{Cd}(\text{NO}_3)_2$, $\text{Ni}(\text{NO}_3)_2$ or $\text{Pb}(\text{NO}_3)_2$ and either Fe(III) citrate or Fe(III) EDTA as iron supply for additional two weeks (controls without heavy metal treatment). Nutrient solutions were replaced three times a week. The heavy metal content of the basic solution was checked by graphite-furnace atomic absorption spectrometry, and it proved to be free of any heavy metal contamination at the $\text{ng} \cdot \text{cm}^{-3}$ concentration level. Leaves below and above the mark were assigned with negative and positive Arabic numbers starting from the mark. When data for different leaf storeys are not given separately, it is referred to lower or upper leaves showing the mean of the data obtained either from leaves marked with plus for plants exposed

to heavy metal treatment or with minus for leaves grown before the treatment was started. Experiments were repeated twice.

3.3.2 Sampling and sample preparation of plant material

The plants were harvested and separated into root, stem, old leaves, leaves and tip with the aid of a silicon knife. The roots were washed with a 0.05 M CaSO₄ solution in order to remove the remnants of the nutrient solution. According to the type of heavy metal or iron(III) treatment, the harvested plants were divided into eight groups.

The leaves belonging to the same leaf-storey were stored together in filter paper prior to their subsequent sample preparation. Root and leaf samples were dried in an oven at 80 °C until constant weight was achieved. The microwave-assisted digestion of the dried plant material as well the subsequent elemental determination was achieved according to our previous work (Csog et al., (33)). Unpaired t-tests and ANOVA were performed by Microsoft Office Excel 2007 and InStat v. 3.00 (GraphPad Software Inc.). The term ‘significantly different’ means that the similarity of samples were less than $P=0.05$.

3.3.3 Results and Discussion

Independently of the Fe(III) supply, micropropagated poplars bearing 4 or 5 lower leaves developed about 6 upper leaves during the growth period while exposed to heavy metal (Cd(II), Pb(II) or Ni(II)) treatments and two different iron(III) supplies in 1 : 1 molar ratio. Only, in the case of Fe(III) EDTA treatment, poplars grown in control and Pb(II) containing nutrient solution developed 7 upper leaves but significant biomass stimulation or decrease upon heavy metal treatment was not observed.

The dry weight of the whole plants grown in Fe(III) citrate were slightly higher (by 10–30%) than that of plants grown in Fe(III) EDTA containing nutrient solution (Table 4). Compared with the dry weight of the control plants, 6–30% decrease was observed in the total mass of the plants exposed to Ni(II) and Pb(II), independently of the iron(III) supply. The Cd(II) treatment resulted in a roughly 30% decrease in the dry weight of the whole plants in the case of both Fe(III) treatments.

Treatment	DW (g) ± SD	Fe (µmol/g DW) ± SD				Heavy metal (µmol/DW) ± SD			
		Total		Shoot		Total		Shoot	
Fe(III) citrate as iron source									
	poplar*	cucumber**	poplar*	poplar*		cucumber**	poplar*	poplar*	
control	7.0 ± 1.2 a	12.0 ± 0.7 a	12.4 ± 1.2 a	0.7 ± 0.1 b					
+Cd(II)	5.0 ± 1.0 a	61.5 ± 4.1 b	30.4 ± 6.2 c	0.4 ± 0.1 a	Cd	12.4 ± 0.7	10.9 ± 1.1	Cd: 5.9 ± 0.5	
+Ni(II)	6.6 ± 0.9 a	12.1 ± 0.6 a	11.9 ± 1.1 a	2.5 ± 0.2 d	Ni	7.7 ± 0.5	6.9 ± 0.8	Ni: 1.8 ± 0.2	
+Pb(II)	6.5 ± 1.1 a	12.6 ± 0.5 a	18.6 ± 1.7 b	1.2 ± 0.1 c	Pb	17.4 ± 1.5	15.7 ± 1.3	Pb: 0.2 ± 0.04	
Fe(III) EDTA as iron source									
	poplar *	cucumber**	poplar*	poplar*		cucumber**	poplar*	poplar*	
control	6.3 ± 0.7 b	2.7 ± 0.2 a	2.5 ± 0.2 a	1.0 ± 0.1 b					
+Cd(II)	4.2 ± 0.9 a	9.3 ± 1.2 d	4.1 ± 0.6 b	0.5 ± 0.07 a	Cd	10.9 ± 0.9	6.8 ± 0.8	Cd: 3.5 ± 0.3	
+Ni(II)	5.3 ± 0.4 a	7.0 ± 0.8 c	5.2 ± 0.4 c	1.9 ± 0.2 c	Ni	6.8 ± 0.3	3.3 ± 0.2	Ni: 1.6 ± 0.2	
+Pb(II)	5.0 ± 0.5 a	4.6 ± 0.3 b	4.3 ± 0.5 b	1.2 ± 0.1 b	Pb	6.7 ± 0.4	4.7 ± 0.5	Pb: 0.05 ± 0.01	

Table 3 Dry weight, total and shoot Fe and heavy metal content of poplar plants grown in control or 10 µM of Cd(II), Ni(II) and Pb(II), Contained nutrient solutions, respectively, and Fe(III) citrate or Fe(III) EDTA as the iron supply compared to some previous data on cucumber cf. Ref Csog et al (2011). DW = dry weight of plant material SD = data standard deviation; n=3. The values indicated by different letters in a column are significantly different at P<0.05 (ANOVA with Tukey-Kramer post test)

*present study

**according to our previous report cf. ref. Csog et al. (2011)

Iron uptake was not hampered by the heavy metal treatment. In the case of Ni(II)-treated poplar plants having Fe(III) EDTA as iron source, the iron uptake was even double than that of the control plants. However, iron uptake was higher by factor 2–7 in the case of poplar plants having Fe(III) citrate as iron supply compared to those having Fe(III) EDTA. Although iron uptake was higher when the nutrient solution contained Fe(III) citrate, except for Ni, the iron concentration of the shoot was higher in the case of heavy metal and Fe(III) EDTA treatments. Thus, the enhancement of Fe transport in the presence of Ni(II) was about 3.5 times higher compared to the transport in control poplar grown with Fe(III) citrate. Lead did not affect the Fe transport in this iron(III) chelate group of plants. Cadmium decreased the Fe transport independently of the Fe(III) form used in the nutrient solution. In the case of Fe(III) EDTA, Ni(II) did not alter the iron(III) transport and Pb(II) slightly decreased it. The iron

distribution within poplar plants as a function of the Fe(III) chelate of the nutrient solutions can be seen in

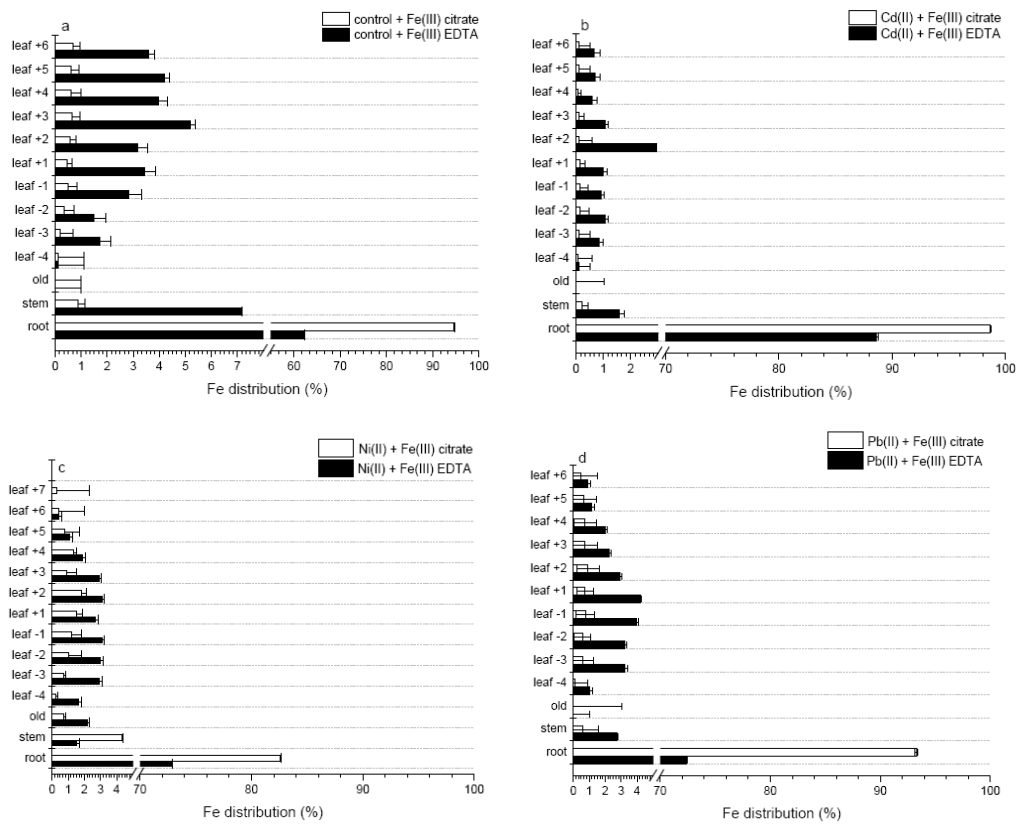


Figure 4 Iron distribution in control poplar grown containing nutrient solutions,

The distribution pattern of the heavy metals investigated within the different plant compartments (root, stem, old leaves, lower leaves, upper leaves and tip) in the case of Fe(III) citrate and Fe(III) EDTA as iron sources can be seen in Figure 23-25.

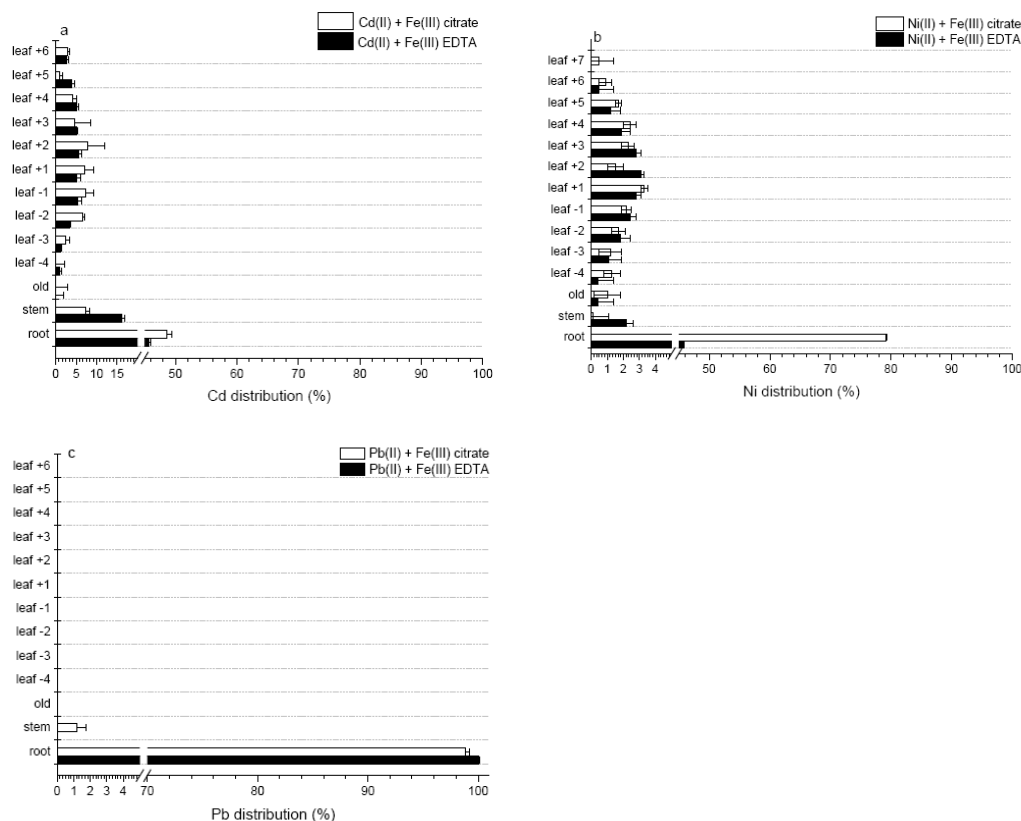


Figure 5 heavy metal distribution in poplar grown in nutrient solutions containing 10 μ M Cd(II) Fe(III) citrate or Fe(III) EDTA as iron supply. RSD = relative data standard deviation; n=3

The heavy metal accumulation was Ni < Cd < Pb for the Fe(III) citrate treatment and Ni < Pb < Cd for Fe(III) EDTA treatment if heavy metal amounts were expressed in micromoles / g dry weight of total plants. Depending on the type of heavy metal treatment, in the case of Fe(III) citrate as iron source, the heavy metal accumulation was higher by factor 1.6–3.3 compared to plants growing in nutrient solution containing the investigated heavy metals and Fe(III) EDTA. Concerning the heavy metal transport, it can be stated that Pb(II) was practically accumulated in the root independently of the Fe(III) source. Poplar treated with Ni(II) and Fe(III) EDTA showed about 50% transport toward the shoot. Cadmium proved to be the most mobile within the plants independently of the Fe(III) source of the nutrient solution, as 51– 54% could be determined in the shoot (Table x).

Similarity of the iron and heavy metal uptake for cucumber and poplar As poplar belongs to the strategy I group of plants in terms of iron uptake, reduction of iron(III) to iron(II) occurs prior to its uptake. The 1: 1 heavy metal and iron(III) molar ratio was set in order to exclude any competition between the divalent heavy metals and the iron for the

complex forming agent, citrate or EDTA. Moreover, special attention was paid to the Fe(III) : citrate ratio to be also 1:1 like in the case of Fe(III) EDTA in accordance to our previous work (Csog et al., (33)) conducted on cucumber plants grown in a similar way. As both plants belong to the strategy I according to iron uptake, it was expected to get higher iron and heavy metal uptake when the nutrient solution contains Fe(III) citrate and our results proved this hypothesis. However, we found that, except for Cd, the total amounts of iron determined in both plants were very similar especially if iron was supplied to the nutrient solution in form of Fe(III) citrate. Thus, the differences registered in the Fe uptake of these two plants ranged between 2% and 47%. These deviations for the Fe uptake of cucumber and poplar varied between 7% and 35% if Fe(III) EDTA was applied. A possible explanation for the anomaly observed in the case of Cd is that this element causes perturbation of root metal uptake and it interferes with the metal translocation to the shoot. In the case of cucumber, this phenomenon occurs at a higher extent than in the case of poplar manifested by the reduced root system (33), and consequently, total dry weight of the plants, which is not so pronounced for poplar plants. In the case of cucumber and poplar plants exposed to heavy metal treatment and Fe(III) citrate, the uptake of the investigated heavy metals correlates even more the above-calculated differences not exceeding 15%. For the heavy metal uptake of cucumber and poplar in the presence of EDTA, higher deviations were observed. This is understandable as EDTA is a strong chelating agent and it interferes more with the heavy metal uptake especially with the reduction of Fe(III) to Fe(II) accompanied by a dramatic decrease of 10 orders of magnitude in the stability constant of their respective chelate complexes. Consequently, Cd(II), Ni(II) or Pb(II) complexation with EDTA is more pronounced even in the nutrient solution rendering the heavy metal uptake unpredictable. Although cucumber and poplar belong to the same iron uptake strategy group, it is surprising that this Fe acquisition mechanism results in similar Fe, Cd, Ni and Pb uptake for a cultivar and a woody plant if plants were subjected to identical plant growth treatments. In the case of control cucumber and poplar plants, the iron uptake from Fe(III) citrate or Fe(III) EDTA was very similar.

Meanwhile the Fe distribution in poplar was similar to that observed in identically grown cucumber (Csog et. al, 2011), the heavy metal distribution within poplar was significantly different compared to cucumber. For example, Pb(II) had a low mobility within cucumber (33), but it was practically not detected in the shoot of poplar. This latter finding about the accumulation of Pb in the poplar root contradicts the report of Komárek et al. (34) that established a high translocation of Pb towards the shoot in the case of a hybrid poplar

species grown on Pb-contaminated mining and smelting sites in the presence of EDTA. However, in the cited work soils were amended with 3–6 mmol EDTA / kg soil. At this high complexing agent concentration, transport of Pb is expected in the form of chelate complex. The nickel distribution profile was very similar in poplar and cucumber plants (see also Csog et al., (33)), maybe this is related to the essentiality of this element.

3.3.4 Conclusion and Original Contribution

If a potential of a plant for phytoremediation purposes is closely connected with its root-to shoot translocation ability for a certain heavy metal, extraction of cadmium from contaminated sites cannot be excluded. The translocation factor obtained for poplar in the present study is far better than that calculated by Zacchini et al. (35). In the abovementioned work, translocation factor was 10% for poplar clone cuttings grown for 3 weeks in nutrient solution containing 50 μM of Cd(II) and Fe(II) and EDTA in 1 : 1 molar ratio at the 10 μM concentration level, meanwhile in the present work the Cd concentration was only 10 μM . Our results are much closer to that of Dos Santos Utmazian et al. (31) conducted on *Populus nigra* L clones grown also in nutrient solution with single Cd and Zn and a mixture of Cu, Cd, Pb and Zn solutions, where the Cd concentration was 4.45 μM in all cases. The results obtained by Dos Santos Utmazian et al (31) indicated that poplar is rather good for phytostabilisation than for phytoremediation purposes due to the high tolerance of the investigated plants to Cd associated with a low metal accumulation. This finding was recently confirmed by field experiments conducted on poplar over 6 years by Lettens et al. (36). However, the ability of poplar to take up and transport Cd toward the shoot in higher amounts should not be discarded for phytoremediation purposes. Although, in a recent work, Komárek et al. (37) concluded that a two-year-long phytoextraction process of Cd, Pb and Zn enhanced with NH_4Cl or EDTA in a real contaminated site resulted in a dramatic decrease in the biomass of the poplar species used, it is also emphasized in this work that the success of the phytoremediation depends also on the heavy metal concentration of the polluted soil. For example, Stobrawa and Lorenc-Plucinska (38) established a full tolerance of 30–70 $\mu\text{g} \cdot \text{g}^{-1}$ of Pb in soils for fine root cuttings of poplar (*Populus nigra* L.).

The outcomes of the present study seem to be more promising for phytoremediation of areas polluted with Ni that showed, in our experiments, a remarkable mobility within the plant regardless of the iron(III) chelate used in the nutrient solution. Maybe this is related to the fact that Ni is a micronutrient in low concentrations. Nevertheless, further pot and field

experiments should be conducted with increasing Ni and chelating agent dosage, etc. in order to prove the potential of poplar for phytoremediation of areas possibly affected by high Ni concentrations.

3.4 Heavy metal phytoaccumulation by aquatic plants (*Cabomba Aquatica*, *Vallisneria Spiralis*, *Echinodorus Cordifolius*)

Pollution of air, soil, and water with heavy metals is a major environmental problem. There is a need to search for alternative methods to solve the problem. Metals cannot be easily degraded and the cleanup usually requires their removal. Aquatic macrophytes are taxonomically closely related to terrestrial plants, but are aquatic phanerogams, which live in a completely different environment. These macrophytes are growing in or near water that are emergent, submerged or floating. They are beneficial to lakes because they produce oxygen, which helps in overall lake functioning, and provide food for some fish and other wildlife. They are unchangeable biological filters and play an important role in the maintenance of the aquatic ecosystem. (39) Aquatic macrophytes are considered as important components of the aquatic ecosystem because they act as an efficient accumulator of heavy metals. They have great potential for the phytoremediation of water contaminated with heavy metals. Their characteristics to accumulate metals make them an interesting research objects for testing and modeling ecological theories on evolution and plant succession, as well as on nutrient and metal cycling. The Fanwort (*Cabomba aquatica*), Tape Grass (*Valisneria spiralis*) and Marble Queen (*Echinodorus cordifolius*) are easy to culture in the laboratory and are thus a convenient plant material for ecotoxicological investigations.

3.4.1 Materials and Methods

Plant growth experiments using hydroponics were conducted on Fanwort (*Cabomba aquatica*), Tape grass (*Vallisneria spiralis*), and Marble Queen (*Echinodorus cordifolius*), grown plants were transferred to nutrient solution and were further grown in aquariums with dimension 21cm*38 cm *28 cm; equipped with fluorescent tubes ensuring controlled environment of 14/10 h light (1,5 W/liter of water, (3 T5 PHILIPS 15W)) / dark photoperiods; at a temperature of 24-28 °C. The plants were growing in modified Hoaglan nutrient solution containing either Fe(III) citrate or Fe(III) EDTA as iron source. The plants were left for 3 days for acclimatization. Then, groups were transferred in basic nutrient

solution containing 10 mg of Cd(NO₃)₂, ZnSO₄ × 7H₂O or CuSO₄ × 5H₂O and either Fe(III) citrate or Fe(III) EDTA as iron supply for 6 days, controls were left without heavy metal treatment. The heavy metal content of the basic solution was checked by atomic absorption spectrometry. Experiments were repeated twice.

The plants were harvested and separated. They were washed with distilled water in order to remove the remnants of the nutrient solution. According to the type of heavy metal or iron(III) treatment, the plants were divided into groups. Samples were dried in an oven at 80°C until constant weight was achieved.

3.4.2 Sampling and sample preparation of plant material

The dried plant samples were digested in 10 ml of concentrated nitric acid, supplied by Merck. The mass of the plants was approximately 400–1500 mg. If the mass of the plant samples exceeded these values, another 10 ml of concentrated nitric acid of Suprapur[®] grade was added. The digestion took place at 20 °C for 24h and at 100 °C for 8h. After digestion, the solutions were filled up to 100 ml with distilled water.

The analysis of the digested samples was carried out by an GBC SensAA Dual Atomic Adsorption Spectrophotometer with flame atomization system. Lamp used was a Photron P410 HCL-D2 at 307,6 nm and 213,9 nm for Zn; 326,1 nm and 228,8 nm for Cd; 244,2 nm and 249,2 nm for Cu.

3.4.3 Results and Discussion

A. Mono metal uptake

Copper uptake

The concentration of Cu in the water initially was at a concentration of 10.0 mg/l (10,56 mg/l). The duration of the experiment was 6 days; all plants have accumulated copper as can be seen in Table 5. The highest removal of Cu from the water was obtained from *Valisneria Spiralis* (Table 5).

Cabomba Aquatica						
	Zn		Cd		Cu	
	Control	Contaminated	Control	Contaminated	Control	Contaminated
Plant mass	1,538	1,925	1,538	1,818	1,538	0,839

Heavy metal (mg)	0,105	0,688	0,003	0,440	0,268	7,099
Valisneria Spiralis						
	Zn		Cd		Cu	
	Control	Contaminated	Control	Contaminated	Control	Contaminated
Plant mass	0,548	0,834	0,548	0,655	0,548	0,465
Heavy metal (mg)	0,068	1,967	0,006	2,287	0,315	13,232
Echinodorus Cordifolius						
	Zn		Cd		Cu	
	Control	Contaminated	Control	Contaminated	Control	Contaminated
Plant mass	1,092	1,442	1,092	3,207	1,092	2,138
Heavy metal (mg)	0,081	2,829	0,003	6,017	0,032	34,440

Table 4 Accumulated heavy metals in *Cabomba aquatica*, *Valisneria spiralis* and *Echinodorus cordifolius* after 6 days in modified Hoagland nutrient solution, at an ambient temperature of 24-28 °C and 14/10 light/dark period (1,5 W/l of water)

Cabomba Aquatica						
	Zn		Cd		Cu	
	Control	Contaminated	Control	Contaminated	Control	Contaminated
Plant mass	1,538	1,925	1,538	1,818	1,538	0,839
Heavy metal (mg/g)	0,068	0,357	0,020	0,242	0,174	8,461
Valisneria Spiralis						
	Zn		Cd		Cu	
	Control	Contaminated	Control	Contaminated	Control	Contaminated
Plant mass	0,548	0,834	0,548	0,655	0,548	0,465
Heavy metal (mg/g)	0,124	2,359	0,011	3,491	0,575	28,456
Echinodorus Cordifolius						
	Zn		Cd		Cu	
	Control	Contaminated	Control	Contaminated	Control	Contaminated
Plant mass	1,092	1,442	1,092	3,207	1,092	2,138
Heavy metal (mg/g)	0,074	1,962	0,003	1,876	0,029	16,109

Table 5 Calculated heavy metal/ dry weight plant biomass (*Cabomba aquatica*, *Valisneria spiralis* and *Echinodorus cordifolius*) after 6 days in modified Hoagland nutrient solution, at an ambient temperature of 24-28 °C and 14/10 light/dark period (1,5 W/l of water)

The concentration of copper was 28,456 mg/g dry wt. biomass after 6 days of treatment, in *Valisneria Spiralis* from water containing 10 mg Cu/l. This shows that *Cabomba Aquatica* and *Echinodorus Cordifolius* is less effective accumulator of Cu with 8,461mg/g and 16,109 mg/g. However these values that we measured in the plants grown with the above mentioned method, are much higher than those reported for other aquatic plants. (40) (41)

Cadmium uptake

The concentrations of Cd in the water initially was at a concentration of 10,0 mg/l (9,07 mg/l). The highest concentration of Cd was 3,491 mg/g dry wt. in *Valisneria Spiralis*. The maximum concentration in *Cabomba Aquatica* and *Echinodorus Cordifolius* was 0,242 respectively 1,876 mg/g dry wt. which considering the survival rate of the plants is low and only effective if more research is done to determine the ideal heavy metal concentration/living biomass ratio.

Zinc uptake

The concentrations of Zn in the water initially was at a concentration of 10,0 mg/l (9,87 mg/l). Like other heavy metals in the present study, the highest removals of Zn from the water were obtained from *Valisneria spiralis* after 6 days of treatment,(Table 1). The highest concentration of Zn was 2,359 mg/g dry wt., in *Valisneria spiralis*. The accumulation in the other plants was at 0,357 mg/g dry wt. for *Cabomba aquatic*, respectively 1,962 mg/g dry wt. for *Echinodorus cordifolius*.

Mishra and Tripathi (2008) (40) reported that the highest concentrations of Cu in three aquatic macrophytes (*P. stratiotes L.*, *Spirodela polyrhiza*, and *E. crassipes*) were 0.875, 0.186, and 2.75 mg/g dry wt., respectively, over 15 days. Kamal et al. (2004) (41) observed maximum Cu concentrations of 0,304; 0,848 and 0,314 mg/g dry wt. in parrot feather (*Myriophyllum aquaticum*), creeping primrose (*Ludwigina palustris*), and water mint (*Mentha aquatic*), after 21 days of incubation. After these result we can say that 28,456 mg/g dw is above expectations and further exploration can show results worthy to be applied in the field.

According to the literature, when applied at lower concentration, Cd is found to be more easily accumulated in the plant and, the opposite can happen at higher Cd level (42) (43) (44) (45). The greatest amount of Cd accumulated by a wetland plant species is 36 mg/g, by *E. crassipes* (46). Zayed et al. in 1998 (47) reported high concentrations of Cd (13 mg/g) in *L. minor L.* when supplied with 10 mg Cd/l. Zhu et al. in 1999 (48) reported that *E. crassipes* accumulated 6,103 mg/g dry wt. of Cd in roots and 0,371 mg/g dry wt. of Cd in shoots, cumulatively 6,474 mg/g dry wt. of Cd in the whole plant, when 10 mg Cd/l was supplied. These values far exceed our own results of 3,491 mg/g dry wt. in *Valisneria spiralis*, 0,242 mg/g dry wt. in *Cabomba aquatica* and 1,876 mg/g dry wt. in *Echinodorus cordifolius*. But by manipulating the surroundings of the plants or even the content of the nutrient solution we

could control the heavy metal uptake. As reported by Csog et al and Mihutcz et al (33)(...) by changing the iron supplier the plant can be determined to accumulate a specific heavy metal in dry land plants.

The highest reported concentrations of Zn in duckweed are as much as 30 mg/g dry wt. when grown in medium containing 10 mg Zn/l (49). Kamal et al. (41) reported that the highest concentrations of Zn reached 549, 1243, and 1498 mg/g dry wt. for parrot feather (*M. aquaticum*), creeping primrose (*L. palustris*), and water mint (*M. aquatic*), respectively, after 21 days of incubation. Mishra and Tripathi (40) reported maximum concentrations of Zn in three aquatic macrophytes (*P. stratiotes* L., *S. polyrhiza*, and *E. crassipes*) of 0.98, 1.5, and 6.51 mg/g dry wt., respectively, over 15 days. (50) Conform this study, *Cabomba aquatic* (0,357 mg/g dry wt.), *Valisneria spiralis* (2,359 mg/g dry wt.) and *Echinodorus cordifolius* (1,962 mg/g dry wt.) appear to be a poor accumulator of Zn. This might be contributed to the fact that zinc is a metabolic metal and its uptake is strictly regulated and only by changing the parameters can these plants become zinc hyper accumulators. However, it has been reported that another aquatic plant, *Eleocharis acicularis*, accumulated 13,7 mg/g dry wt. of Zn after 2 months of transplanting at an abandoned mine drainage site in Japan (51).

When we look at the plants vital signs during the experiment (coloration of leaves, flexibility of plant parts) we can see the toxicity effect of the heavy metals exerted on the plants metabolic system. In case of copper the plants discoloration showed that keeping it more time than 6 days in the solution would leave to the plants death. The discoloration and swooning of the plants parts shows that chronic damage occurs with Cd after 5 days. But for the unity of the experiment the plants were left in the solution for 6 days. In case of zinc the vital signs of the plant remained constant thru the entire study. This may reflect that the plant didn't saturate itself with Zn. *Valisneria spiralis* showed a slight increase for plants mass in the experiments which suggest a higher heavy metal tolerance for Zn than that of Cu and Cd.

To compare the metal uptake between each plant species a ratio a coefficient was calculated for each metal. This showed that the copper concentrations in *Valisneria spiralis* were 1,77-3,36 times higher than in *Cabomba aquatica* and *Echinodorus cordifolius*. Cadmium concentration in *Valisneria spiralis* was much higher by approximately 1,9-14,4 than in case of *Cabomba aquatica* and *Echinodorus cordifolius*. In case of zinc the concentration in *Valisneria spiralis* varied between 1,2-6,6 for *Cabomba aquatica* and *Echinodorus cordifolius*. (Table 1)

B. Competitive metal uptake

This experiment was also conducted using hydroponics. Plants were put to nutrient solution in aquariums equipped with fluorescent of 14/10 h light/ dark photoperiods; at a temperature of 24-28 °C. The nutrient solution was as in the previous part the modified Hoaglan nutrient solution containing Fe(III)EDTA as iron source. The plants were left for 3 days for acclimatization. Then, they were transferred in basic nutrient solution containing 10 mg/l of Cd(NO₃)₂, ZnSO₄ × 7H₂O and CuSO₄ × 5H₂O; and Fe(III) EDTA as iron supply for 6 days, controls were left without heavy metal treatment. The heavy metal content of the basic solution was checked by atomic absorption spectrometry. The plants were harvested and washed with distilled water. The samples were dried in an oven at 80°C until constant weight was achieved.

The dried plant samples were digested in 10 ml of concentrated nitric acid, supplied by Merck. The mass of the plants was approximately 300–1000 mg. The digestion took place at 20 °C for 24h and at 100 °C for 8h. After digestion, the solutions were filled up to 100 ml with distilled water. The analysis of the digested samples was carried out by the GBC SensAA Dual Atomic Adsorption Spectrophotometer with flame atomization system. The results are shown in Table 7.

In the case of competitive uptake, Cu concentrations changed in function of the plant species. In *Cabomba aquatica* the amount of copper was almost the same as in mono metal uptake, as was in *Valisneria spiralis*. But compared with the other plants Cu concentrations increased by 1,78 times in *Echinodorus cordifolius*. These results indicate that, depending on the species Cu uptake can be enhanced by the composition of the nutrient solution and the subsequent accumulation. This also depends on the plants natural tendency to hyperaccumulate copper. (Table 8)

Cd is the element whose accumulation in the *Cabomba aquatica* plant was most obviously affected by the competitive uptake, the difference was even 10 times greater. In *Valisneria spiralis* the uptake of Cd in competitive surroundings was almost the same than in the case of mono metal uptake. The accumulation in *Echinodorus cordifolius* was noticeably higher but not as high as in *Cabomba aquatica*. (Table 8)

In competitive heavy metal uptake Zn concentrations in all three plants were affected. Compared with the mono metal uptake, the accumulation of Zn concentrations increased by

2,02; 2,78 and 1,49 times in *Valisneria spiralis* and *Echinodorus cordifolius*. Compared to eche other *Valisneria spiralis* accumulated by 2,24 times more than *Echinodorus cordifolius* and 8,81 times more than *Cabomba aquatica*. These results indicate that, depending on the species Zn uptake and the subsequent accumulation can be enhanced by the heavy metal content of the nutrient solution and the species natural tendency to accumulate zinc. (Table 8)

Plant Type	Cabomba Aquatica		Valisneria Spiralis		Echinodorus Cordifolius	
	Control	Competitive	Control	Competitive	Control	Competitive
Heavy Metals	mg	mg	mg	mg	mg	mg
Zn	0,160	0,508	0,085	2,105	0,010	2,640
Cd	0,026	0,979	0,009	0,871	0,104	2,406
Cu	0,214	5,419	0,146	9,285	0,020	26,010

Table 6 Competitively accumulated heavy metals in *Cabomba aquatica*, *Valisneria spiralis* and *Echinodorus cordifolius* after 6 days in modified Hoagland nutrient solution, at an ambient temperature of 24-28 °C and 14/10 light/dark period (1,5 W/l of water)

Plant mass	Cabomba Aquatica		Valisneria Spiralis		Echinodorus Cordifolius	
	Control	Competitive	Control	Competitive	Control	Competitive
Heavy Metals	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g
Zn	0,254	0,745	0,243	6,559	0,010	2,920
Cd	0,041	1,438	0,026	2,713	0,101	2,661
Cu	0,339	7,957	0,417	28,924	0,019	28,772

Table 7 Calculated heavy metal content in plant biomass (*Cabomba aquatica*, *Valisneria spiralis* and *Echinodorus cordifolius*) after competitive accumulation in 6 days in modified Hoagland nutrient solution, at an ambient temperature of 24-28 °C and 14/10 light/dark period (1,5 W/l of water)

The results from this research have revealed a complex interaction among different heavy metals in terms of affecting plant growth and the accumulation of various elements. A paper reviewed by Dube et al. (52) shows that the binding forces between heavy metals and environment are dependent on ion properties such as charge and ionic radius. Ions with higher charges will be more strongly bound in the nutrient solution than lower charged ions. As for metal ions with the same charges, the most important factors are ionic radius and rank of hydration.

C. Intracellular heavy metal uptake

This experiment was done using hydroponics on *Valisneria spiralis*. This has been done because the best yield for all heavy metals was shown by *Valisneria spiralis*. The plants were put to nutrient solution in aquariums equipped with fluorescent of 14/10 h light/ dark photoperiods; at a temperature of 24-28 °C. The nutrient solution was as in the previous part

the modified Hoaglan nutrient solution. To assess the influence of chelating agents one round of plant growth was done without Fe(III)EDTA and one containing Fe(III)EDTA as iron source. The plants were left for 3 days for acclimatization. Then, they were transferred in basic nutrient solution containing 10 mg/l of Cd(NO₃)₂, ZnSO₄ × 7H₂O and CuSO₄ × 5H₂O; and Fe(III) EDTA as iron supply for 6 days, controls were left without heavy metal treatment. The heavy metal content of the basic solution was checked by atomic absorption spectrometry. Heavy metal exposed samples were rinsed several times with distilled water as before. For the displacement of the extracellular metals accumulated on the surface during the treatment we used NiCl₂ solution. The plants were washed three times for 30 min with 200 mL 20 mM NiCl₂, dried overnight at 80 °C and digested in 10 ml of concentrated nitric acid of Suprapur[®] 20 °C for 24h and at 100 °C for 8h. After digestion samples were filled with distilled water to a final volume of 100 ml. Heavy metal concentrations were measured by atomic absorption spectroscopy (GBC SensAA Dual Atomic Adsorption Spectrophotometer with flame atomization system). Comparing the metal content of the rinsed and unrinsed plants we could calculate the extracellular metal content. The result can be seen in Table 9. The comparison of the results is in Figure 29.

Table 8 Intracellular metal content in *Valisneria Spiralis* with and without Fe-EDTA after 6 days in modified Hoagland nutrient solution, at an ambient temperature of 24-28 °C and 14/10 light/dark period (1,5 W/l of water)

With no Fe-EDTA added

	No NiCl2				With NiCl2									
Plant mass	0,596		0,227		0,224		0,159		0,204		0,211		0,309	
	Control		Zn		Cd		Cu		Zn		Cd		Cu	
	mg	mg/g	mg	mg/g	mg	mg/g	mg	mg/g	mg	mg/g	mg	mg/g	mg	mg/g
Zn	0,166	0,279	0,175	0,771					0,107	0,525				
Cd	0,005	0,008			0,435	1,942					0,336	1,592		
Cu	0,054	0,091					3,841	24,157					5,170	16,731

With Fe-EDTA added

	No NiCl2				With NiCl2									
Plant mass	0,433		0,134		0,146		0,149		0,145		0,224		0,156	
	Control		Zn		Cd		Cu		Zn		Cd		Cu	
	mg	mg/g	mg	mg/g	mg	mg/g	mg	mg/g	mg	mg/g	mg	mg/g	mg	mg/g
Zn	0,137	0,316	0,328	2,448					0,144	0,993				
Cd	0,006	0,014			0,775	5,308					0,307	1,371		
Cu	0,152	0,351					5,262	35,315					4,145	26,571

Intracellular metal content

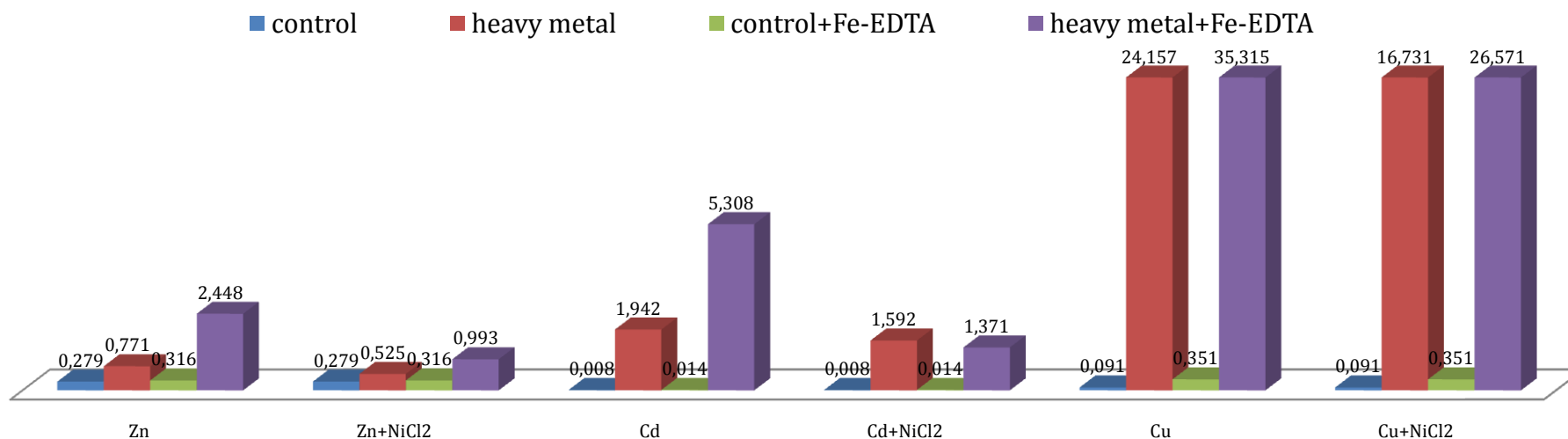


Figure 6 Intracellular metal content in *Valisneria Spiralis* with and without Fe-EDTA

The heavy metal content in *Valisneria Spiralis* increased with and without the chelating agents. The intracellular content of the heavy metals reached 0,525 mg/g dry wt. for Zn; 1,592 mg/g dry wt. for Cd and 16,731 mg/g dry wt. for Cu, when no chelating agent was added. In case of Fe-EDTA addition the content of the metals reached 0,993 mg/g dry wt. for Zn; 1,371 mg/g dry wt. for Cd; 26,571 mg/g dry wt. for Cu. The intracellular uptake of heavy metals can be characterised as follows: Cu > Zn > Cd. Without the addition of chelating agents the rinsing of the plants brought a constant 30% wash away, with the addition of chelating agents the washed away heavy metal varied: 59% for Zn; 74% for Cd; 25% for Cu. According to these results we can conclude that the chelating agents play a significant part in a plants metabolism. When chelating agents are present the uptake of heavy metals can be regulated by the plants metabolism as shown in previous works (), which helps with the selective phytoremediation of its surroundings.

Several authors believe that bioaccumulation proceeds slowly whereas extracellular biosorption is an extremely rapid and passive process. (53) (54) (55) The different intracellular accumulation levels under identical conditions may be caused by metal-specific transport mechanisms (53), the details of which are still largely unknown. Notably, these differences in accumulation capacity were observed only at high non-physiological metal concentrations.

3.4.4 Conclusion and Original Contribution

All three plants accumulated the heavy metals from their solution, which renders them a possible subject for phytoremediation/ phytoextraction experiments. The concentration of all three metals in all plants increased both when added solely and all together. With few exceptions, there were differences between the uptakes of heavy metals whether in the presence or absence of the other metals, indicating that there was no competition between the metals at uptake (this could be because there are specific uptake and binding sites for each of the metals, thus precluding interaction (56)). The exceptions were that the Cd concentration in *Valisneria spiralis* did not show much change and the Cu concentration in *Echinodorus cordifolius* significantly increased in the presence of the other metals. Copper concentrations were almost the same in *Cabomba aquatica* when treated with Cd and Zn.

Metal concentration was the highest in *Valisneria Spiralis* rendering the species of interest for use in phytoremediation. From these experiment it can be concluded that Zn, Cu and Cd, and are taken up directly from the water by the whole plant; this, together with the

lack of translocation from root to shoot, would be beneficial in a water treatment. The lack of root-to-shoot translocation helps with the problem of dispersion of metals in the plant. Neither extra- nor intracellular uptake was limited; furthermore, competition between the investigated metals did not limit their accumulation in total. The species *Valisneria Spiralis* seems promising for use in phytoremediating water. This is because it can be expected to take up metals directly from the water with great efficiency, even if contamination is by a mixture of metals.

After the displacement of extracellular metals using NiCl_2 , the highest rate of intracellular accumulation was achieved by Cu followed by Zn and Cd after 6 days of 10,0 mg/l metal. Cu accumulation was associated with a reduced sulphur level in the electron-dense precipitates of the cytoplasm and an increasing level of P in the vacuole. The probability of Cu chelation by SH-groups of GSH in the cytoplasm and the Cu-binding as phosphates in vacuoles has been shown also by Rau et al. (57)

4 General Conclusion

- The hydroponic method helps achieve the ideal parameters for plants growth and this way it is suited to create an iron and heavy metal uptake and distribution model with cucumber.
- The iron supply depends on the thoughtful selection of complexing ligands.
- The complexation/chelation processes in the root/medium interface determines mainly the accumulation of iron. The stability of the iron/heavy metal ligands influences the iron/heavy metal uptake which can be beneficial to influence the exact type of contaminants being removed
- The distribution of iron is affected by his internal competition with heavy metals and the transport systems involved.
- Translocation factor for poplar grown in hydroponics shows a potential for phytostabilisation and phytoremediation.
- The high tolerance for Cd and Pb in a low metal concentration surrounding qualifies for phytostabilisation.
- The ability to take up and transport Cd toward the shoot in higher amounts is promising for phytoremediation purposes. The success of the phytoremediation depends on the heavy metal concentration of the polluted soil, to control the decrease of the biomass.

- Phytoremediation of areas polluted with Ni is promising for poplar. The accumulation factor can be influenced by the right chelating agent.
- Aquatic plants grown in an ideal environment can accumulate heavy metals, even when a mixture of metals is present.
- There were differences between the uptakes of heavy metals, between the presence, or absence of the other metals, with few exceptions.
- The uptake of Zn, Cu and Cd, directly from the water by both leaves and roots ensures a greater yield in a water treatment method.
- *Valisneria Spiralis* can become a species of interest for use in phytoremediating water. The amount of heavy metals accumulated shows a potential use for application in waters contaminated with Cd and Cu which are widely used from basic household items to complex industrial technologies.
- The high rate of Cu accumulation can be achieved, because the probability of Cu chelation by SH-groups, of GSH in the cytoplasm and the Cu binding as phosphates in vacuoles. These require more research on the plants response on cellular level and stress proteins.

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