Heavy Metal Stress

Ecology of organisms on heavy metal sites: mechanisms of stress management

Content

Abstract:

Dealing with high concentrations of heavy metals poses a big problem to most organisms, but some plants have developed strategies to tolerate toxic levels of contamination. It was our goal to find out which plants are able to tolerate different metals and getting to know various techniques used in modern laboratories. We chose two different sites in Austria; one contaminated by an old copper mine, the other one containing natural serpentinite, which gave us the possibility to see both sources of heavy metals; natural and anthropogenic. Throughout or work we had a special focus on the species Nocceae goesingense & Nocceae caerulescens and tried to examine how these plants are able to live with toxic metal concentrations

1. **Introduction**

Dealing with soils contaminated by heavy metals is a task growing more and more important, because modern life style is deeply dependent on resources won through mining. Every mine produces tons of colliery waste, which is in many cases stored on open spoil heaps. These heaps still contain high concentrations of toxic heavy metals and can be a threat to surrounding villages or agricultural areas if the heap material is washed away by water or spread by wind. Because of these dangers it is crucial to find ways to secure those wastes and stop further spreading.
But it's not just mining, that causes pollution by heavy metals; other significant sources include smelting and processing of ores, combustion engines, agriculture, sewage sludge and natural occurring heaps.
One approach to stop the spreading of material stored in heaps is to find a way, to create a protective layer of plants to minimize erosion and elution. To reach this goal it is crucial to know which plants are able to tolerate high metal concentrations and why they are able to do so (Salt, Smith and Raskin 1998).

This specific practical is about two heavy metal sites located in Austria:
-Hirschwang an der Rax – lower Austria (Lat: 47.703148, Long: 15.791570)

- spoil heap of an old copper mine (closed in 1890)
- Greywacke with copper contamination
- pH around 4
- high percentage of rough gravel
- Problems additional to metal contamination:
    - low humus content
    - low water capacity
    - high radiation stress

In Hirschwang we examined the heap itself, a small area about 20 meters further down the hill and a small patch of earth next to the Törlweg, which was not directly influenced by the heap.

-Redlschlag – Burgenland (Lat: 47.441963, Long: 16.299462)

- natural serpentinite (Ni, Cr, Co)

In Redlschlag we examined the "Steinstückel", the "Ochsenriegel" and a small patch of soil in a conifer wood with some ferns growing on it
Plant and soil samples from both sites were taken and analysed via different methods to find out how the vegetation, growing on contaminated soil, deals with the toxicity of the heavy metals.
Already known to be hyperaccumulators of heavy metals are the two species *Nocceae caerulescens* and *Nocceae goesingense* (Reeves 1988), so they were analysed to find out where and in which proportion the metals are taken up.

2. Material & Techniques

2.1: EDX – Analysis

***E**nergy-**d**ispersive **X**-ray spectroscopy*

The EDX – technique is especially useful to find out in which parts of the plant accumulation of specific elements occurs. The detection is not limited to the level of organs, but allows scanning individual tissue layers and thus gives a detailed view of the preferred storage locations for metals within the plant.

2.1.1. Equipment needed:

-Electron Microscope (TEM – transmission electron microscope or SEM – scanning electron microscope)

-EDX – Unit:

- X-Ray detector
- pulse processor
- analyser

2.1.2. How it works:

The electron beam of the EM hits the atoms of the sample, interacting with them in a special way. The highly energized incoming electrons of the beam collide with the electrons contained within the different electron shells of the atoms contained in the sample.

The incident beam may excite an electron in an inner shell, ejecting it from the shell while creating an electron hole where the electron was. An electron from an outer, higher-energy shell then fills the hole, and the difference in energy between the higher-energy shell and the lower energy shell may be released in the form of an X-ray. These X- rays are then recognized by the X- ray detector in the EDX- unit, which then creates a signal and passes it on to the pulse processor. There the signals are measured and given to the analyser.

The analyser then processes the signals for data display and further analysis via specialized software.

The amount of energy released by the transferring electron depends on which shell it is transferring from, as well as which shell it is transferring to. Furthermore, the atom of every element releases X-rays with unique amounts of energy during the transferring process. Thus, by measuring the amounts of energy present in the X-rays being released by a specimen during electron beam bombardment, the identity of the atom from which the X-ray was emitted can be established.

The output of an EDX analysis is an EDX spectrum. The EDX spectrum is just a plot of how frequently an X-ray is received for each energy level. An EDX spectrum normally displays peaks corresponding to the energy levels for which the most X-rays had been received. Each of these peaks is unique to an atom, and therefore corresponds to a single element. The higher a peak in a spectrum, the more concentrated the element is in the specimen.

2.1.3. How it's done:

The samples have to be completely water free for using them in the SEM, so they are air dried at room temperature (~24 °C). Then they are cut and arranged on a stub, which is coated with a sticky coal foil. For better image contrast in the SEM the samples have to be coated, in this case carbon is used, because in the element analysis it's easier to differentiate from the elements found in the sample, than any other coating.
After coating the samples are examined in the SEM, where the spots used for measuring are determined. Then the EDX- Measurement is started, allowing 100 seconds of measuring per spot and 5- 10 spots per sample. The raw data were processed with the software "GENESIS – SPECTRUM" by EDAX INC. ©.

2.2: AAS & ICP-MS

Atomic **a**bsorption **s**pectroscopy &**I**nductively **C**oupled **P**lasma- **M**ass **S**pectrometry

These techniques were used to measure the total metal content of plant and soil samples collected on the two examined areas. We used two methods to extract the metals from the collected soil samples. If the sample is extracted with aqua regia, the results show the total amount of metals stored in the soil; if the extraction is done with ammonium nitrate the results show the soluble content of the metals, which is usually the portion, which is available for plants.
Furthermore it is a very good method to find out if the plants growing on the contaminated soils hyperaccumulate, accumulate or exclude the metals to survive.
In respect to nickel the term hyperaccumulator was defined by R.R. Brooks (1977) to describe plants that show Ni concentrations higher than 1000 µg g-1.
For Zink, plants are called hyperaccumulators if they store the metal in concentrations higher than 10000 µg g-1 (J.M. Baker 1989).

2.1.1. How it works

AAS:

The sample solution is fogged and sprayed into a sample chamber where it's burned by a air-acetylene flame at about 2800 °C, which atomizes all molecules contained in the solution. Then the light of a hollow cathode lamp is divided in two beams: the reference beam and the sample beam. The sample beam passes through the sample chamber and is analysed by a detector. Because every element has a specific absorption of light it is possible to determine which elements, and in which concentration were in the solution. The AAS measures only one element per measurement, so in soil samples only copper and nickel were measured, and in plant samples copper, nickel, and zinc levels were determined.

ICP:

The ICP works with Argon- plasma consisting of gas, containing a concentration of ions high enough to make it electrically conductive. This plasma flame is the used to atomize any molecules in the sample solution, which is sprayed into the test chamber. In ICP – MS the resulting Ions are channelled into a mass spectrometer, usually a quadrupole, where they are analysed.

2.1.2. How it's done:

Soil:

All samples were air dried and sieved to get out all particles bigger than 2 mm.

Ammonium Nitrate Extraction:

The soil [2,5 parts] was mixed with 1 M NH_4NO_3 Solution [1 part] and put on a laboratory – shaker for about 2 hours. After the extraction the solution was filtered and stabilized with HNO_3.

Aqua Regia Extraction:

Aqua regia is a mixture containing 3 parts of concentrated HCL and 1 part of concentrated HNO_3.

This extraction is done by adding 30 ml of aqua regia to 2 g of each soil sample and then boiling the mixture for about 3 hours. During this time the acids get neutralized-

After cooking each of the tubes is filled up with distilled water to reach a volume of 100 ml.

Plants:

All plant samples were dried, separated in parts growing over the ground and roots and grounded.

Then 2 g of dry matter were cooked with 24 ml of a mixture consisting of 5 parts nitric acid and 1 part perchloric acid.

2.3: Anatomical analysis

To find out if the plants living on soils with toxic heavy metal levels have any notable anatomic features we examined them in a light microscope.

Parts of each plant were sectioned and examined using bright field-, dark field-, phase contras- and polarized light microscopy.

2.3.2 Anatomical features:

If a plant encounters heavy metal contaminated soil the usual way of taking up these metals is if they are diluted in water and are transported from the root to the shoot. The first barrier these metals encounter within the root is the endodermis which forces water to pass through the symplast, where transport is often regulated by carrier proteins in the plasmalemma.

If metals pass this barrier they are often transported through the phloem in complexed form and then stored in various organs or sometimes even excreted through specialized glands. Especially suspicious as anatomical adaptations to heavy metal stress are well developed trichomes, enclosures or crystal- structures in vacuoles or symbiosis with mycorrhizal fungi.

2.3.2 Plants analyzed:

-*Arabidopsis halleri [*Brassicaceae*]*
-*Rumex acetosella [*Polygonaceae*]*
-*Vaccinium myrtillus [*Ericaceae*]*
-*Nocceae caerulescens [*Brassicaceae*]*
-*Nocceae goesingense [*Brassicaceae*]*
-*Silene nutans [*Caryophyllaceae*]*

2.4: Plasmolytic tolerance analysis

A very good and simple method to test the heavy metal tolerance of individual plant cells of different species is the plasmolytic tolerance test after Höfler (Höfler 1932).
In this test sections of plant organs are incubated in heavy metal solutions for ~48 hours, and then a vitality test via plasmolysis is performed.

2.4.1 Plants analyzed:

Rumex acetosella, Rumex acetosa [Polygonaceae]
Nocceae goesingense, N. caerulescens, N. minima, Arabidopsis halleri [Brassicaceae]
Allium cepa [Alliaceae]
Triticum aestivum [Poaceae]
Cynodontium sp [Dicranaceae]
Armeria obir, Armeria wales [Plumbaginaceae]

2.4.2 Preparation &Execution:

Preparation of heavy metal solutions:
Stock solutions with a concentration of 0,1 M were prepared using sulphates of nickel, zinc, copper and chrome. Metal- sulphates are preferred because in solution the sulphate has very little effects on the plant cells, so if a plant cell dies during incubation it's very likely that this was caused by the metal concentration.
The stock solutions were diluted to create serials of concentrations ranging from 10^{-1} M to 10^{-7} M.

Sections of all plants with at least two undamaged cell rows were made and then incubated in the solutions for 48 hours.

After this incubation time all sections were examined using light microscopy. The sections were looked at right after taking them out of the metal solutions to search for general sign of dead cells like discoloration of chloroplasts or disfigured nuclei.
If the cells were seemingly alive they were placed in a 1M sucrose solution for 15 Minutes to induce plasmolysis.
If the cells showed plasmolysis after this treatment, it meant that they were still alive.

2.5: Germination tests

To find out if heavy metals influence germination and growth of non resistant plants, it is important to create stable basic conditions, so that all other factors can be disregarded. In the natural habitat, there are a lot of factors that have influence on plants, for example temperature, exposition, and water- and nutrient availability. If it is just the heavy metal influence, which is to be examined, all the other factors have to be banned, because otherwise the results would not be significant. For this purpose germination rolls are very common in laboratories, because they are easy to produce, don't take much space, and the experiment is easy to redo. In a laboratory it is simple to create stable basic conditions, so the plants, used in the experiments should all have more or less the same vitality, size and weight.

2.5.1: How it´s done:

A filter paper (A3 format) is folded in half, and six centimetres under the fold seeds are placed in constant distance, and then the paper is rolled, and fixed with a rubber band (in our case, ten wheat caryopsis were placed in each roll).
Those germination rolls were put into different heavy metal solutions.
The heavy metals we wanted to test were nickel copper zinc and chrome. These metals would not dissolve in water, so we used their sulphates. We produced solutions in different concentration, from 10^{-1} to 10^{-8} and a control with distilled water each. Then we put our germination rolls in those solutions, and left them there for twelve days. It is crucial to mark the initial fluid level and refill with distilled water to replace any liquid lost by evaporation and therefore avoid an increase in metal concentration.
After those twelve days the germination rolls were unrolled, and we did our measurements.
At first we counted how many of the caryopsis germinated, then we measured the length of root and shoot of every plant. If the shoot was big enough, we also measured the chlorophyll- fluorescence. At last we determined the fresh- and the dry weight.

2.6.: Soil analysis - photometric determination of humus content

2.6.1: How it works:

The content of organic substances in soil can be detected trough wet oxidation with potassium dichromate and sulphuric acid.
The organic substances get oxidised, potassium dichromate gets reduced from Cr^{6+} to Cr^{3+}. With a photometer it is possible to measure the colouration of the Cr^{3+}.

2.6.2: How it´s done:

The samples have to be absolutely dry, and sieved to get rid of the particles bigger than 2 mm.
Dependent on the amount of humus 0,5 g to 2 g soil was mixed with 20 ml $K_2Cr_2O_7$. Then 15 ml of concentrated H_2SO_4 are added. The mixture has to be left under the fume hood for two to three hours. After that time the mixture is filled up with distilled water until volume reaches 100 ml. The sample should now rest over night, so that the particles can sink down.
On the next day 1 ml of the sample is mixed with 24 ml of distilled water, and shaken. It is important that no soil particles are in the solution, otherwise the measurements would be wrong. Now the sample is ready for the photometer.
For the photometer a calibration solution is important, so we made calibration solutions with 0, 116, 232, and 348 mg myo- inosit, which got mixed with 100 ml distilled water, and afterwards also with 20 ml $K_2Cr_2O_7$ and 15 ml H_2SO_4. Those different solutions correlate with 0, 4, 8 and 12% humus in our samples.

The results the photometer produces can be translated with following formula:

$$\frac{(VP - BW) \times 2}{EW}$$

BW… blank value
EW… net weight
VP … humus content (%)

3. Results& conclusions

3.1: EDX - Analysis

Examined by EDX- analysis, measuring the levels of copper, zinc, iron and chromium were parts of *N. caerulescens* and *N. goesingense*, collected in Redlschlag.

Respectively:

- rosette leaves (upper side, bottom and cross sections)
- stem leaves (upper side, bottom and cross sections)
- stem (cross sections)

The metal concentrations were measured on 5 to 10 spots per plant sample and then the median was calculated.

The big questions for this analysis method were to find out where metals are stored within the plant and in which relations they are taken up.

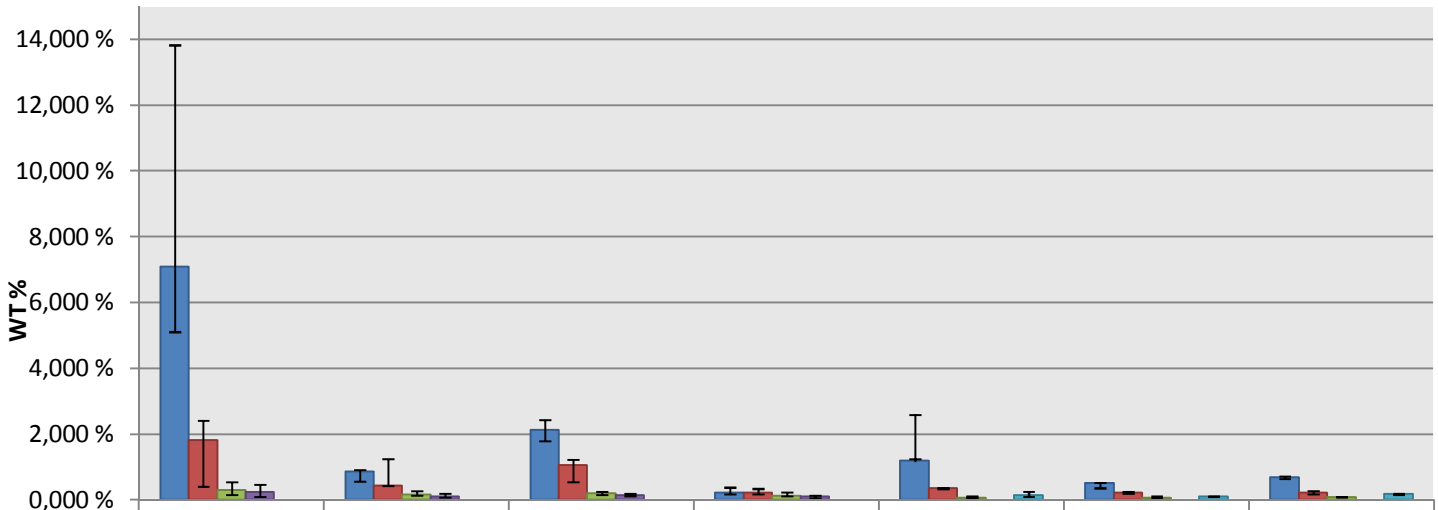
3.1.1. N. caerulescens:

For better understanding of following figures and tables, the abbreviations are explained in Legend 1.

Rsl	Rosette leaf
St	Stem
Stl	Stem leaf
US	Upside
B	Bottom
C	Cross-section

Legend 1- Abbreviations used in Table 1

As seen in Table 1 the preferred metals nickel and zinc are mainly stored in the rosette leaves. Notably there is a tendency that metals are more likely to be stored in the upper epidermal layers of leaves.



	Rsl. US	Rsl. C	Rsl. B	St. C	Stl. US	Stl. C	Stl. B
NiK	7,095 %	0,860 %	2,125 %	0,230 %	1,200 %	0,510 %	0,690 %
ZnK	1,810 %	0,440 %	1,050 %	0,220 %	0,350 %	0,230 %	0,230 %
FeK	0,295 %	0,160 %	0,195 %	0,130 %	0,075 %	0,070 %	0,090 %
CrK	0,235 %	0,110 %	0,140 %	0,100 %			
CuK					0,135 %	0,110 %	0,175 %

Table 1 – Medians of weight percent (5 or 10 measurements per organ section); error bars show 1st and 3rd quartile *[N. caerulescens]*

The big error bars are not a sign of inaccurate measurements, but of the heterogeneity of the leaf epidermis.

It is clearly visible, that metals (especially Ni & Zn) are stored preferably in the epidermal layers of rosette leaves.

To answer the question of whether there is a correlation between the accumulation of the different metals the data were put into a graph and the trend lines were calculated.

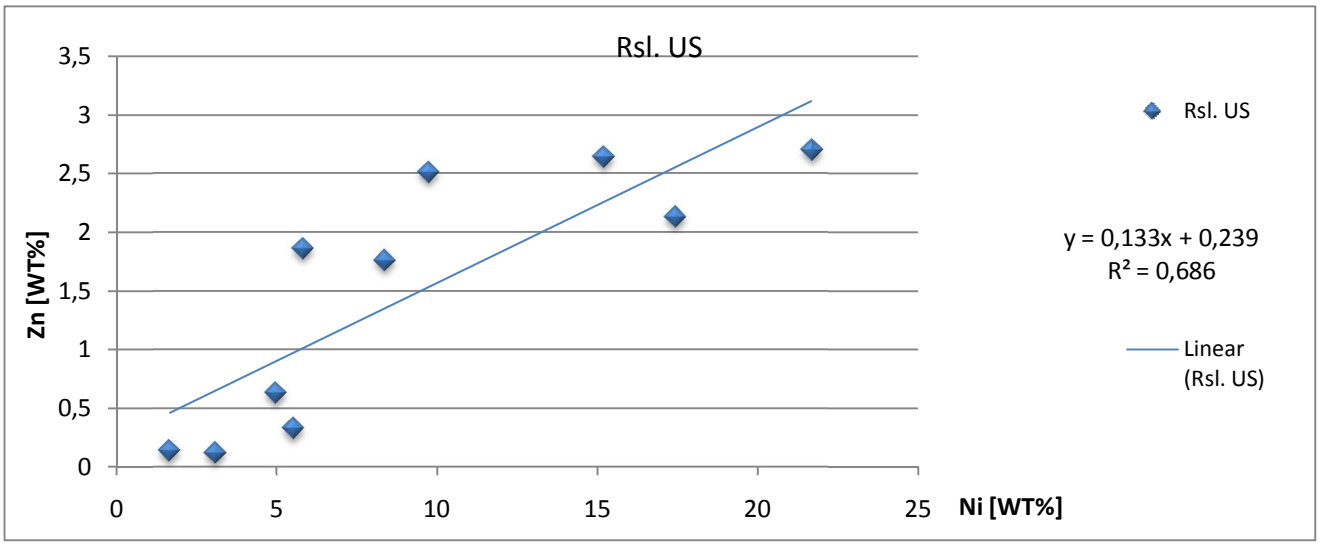


Table 2 – Correlation trend line between uptake of Zn and Ni in epidermal layers of the upside of a rosette leaves *[N. caerulescens]*

Table 2 shows that there is a significant correlation in the uptake of zinc and nickel, which means that the total uptake of metals within the leaf varies, but the composition of metals stays more or less the same.

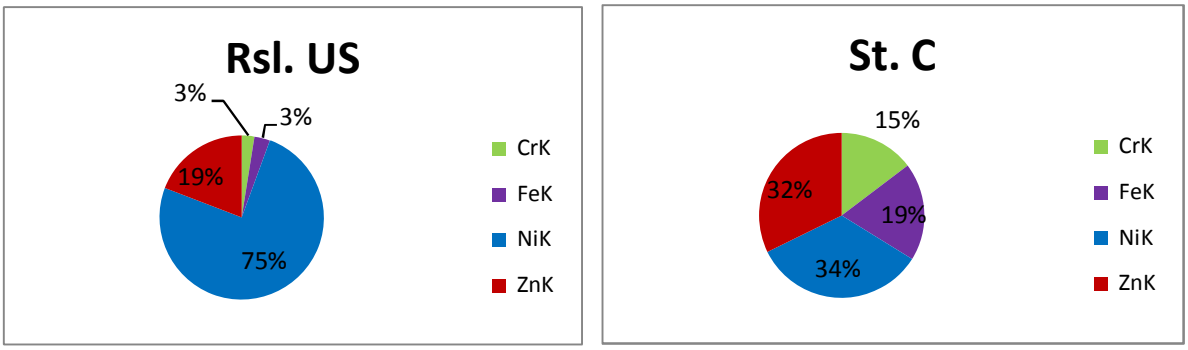


Fig.1 – Comparison of metal composition between rosette leaf and stem; Percentages of total metal content are shown [*N. caerulescens*]

In Fig.1 it can be clearly seen, that in the leaf nickel and zinc are preferably stored, but in the stem there is nearly no preference for any metal.

3.1.2. N. goesingense:

Table 3 shows that N. goesingense accumulates nickel more than any other metal and stores most of the metal in the epidermal layers of leaves.

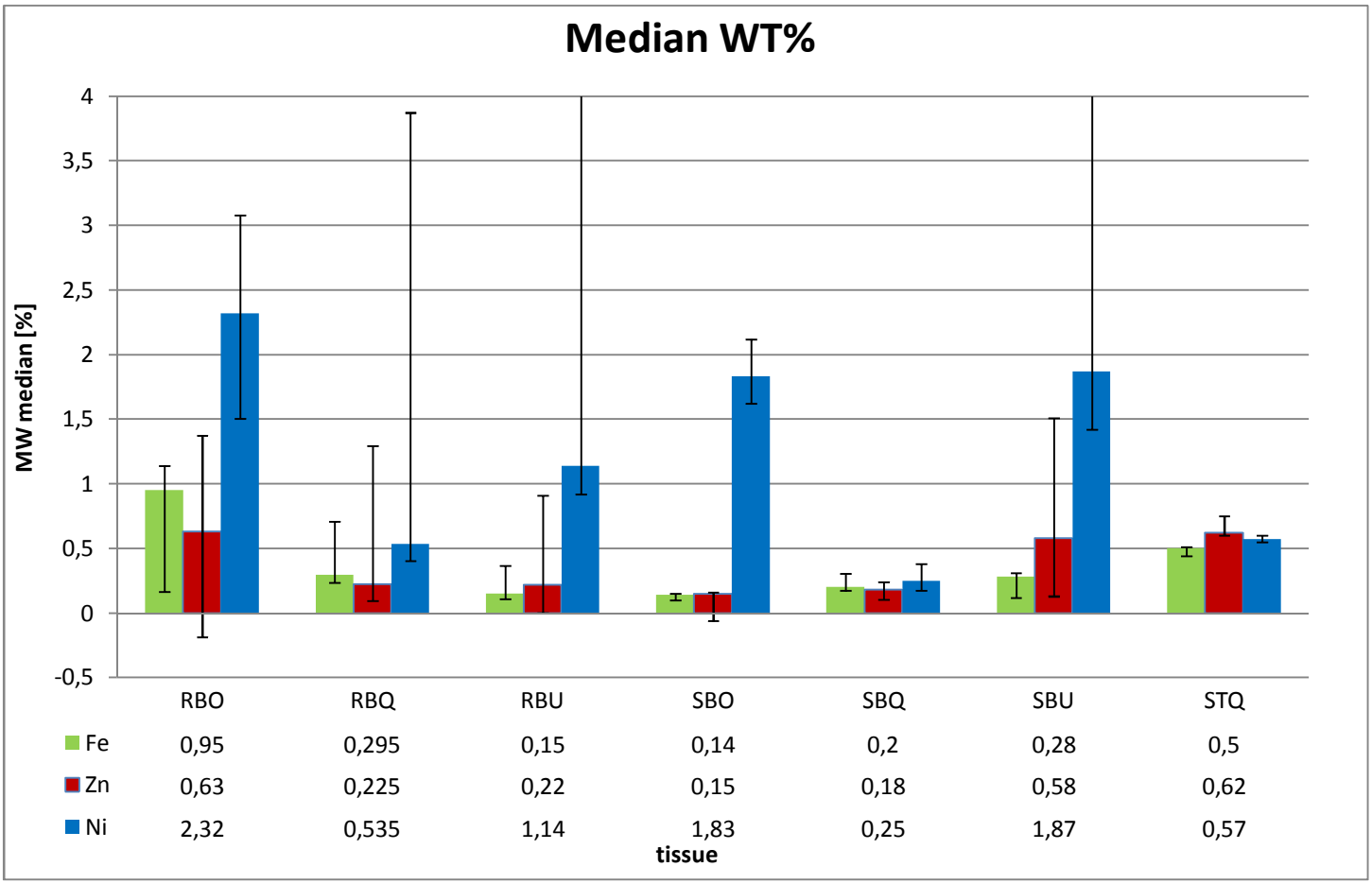


Table 3 - Medians of weight percent (5 or 10 measurements per organ section); error bars show 1st and 3rd quartile *[N. goesingense]*

As well as in N. caerulescens there was the question if there is a correlation in the uptake of Ni and Zn.

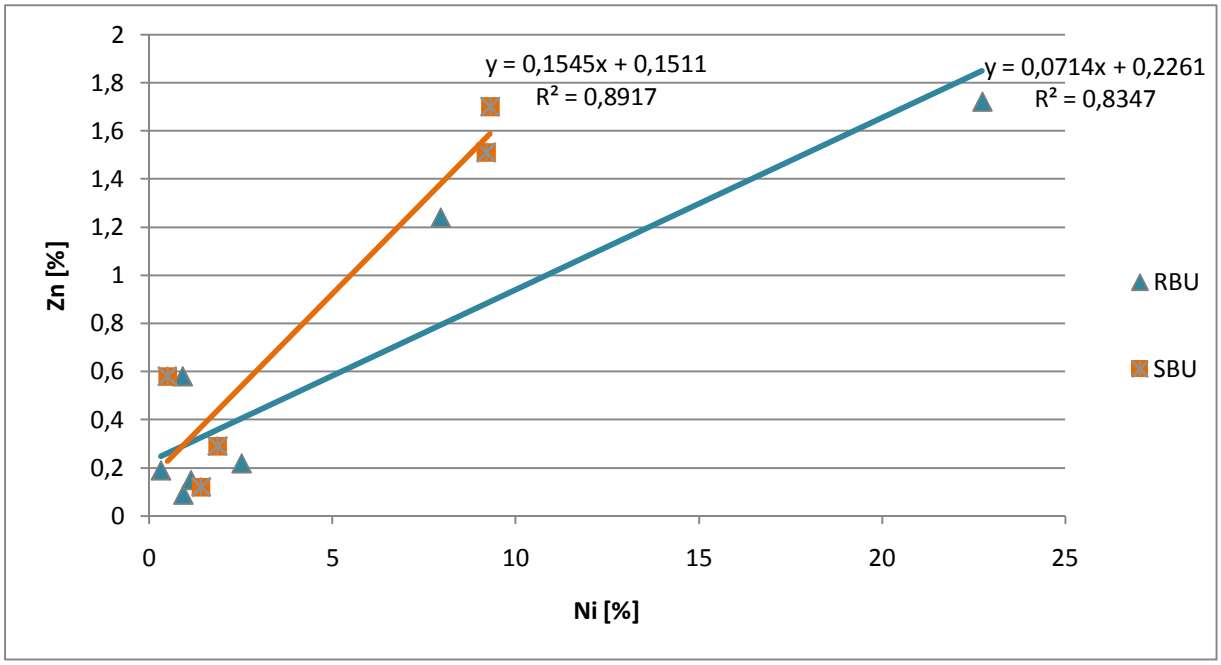


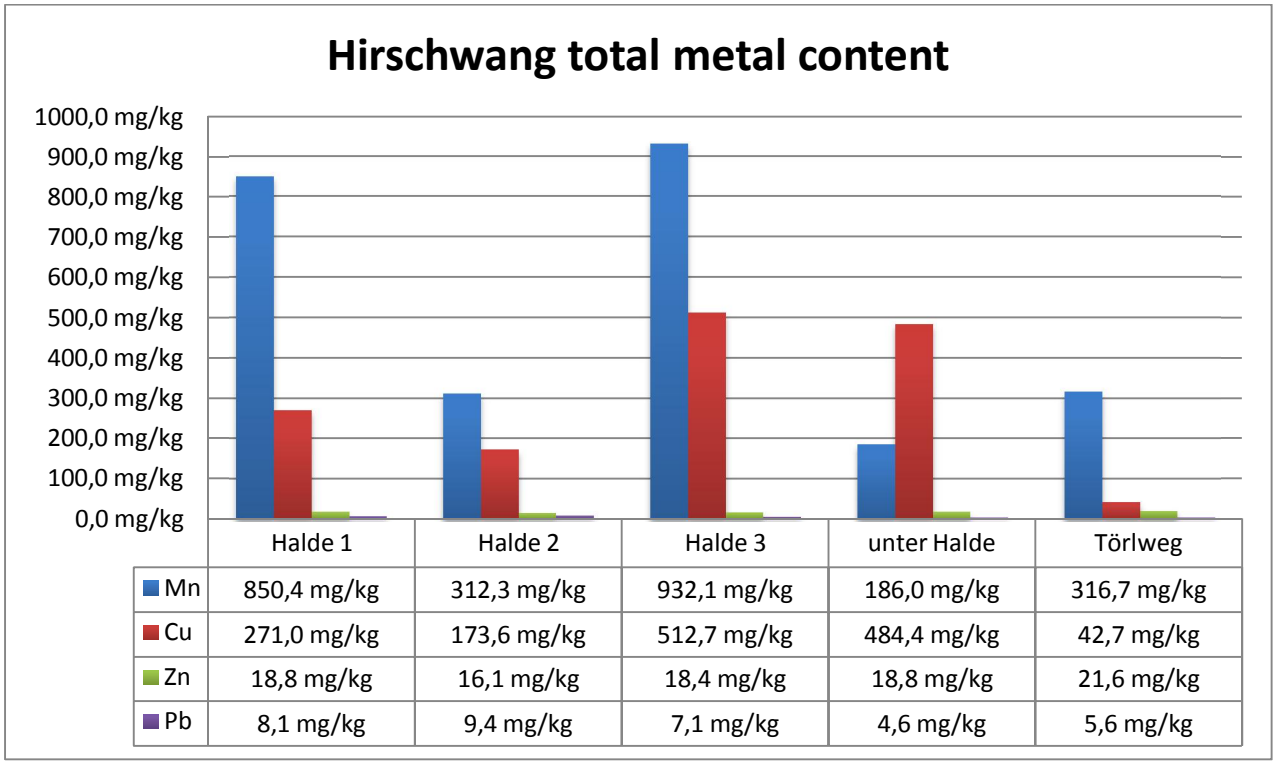
Table 4 - Correlation trend lines between uptake of Zn and Ni in different Organs *[N. goesingense]*

There is actually a significant correlation between Zn and Ni uptake in different leaves of *N. goesingense*.

3.2: AAS & ICP-MS

Because of technical errors the values of the AAS copper- Measurements are not trustable and will not be used here.

Results of ICP measurements of plant and soil extracts from Hirschwang:

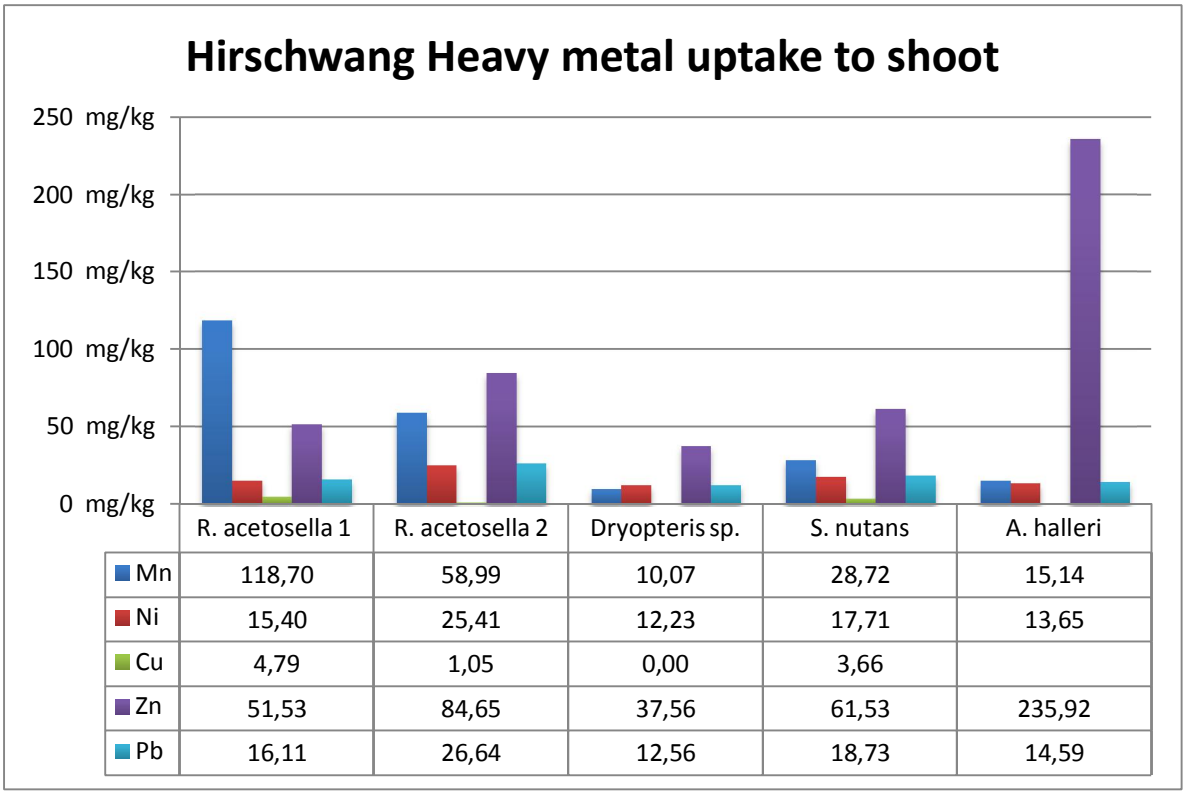


	Halde 1	Halde 2	Halde 3	unter Halde	Törlweg
Mn	850,4 mg/kg	312,3 mg/kg	932,1 mg/kg	186,0 mg/kg	316,7 mg/kg
Cu	271,0 mg/kg	173,6 mg/kg	512,7 mg/kg	484,4 mg/kg	42,7 mg/kg
Zn	18,8 mg/kg	16,1 mg/kg	18,4 mg/kg	18,8 mg/kg	21,6 mg/kg
Pb	8,1 mg/kg	9,4 mg/kg	7,1 mg/kg	4,6 mg/kg	5,6 mg/kg

Table 5 - Total metal contents of the different sites in Hirschwang

In Hirschwang the two dominating metals were manganese and copper. The site “Törlweg” was a few hundred meters away from the spoil heap and therefore doesn’t contain much copper.

The sample point “untere Halde” is not directly on the spoil heap but about 20 metres further down the hill. That the copper concentration here is still very high shows that the copper is washed out by rainwater and contaminates the area around the actual heap.



	R. acetosella 1	R. acetosella 2	Dryopteris sp.	S. nutans	A. halleri
Mn	118,70	58,99	10,07	28,72	15,14
Ni	15,40	25,41	12,23	17,71	13,65
Cu	4,79	1,05	0,00	3,66	
Zn	51,53	84,65	37,56	61,53	235,92
Pb	16,11	26,64	12,56	18,73	14,59

Table 6 – Heavy metal uptake by different plants in Hirschwang

R. acetosella and S. nutans grew on the copper contaminated spoil heap with copper concentrations between Cu concentrations between 300 and 500 mg/kg but nearly nothing was taken up by these plants. The Manganese concentrations were also much higher in the soil than in the plants, which is also confirmed by the bioconcentration factors. This marks these plants as excluders of Cu and Mn.

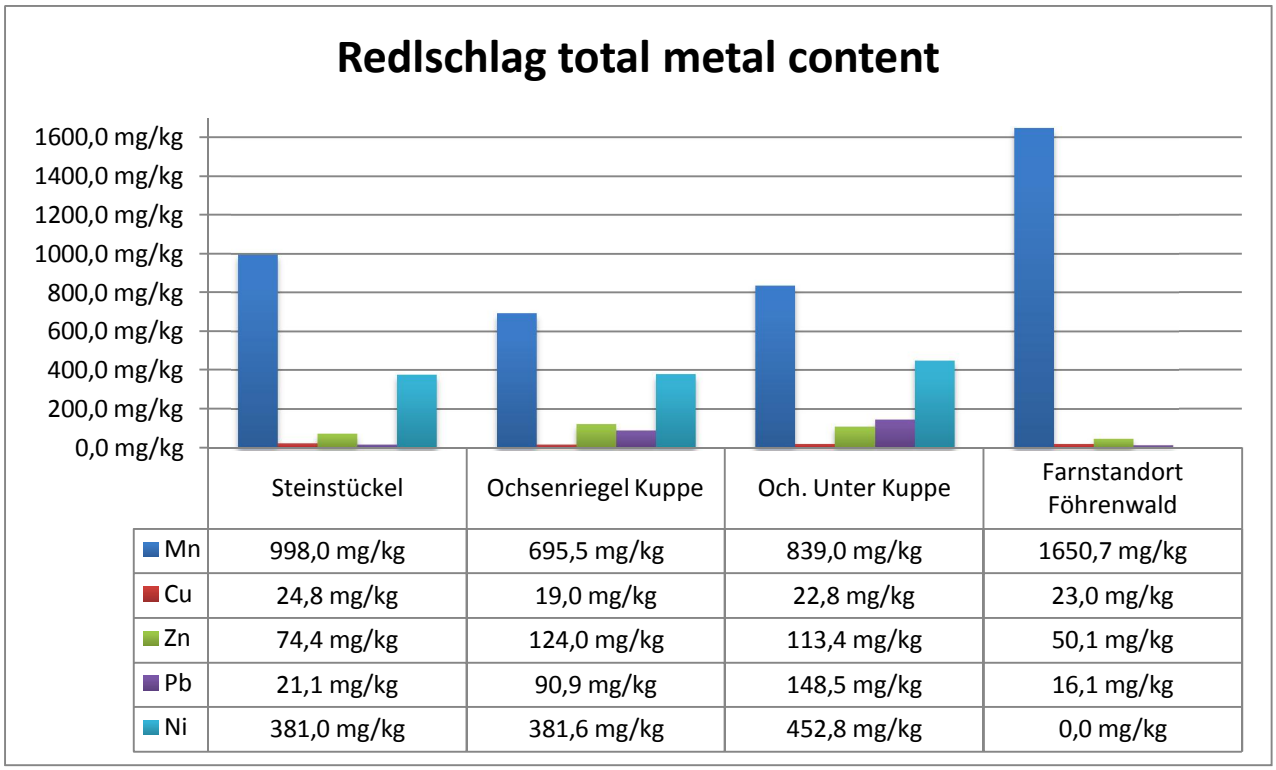
Even in Excluder plants there are always traces of these metals in the plants, because in small doses they are important micronutrients to the plant.

All of these plants accumulate Zinc and lead.

Species	**Mn**	**Cu**	**Zn**	**Pb**
R. acetosella 1	0,14	0,02	2,89	2,07
R. acetosella 2	0,07	0,00	4,74	3,42
Dryopterissp.	0,05	<det. Limit	2,00	2,75
S. nutans	0,15	0,01	3,28	4,10
A. halleri	0,05	<det. Limit	10,94	2,60

Table 7 – BCF factors of different plants collected in Hirschwang

Results of ICP measurements of plant and soil extracts from Redlschlag:

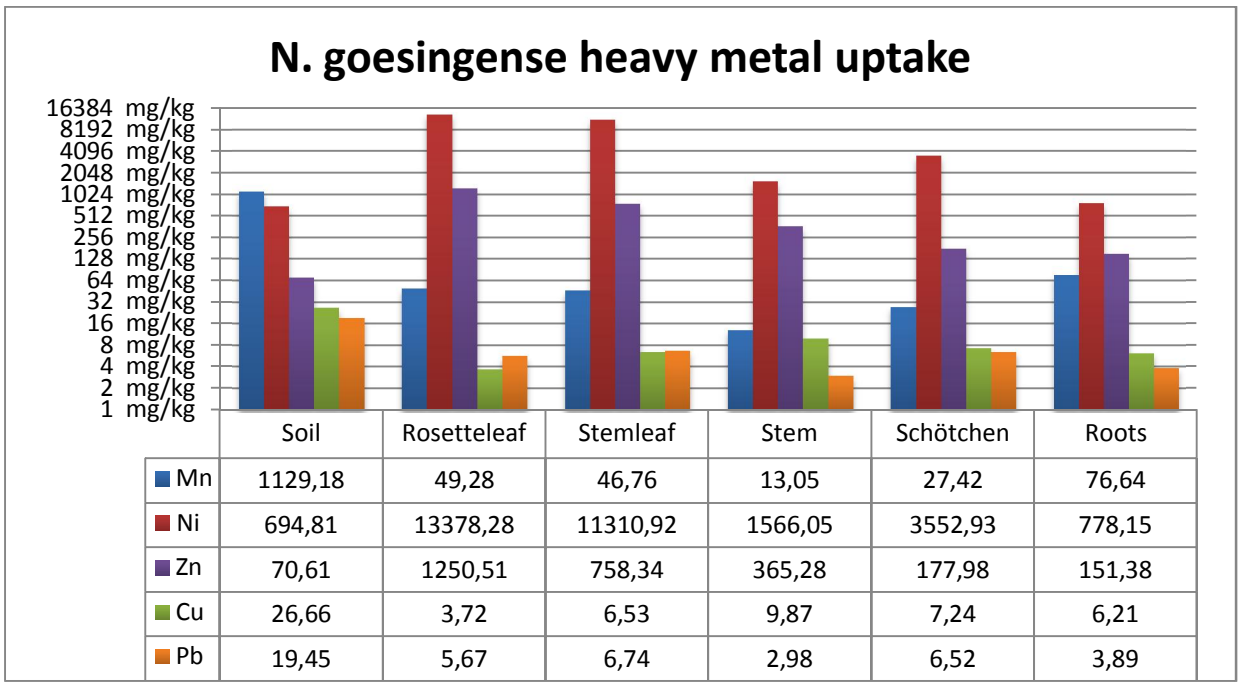


	Steinstückel	Ochsenriegel Kuppe	Och. Unter Kuppe	Farnstandort Föhrenwald
Mn	998,0 mg/kg	695,5 mg/kg	839,0 mg/kg	1650,7 mg/kg
Cu	24,8 mg/kg	19,0 mg/kg	22,8 mg/kg	23,0 mg/kg
Zn	74,4 mg/kg	124,0 mg/kg	113,4 mg/kg	50,1 mg/kg
Pb	21,1 mg/kg	90,9 mg/kg	148,5 mg/kg	16,1 mg/kg
Ni	381,0 mg/kg	381,6 mg/kg	452,8 mg/kg	0,0 mg/kg

Table 8 - Total metal contents of the different sites in Redlschlag

Table 8 shows the composition of metals on the different sites of Redlschlag, using the values from aqua regia extracts measured by ICP-MS. The most abundant heavy metal throughout all sites was manganese, followed by nickel (excepting "Farnstandort Föhrenwald").

To find out which metals were taken up by plants, we took a closer look at one of the *N. goesingense* plants we collected at "Steinstückel".



	Soil	Rosetteleaf	Stemleaf	Stem	Schötchen	Roots
Mn	1129,18	49,28	46,76	13,05	27,42	76,64
Ni	694,81	13378,28	11310,92	1566,05	3552,93	778,15
Zn	70,61	1250,51	758,34	365,28	177,98	151,38
Cu	26,66	3,72	6,53	9,87	7,24	6,21
Pb	19,45	5,67	6,74	2,98	6,52	3,89

Fig. 2 – Uptake of heavy metals of the different organs in *N. goesingense* in comparison to the soil.

Fig. 2 shows the organ specific metal content of a N. goesingense plant collected in Redlschlag. As seen in the EDX- analysis most of the metals are stored in the leaves, especially the rosette (ground) leaves.
This is further supported if the bioconcentration factors (BCF), which allow a comparison of taken up metals, with the concentration of metals contained in the soil are calculated.

tissue	Mn	Ni	Cu	Zn	Pb
Rosette leaf	0,04	19,25	0,14	17,71	0,29
Stem leaf	0,04	16,28	0,24	10,74	0,35
Stem	0,01	2,25	0,37	5,17	0,15
Schötchen	0,02	5,11	0,27	2,52	0,34
Roots	0,07	1,12	0,23	2,14	0,20

Table 9 – BCF factors of individual Organs of *N. goesingense*

If the value for BCF is >1, it's a clear sign of accumulation. Looking at the values of Table 9, showing very high values in all organs for Ni and Zn, we can definitely say that this plant accumulated these elements in high concentrations.

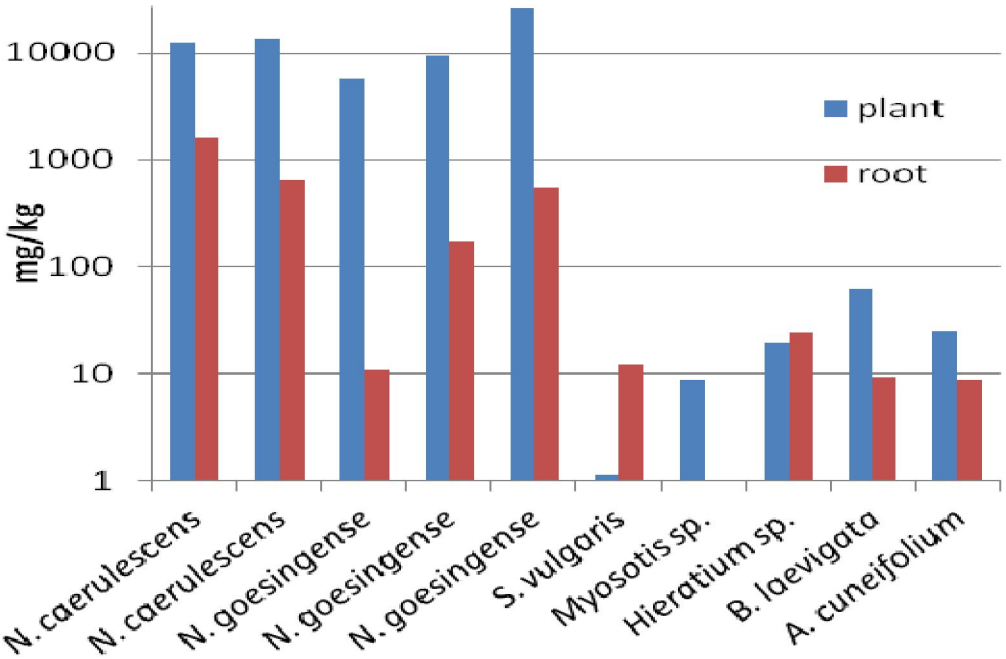
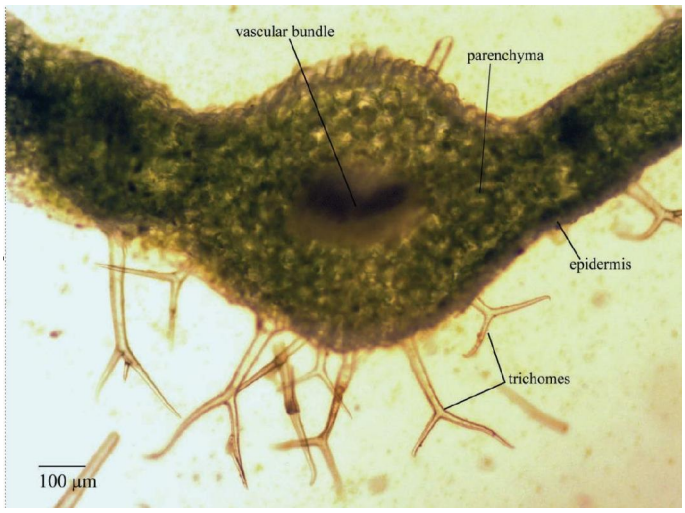


Fig. 3 – Ni content of various plants collected in Redlschlag; split up in values for shoot and root

In Fig. 3 the relation between Ni stored in the roots and Ni stored in the shoot can be seen and it is quite clearly visible that the two *Nocceae* species store more of the metal in the shoot, than in the root. This is due to the fact that these plants actively transport the metals into the leaves to store them there.
Also interesting is that *S. vulgaris* takes up Ni in its root, but does not transport any of it into the shoot.

3.3: Anatomical analysis

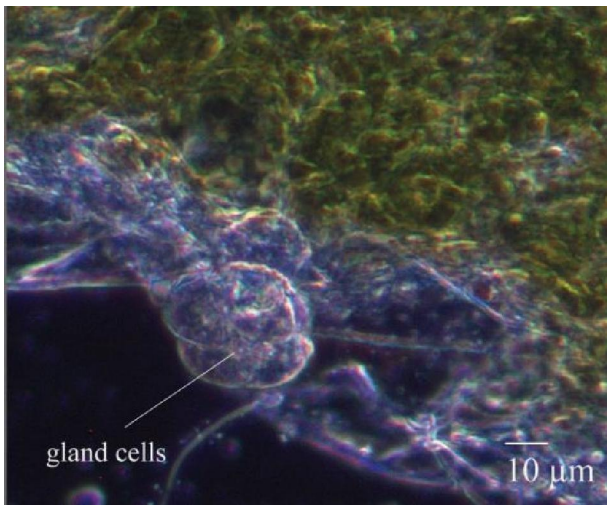
-Arabidopsis halleri:



Prominent anatomical features of A. halleri are big trichomes, which show enclosures in their vacuoles at higher magnifications.

Fig – *Arabidopsis halleri*; leaf cross section

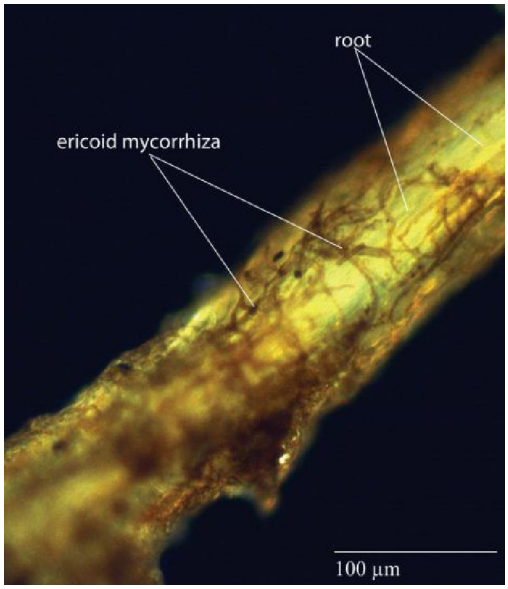
-Rumexacetosella:



It seems to have enclosures in the intercellulars, but it was not possible for us to prove that they are used a storage for heavy metals. Furthermore the leaves are covered in glands, which might play a role in excretion of metals.

Fig.5 – *R. acetosella* leaf with glands

-Vaccinium myrtillus



In *V. myrtillus* ericoid mycorrhiza are quite well developed forming a coat around the root. IT could be that these fungi form a barrier for heavy metals allowing the plant to grow in contaminated soil without having any special resistance mechanisms for its own.

Fig.6 – *V.myrtillus* roots and mycorrhizal

-Nocceae caerulescens & Nocceae goesingense

We could not determine any special anatomical features to explain the heavy metal resistance of the two examined *Nocceae* species.

-Silene nutans

Silene nutans shows big trichomes on its leaves, which are possibly in excreting or storing metals.

Fig.7 – *S. nutans* leaf cross section with trichomes

It is quite notable, that many plants growing on heavy metal contaminated soils show structures like trichomes, gland cells or other excretion organs, and possibly use them to get rid of metals they take up.

3.4: Plasmolytic tolerance analysis

Using the categories explained in Legend 2 a table showing the range of tolerance for each plant was created:

+	most of cells living
+/-	≥ 50% living
-/+	≤ 50% living
-	most of cells dead
P	cell was plasmolysed by metal solution and therefore dead
n.v.	no value

Legend 2 – categories used in Table 10

	Cu							Ni							Zn							Cr						
	-1	-2	-3	-4	-5	-6	-7	-1	-2	-3	-4	-5	-6	-7	-1	-2	-3	-4	-5	-6	-7	-1	-2	-3	-4	-5	-6	-7
Armeria sp.-Obir	-/+	+	+	+	+	+	+	P+/-	P+/-	P+/-	+	+/-	+	+	-	p-	-/+	-/+	+/-	+	+	-	-	+/-	+/-	+	n.v.	+
Armeria sp.-Wales	-	-	P-/+	P+	+	+	+	-	-	-	-	+/-	+	+	-	-	-	-	+/-	+	+	-	-	-	-	-	+/-	+
Rumex acetosella	-	-	-	-	+	+	+	-	-	-	-	-/+	+	+	-	-	-/+	+/-	+/-	+	+	-	-	-	-	-/+	+	+
Rumex acetosa	+	-/+	-/+	-/+	+	+	+	p-/+	p-/+	p-/+	+	p-/+	P+/-	+	+/-	+/-	+/-	+/-	+/-	+/-	+	-	-	+/-	-	-	n.v.	+/-
Nocceae goesingensis	-	-	-	-	+/-	+	+	P -	P -	P -	P -	P-/+	P-/+	-/+	-	-	-	-/+	-/+	+	+	-	-	-	-	-	-/+	-/+
N. caerulescens	P-	P-	P-	P-	+	+	+	P-	P-	p-		p-	p-	P+	-	-	-	-	-	-/+	+	-	-	-	-	-	-	-
N. minimum	-	+/-	+	+	+	+	+	+/-	+	+	+	+	+	+	+/-	+	+	+	+	+	+	-	-	-	-	+/-	+	+
Allium cepa	-/+	+	+	+	+	+	+	n.v.	p-	p-	+/-	p-	p-	p-	-	-	+/-	+/-	+/-	+	+	-	-	-/+	-/+	+/-	n.v.	+
Cynodontium cf.	-	-	-	-	+	+	+	-	+/-	+/-	+	+	+	+	+/-	+/-	+	+	+	+	+	-	-	-	+/-	+/-	+/-	+
Triticum aestivum	-	-	-	-/+	+	+/-	+	P-	-	-/+	+	+	+	+	-	-	-/+	+/-	+	+	+	-	-	-/+	+	+	+	+
Arabidopsis halleri	-	P-/+	P-/+	-/+	-/+	+/-	+	-	-	-	-/+	+/-	+	+	-/+	-/+	+/-	+/-	+	+	+	-	-	-	-	+/-	+	+

Table 10 – Results of plasmolytical analysis

3.5: Germination tests

3.5.1: Germination rate:

Concentration	Cr	Ni	Cu	Zn
1E-01	80%	50%	40%	100%
1E-02	100%	100%	90%	100%
1E-03	90%	100%	90%	100%
1E-04	100%	100%	100%	100%
1E-05	100%	100%	80%	100%
1E-06	90%	100%	100%	100%
1E-07	90%	80%	100%	100%
1E-08	100%	100%	100%	100%
0E+00	100%	100%	100%	100%

Table 11 – percent of seeds germinated

As seen in Table 11, it seems that zinc has no negative effect on the germination of wheat caryopsis. All the other heavy metals which were tested show, the higher the heavy metal concentration, the lower the germination rate were. To make significant conclusions, it would be necessary to repeat the experiment with a bigger number of caryopsis, because within ten caryopsis the possibility of runaway values, which would explain why there were lower germination rates in lower concentrations.

3.5.2: Chlorophyll- fluorescence

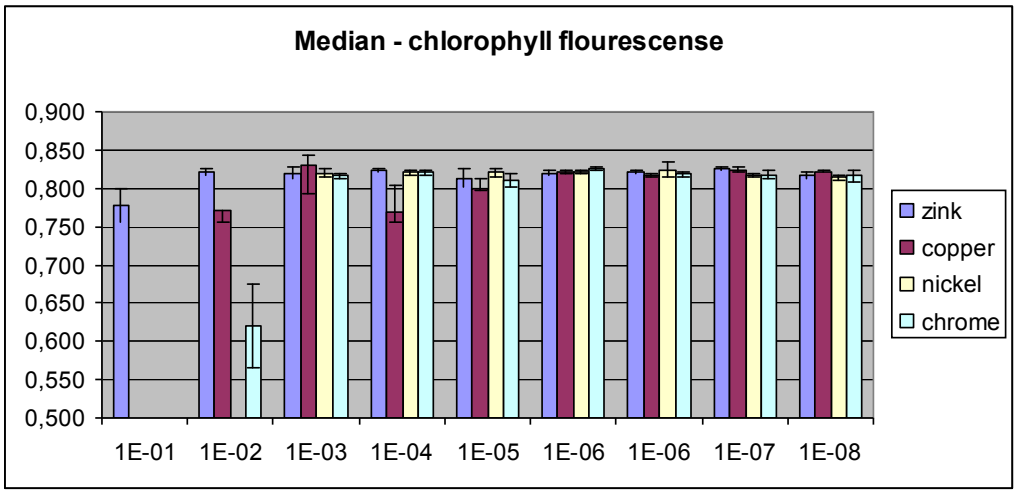


Fig. 8 –Medians of chlorophyll fluorescence measurements

Within the chlorophyll fluorescence there is no trend visible. At high concentrations no data is available, because most of the shoots were too small to make the measurements.

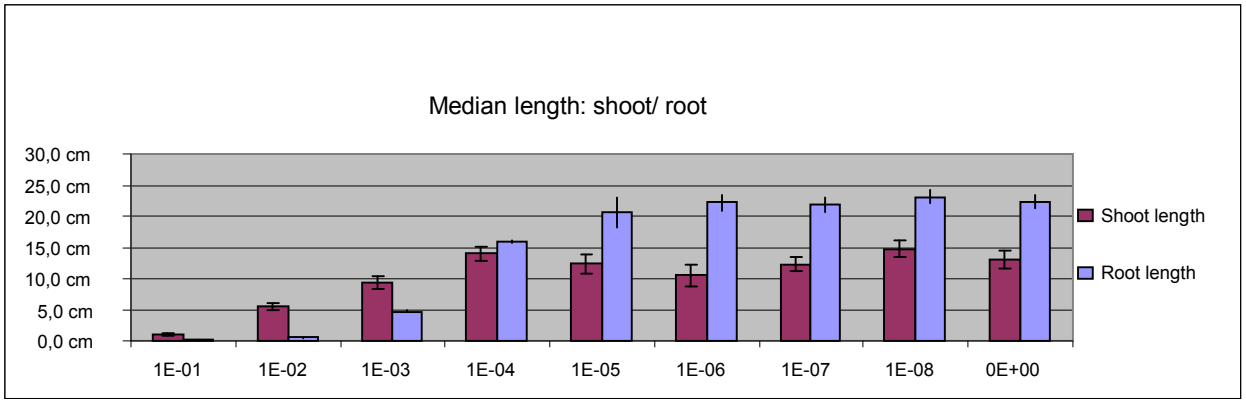


Fig. 9 – Medians of root/ shoot length of wheat seeds germinated in zinc solution

In Fig. 9 it can be seen that the higher concentration the shorter shoot and root grow. It can also be seen that the plants, which grew in a concentration of 10^{-6} had a shorter shoot than those, which grew in higher concentrations.
Relating to the root length it is obvious that the higher the concentration the shorter the roots grow.

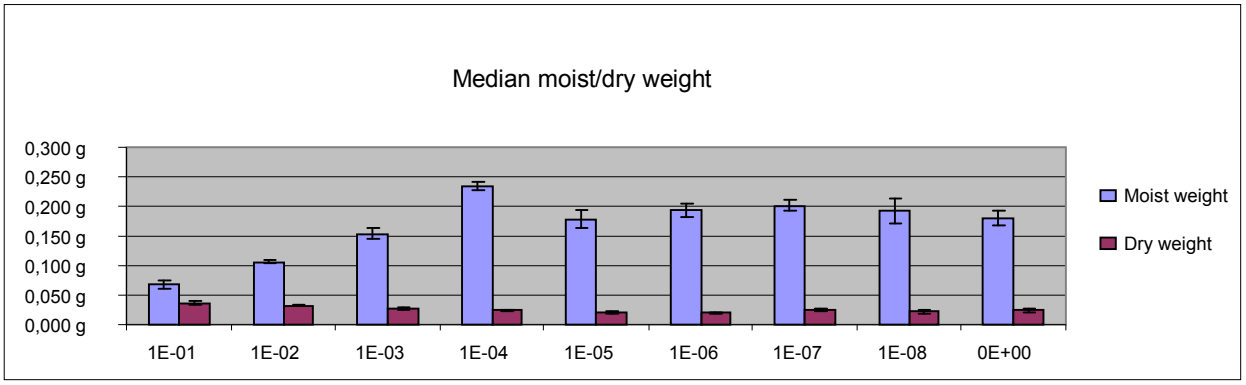


Fig. 10 – Medians of moist/ dry weight of wheat seeds germinated in zinc solution

Fig. 10 shows that the dry weight seems to get higher, if the plants are grown in more concentrated solutions. The moist weight however does not show a linear trend until a concentration of 10^{-4} is reached. In higher concentration the fresh weight reduces drastically with rising heavy metal concentration in the nutrient solution.

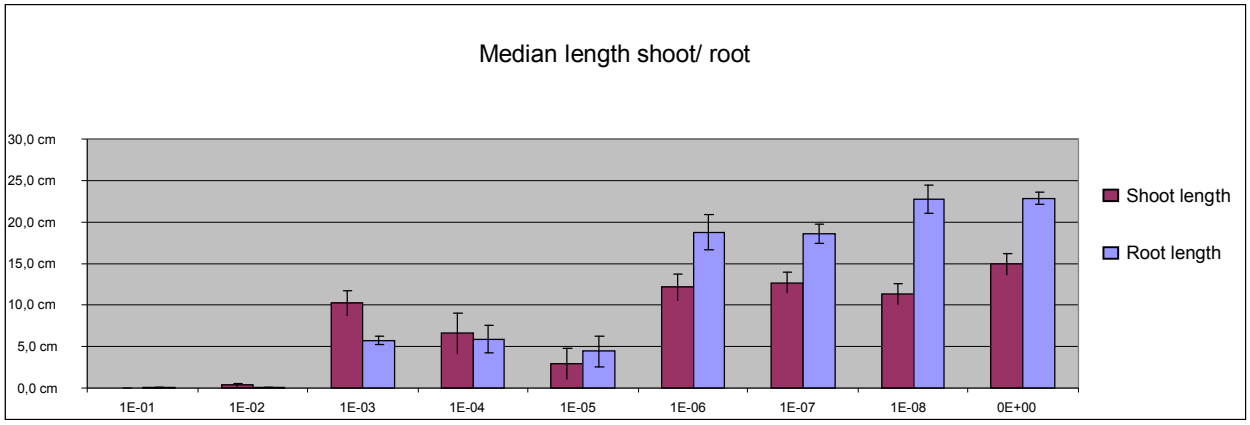


Fig. 11 – Medians of root/ shoot length of wheat seeds germinated in copper solution

Fig. 11 shows that within the copper solutions the root and shoot length are correlating. In the highest concentration the plants show nearly no growth. It can be seen that the plants had bigger problems with the concentration of 10^{-5} than with 10^{-4} or 10^{-3}.

3.6. Soil analysis - photometric determination of humus content

sample no.	site	pH	humus percentage
1	H.W. – H1	3,44	23,7 %
3	H.W. – H1	3,45	12,1 %
4	H.W. – H2	3,02	11,1 %
5	H.W. – H3	3,78	15,8 %
6	H.W. – UH	4,37	12,2 %
7	H.W. – TW	6,95	12,2 %
8	R.S. – SS	5,11	16,0 %
9	R.S. – SS	5,45	10,1 %
10	R.S. – SS	5,14	19,2 %
11	R.S. – SS		21,6 %
12	R.S. – SS	5,45	3,4 %
13	R.S. – SS	5,47	7,1 %
14	R.S. – SS	5,16	18,0 %
16	R.S. – OR	5,52	61,7 %
17	R.S. – OR	4,87	59,6 %
18	R.S. – FF	5,08	4,0 %

Table 12 – humus content& pH values of different soil samples

H.W.	Hirschwang
R.S.	Redlschlag
H1-3	Heap 1-3
UH	under heap
TW	Törlweg
SS	Steinstückl
OR	Ochsenriegel
FF	Föhrenwald

Legend 3 – Abbreviations used in Table 12

AsTable 12 shows, there was a wide range of humus content in the different samples. The lowest percentage was about 3,4% and the highest about 61,7 %. This high difference is caused by the difference between the locations where the samples were collected. Some of them were on the heap, where just a few plants were able to grow; some others were in the forest.

4. Discussion

4.1. EDX- Analysis:

The results show that *N. caerulescens* shows clear hyperaccumulation of zinc and nickel. It was already known that *N. caerulescens* is a hyperaccumulator of zinc, but in the samples we examined, the concentration of Ni was up to 4 times higher than the concentration of Zn.
Because until now N. caerulescens was described as a zinc hyperaccumulator, it should be examined under which conditions *N. caerulescens* stores more nickel than zinc.
As seen in Table 1 the main part of the metals taken up by the plant is stored in the leaves, particularly in the epidermal layers of the rosette leaves. This preference could be due to the plant's ambition to store the toxic metals as far from metabolic activities as possible. The inner cell layers where photosynthesis and other important reactions take place show comparatively small metal concentrations.
In *N. goesingense* the highest concentrations are found in the epidermal layers of rosette and in contrast to *N. caerulescens* also in the stem leaves. In this plant the preferred metal is also Ni.
The higher levels of metals in rosette leaves can be explained partly by the fact that these parts of the plant survive two years or longer and the stem is annual; so the rosette leaves are exposed to a longer duration of metal stress. Furthermore the rosette leaves are closer to the ground and therefore more exposed to resuspension, where metal particles enter the plant through the stoma.
Tables 2 & 4 show the correlation in uptake of Zn and Ni in different organs of *N. caerulescens* and *N. goesingense*. A clear correlation is notable which could mean that these two elements are treated quite similar by the plant.
Furthermore Fig. 1 shows clearly that in the rosette leafs the plant accumulates Ni & Zn in a much higher portion than any other metal, whereas in the stem the portion of metals is nearly the same. This is because the leaves are used as storage areas and the stem just transports material.

For future Projects it would be interesting, to see if metal is also transported and stored in the seeds and possibly passed on to following generations.

4.2 AAS& ICP- MS:

The measurements in AAS & ICP – MS showed that the examined plants had different strategies to cope with the heavy metal stress caused by the contaminated soil.
Those were:

- Hyperaccumulation
- Accumulation
- Exclusion

Hyperaccumulation is a phenomenon spread throughout the Family *Nocceae* (*Thlaspi*) (Reeves 1988), and was confirmed in our experiments, but rather unusual is that *Nocceae caerulescens* hyperaccumulates Ni. The exact circumstances under which this happens are still unknown to us and will be the subject of further research.
Also unexpected was the lead accumulation (BCF <1) of *R. acetosella, A. halleri* and *S. nutans,* which could be because of the elevated concentrations of available lead in the soil, but this could be subject for further research.
Quite generally in accumulators the amount of metals is higher in the shoot than in the roots, because the metals are complexed in the roots and then transported in leaves and sometimes even the seeds to store them. There is even the theory that the plants use heavy metal accumulation as protection against herbivores or pathogens, because metals in these concentrations are toxic for many organisms (Boyd 2007).

A summary of which plant uses which strategy givesTable 13.

Hyperaccumulator:	Accumulator	Excluder:
N. goesingense (Ni) *N. caerulescens* (Ni, Zn)	*R. acetosella* (Zn, Pb) *A. halleri* (Zn, Pb) *S. nutans* (Zn, Pb)	*S. vulgaris* (Ni, Cu, Zn) *R. acetosella* (Cu) *S. nutans* (Cu)

Table 13 – Strategies used by Plants

4.3 Anatomical analysis:

In many plants heavy metal stress causes the development of distinct anatomical features, even though in some cases it's not yet known which advantages these adaptations bring.

Arabidopsis halleri:

Especially the bottom side of the leaves is covered in two- pointed trichomes, which show crystalline enclosures in their vacuoles when closely examined. It seems quite logical that the plant uses these structures to store complexed metals, because there they are far away from any metabolic processes. Furthermore they may be used to deter herbivores.

Rumex acetosella:

In R. Acetosella we discovered that the leaves were covered with small gland cells. Such gland cells can be a good way to excrete unwanted substances and simultaneously deter herbivorous animals.

We also saw some enclosures in the intercellulars between some of the parenchymatic cells, which could serve as storage points for metal ions, but it was not possible for us to prove this thesis.

Vaccinium myrtillus

In *V. myrtillus* the most prominent anatomical feature helping the plant to survive in contaminated soils is not produced by the plant itself, but by ericoid mycorrhizal fungi. The hyphae of these fungi form a dense net around the roots and even grow inside the epidermal cell layers of the plant. So a big portion of the water and the minerals the plant takes up, have already passed through the mycorrhizal hyphae. The fungus itself is quite tolerant against heavy metals, so it either stores the metals and does not pass them on to the plant or completely excludes them in order to help its host to survive even under circumstances normally unsuitable (Martino, et al. 2000). Such forms of symbiosis are quite often observed in plants and fungi.

Nocceae caerulescens & N. goesingense

No special anatomical adaptations were found, which leads us to the conclusion that these plants are heavy metal tolerant because they developed chemical pathways, which allow them to complex the metal ions and therefore show no visible anatomical features.

Silene nutans

S. nutans had quite big trichomes which could be used as a storage or excretion mechanism.

4.4 Plasmolytic tolerance analysis

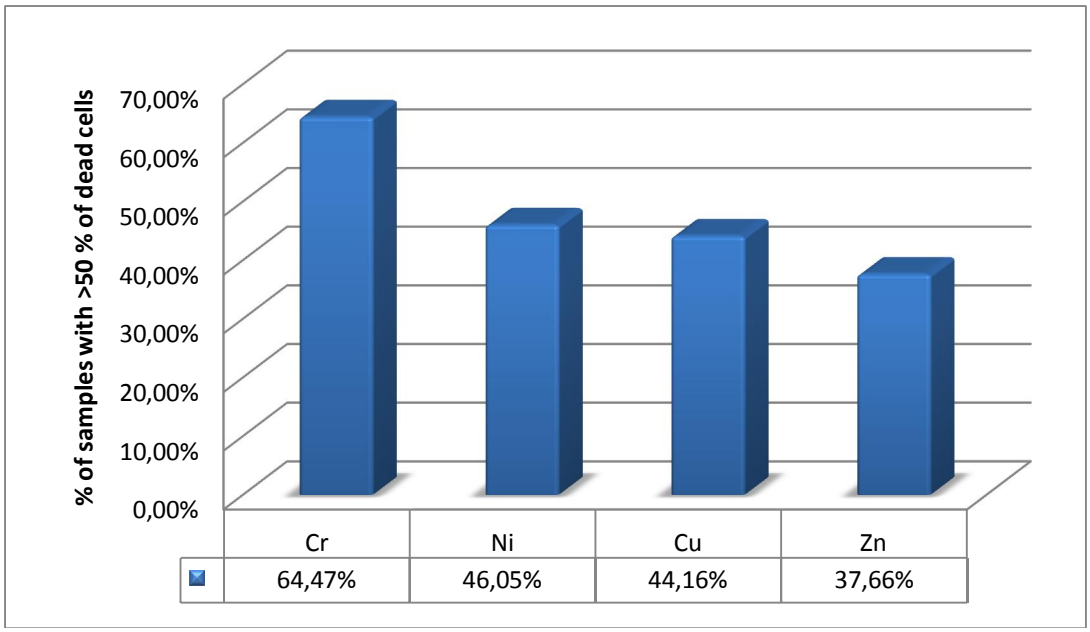


Fig. 12 – Percentage of Samples with mostly dead cells

Looking at Fig. 12 it's quite evident that chromium was the most toxic, even at low concentrations, for the plants we analysed. No plant sample showed any sign of living at concentrations higher than 10^{-3}.

Even *N. minimum*, which seems to be very resistant to all other metals, was not able to survive Concentrations higher than 10^{-4} in the chromium solution.

It is quite notable that the two *Nocceae* species (N*. goesingense and N. caerulescens*), despite their resistance against heavy metal contaminated soils, are quite sensitive if their cells are exposed directly to metal solutions. This is due to the fact that under normal conditions (the plant grows on metal contaminated ground); the metal ions do not reach the leaves in uncomplexed form; this leads to the conclusion that the metals are bound in complexes as soon as they enter the roots.

Copper in low concentrations seems to be quite tolerable on cellular level. Concerning that Cu is a micronutrient for all plants this was expectable.

The same should be and in general is true for zinc, but *N. caerulescens & N. goesingense* are sensitive to zinc even in very low concentrations.

4.5 Germination tests

4.5.1: Zinc

It seems that zinc does not have any negative effects on the germination of wheat caryopsis. On the other hand it is clear that the high concentrations of zinc had a strong negative effect on the shoot and root length. Between the concentration 10^{-5} and 10^{-6} it seems that zinc is even more toxic than at a concentration of 10^{-4}.

It can be seen that at the highest concentrations the dry weight, is higher than at lower concentrations, and on the other side the moist weight gets lower, the higher the concentration gets.

Theories why the values for moist weight are getting lower with rising metal concentration, while the dry weight values are slightly increasing include that the plant grows slower under stressful conditions and therefore produces smaller cells containing comparatively much cell wall material. Another explanation for the rising dry weight values is that the plants take up metal ions, which bind to the cell walls making them notably heavier.

4.5.2: Copper

Our experiments show that copper has a strong negative effect on the germination and growth of non resistant plants like *Triticum aestivum*. At the highest concentrations the caryopsis, which sprout, showed very little shoot and root growth. At a concentration of 10^{-5} the negative effects were even higher than at 10^{-4} and 10^{-3}.

4.5.3: Nickel

The plants which grew in the nickel solution showed as stronger damage as higher the concentration was. The negative effect on the root growth was even stronger than on the shoot growth.

Also the moist weight, was as lower as higher the nickel concentration were.

On the other hand, the dry weight was more or less stabile trough all the concentrations.

4.5.4: Chrome

It is not really clear if chrome has a negative effect on the germination rate. Because the number of caryopsis we used in our test series was just ten per concentration, just one runaway value could manipulate the results a lot.

The root growth was the highest at a concentration of 10^{-6}, which could show a stimulating effect of chrome at the root growth, within a special chrome concentration.

Again the dry weight is the highest within the highest heavy metal concentration.

There is no trend visible at the moist weight, beside the very little weight at the very high concentrations.

All in all it is to say that it seems that heavy metals do not have a strong negative effect on the chlorophyll fluorescence, excepting very high concentrations.
On the other hand it is obvious that heavy metals have a strong influence on germination (except nickel), and growth on non resistant plants.
At the example of chrome, it seems that these effects could also be positive on the growth rate, but in general they are not.
The test series showed that there is a trend, that the higher the heavy metal concentration was, the lower the roots and shoots grew.
Also the moist weight was decreasing the higher the concentrations were.
An interesting result was that the influence of zinc and copper within the concentrations around 10^{-5} was even worse than from a higher concentration. There is the theory that there could be kind of a death zone within a special range of heavy metal concentrations. That theory says that plant cells are able to build kind of a protecting layer, if the heavy metal concentration is strong enough. So it seems that the reason for those death zones is that the concentrations are not high enough for the plants to build their protecting layer, but high enough to harm the plants (Url 1956).

4.6: Soil analysis - photometric determination of humus content

The results of the humus extraction show, that the humus percentage of the soil, on which plants are growing is very diverse.
Trying to look for a correlation between available metal, we found that there is no significant correlation between the available metal content and the percentage of humus in the soil.

For future projects it would be an interesting point, to find out if the humus content is correlating to the influence heavy metals have on the plants.
It is possible that with high humus content, plants are able to tolerate higher heavy metal contents in the soil, or would it be possible that low humus content makes it possible for plants to live with a higher heavy metal concentration?
Perhaps a high percentage of humus makes it easier for the plants to balance the heavy metals, because more nutrients a plant get the healthier it becomes.
So the question is obvious whether high humus content would support plants on heavy metal locations or not.
This would offer the possibility to minimize erosion and elution by a protective layer of plants, just by adding humus.
It could also be that high humus content in the soil makes more heavy metals available for the plants, and they get even bigger problems to grow in such areas. If that would be true high humus percentage could also cause a higher percentage of heavy metals in the ground water, which would not be good.

So there are a lot of questions not answered yet, and a lot of topics for further research.

5. Literature

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