

## BIOTESTING OF HEAVY METAL POLLUTION IN THE SOIL

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**Abstract: Biotesting of heavy metal pollution in the soil.** Chemical pollution of the environment increases sharply globally, and one of the most significant indicator of this increasing pollution is the accumulation of heavy metals in the soil. In mobile forms they can enter the food chain, damaging the environment seriously and posing a risk to human health.

The subject of this work is justified by the fact, that a plant biotest method representing the toxicity of heavy metals to the ecosystem, characterizing the heavy metal pollution of soils and applying plant parameters is not available. Thus, it was to develop a laboratory plant biotest method and assessing system suitable to characterize heavy metal polluted areas, on test soils contaminated by cadmium, lead and copper, applying perennial rye-grass (*Lolium perenne*), as test plant.

The basis for a plant biotest method suitable to characterize heavy metal polluted soils was the rapid seedling biotest method developed by Nooman & Füleky (1991/1992).

In the experiments the number of heavy metal loading levels was increased, applying 0-, 0,75-, 1-, 2- 4× loading levels. 1× loading levels were identical for all three heavy metals with “B” (pollution) limit levels, calculated for air dried soil. A 0× loading means unloaded soil with natural heavy metal content.

In addition to shoot heights other plant physiological parameters were tested as well, i.e. green and dry masses and humidity of the shoots, the mass of the roots and heavy metal content taken up by the shoots and the roots were measured.

In the test plants used, the increase of the heavy metal content could be undoubtedly correlated to heavy metal loading levels.

The greatest impacts of increased heavy metal loading were observed in the heavy metal content taken up by the shoots and the roots. Heavy metal contents taken up by the shoots can be much more sensitive indicators of heavy metal pollution of soils, than the numerical values of soil limit levels.

Our results with Cd, Pb and Cu loading of test soils have shown that shoot height reduction of perennial rye-grass (*Lolium perenne*) can be a well treatable and sensitive indicator of heavy metal pollution level of soils, as it is characteristic for the inhibiting effect of heavy metal pollution and for the acute toxicity level as well. Acute toxicities of heavy metal loading could be easily monitored by the decreasing of green mass.

**Keywords:** ryegrass, copper, cadmium, lead, soil, ecotoxicology

## 1. INTRODUCTION

The certified methods of ecotoxicological analysis currently used in Hungary were almost all elaborated for lower organisms (bacteria, alga, *Daphnia*, fish, nematodes, seedlings) and, with the exception of the *Pseudomonas fluorescens* test, they were designed for establishing the quality of dangerous waste. It is only in recent years that they have been applied in soil analysis for environment protection purposes. Rapid biotest methods based on higher plants have mainly concentrated on analysing the nutrient uptake of the plants (Nooman & Füleky, 1991/1992). The development of methods designed to test the effect of toxic heavy metals on higher plants is still in its infancy. The responses of plants to heavy metal pollution have in most cases been examined in the aboveground plant organs, ignoring the enormous differences in the quantities of heavy metals accumulated in the root systems of various plant species.

The regulations valid in Hungary for the protection of groundwaters and soil (Government Decrees Nos. 10 and 33/2000. and 219/2004., based on the EU Water Framework Directive (WFD) guidelines) contain the following limit values:

(A) background concentration: representative value; the concentrations of various materials that generally occur in subsurface waters or soil in the natural or near-natural state;

(B) lower limit of pollution: the concentration of a contaminant in the subsurface water or soil above which the subsurface water or soil is legally classified as polluted. This value was determined taking into consideration the multifunctionality of the soil and the sensitivity of subsurface waters to pollution.

The environment protection (B) limit values for heavy metals in the soil are: Cd: 1, Cu: 75, Pb: 100 mg/kg.

A more differentiated toxicological or ecotoxicological characterisation can be achieved using the EC<sub>10</sub>, EC<sub>20</sub>, EC<sub>50</sub> and EC<sub>90</sub> values leading to 10, 20, 50 or 90% inhibition. These values indicate the percentage reduction compared with the untreated control.

Opinions differ on several questions related to the effect of plant-specific and environmental factors on the mechanism of heavy metal uptake. In addition, many details of the heavy metal uptake process have yet to be clarified. Metal uptake by plants may depend on the following plant-specific factors (Macnicol & Beckett, 1985): the plant species and variety, the age of the plant, the growth rate, the size and depth distribution of the roots, the transpiration coefficient, the nutrient requirements of the plant.

Based on their element uptake, plant species can be divided into four chemotaxonomic groups: excluders, indicators, accumulators and hyperaccumulators (Whiting, 2000). In excluders the transfer of elements into the shoot is negligible, while the element concentration in the shoots of indicators gives a good indication of the level of soil pollution. Accumulators and particularly hyperaccumulators are able to concentrate large quantities of metals in the shoots (Baker, 1981).

The accumulation of metals in non-hyperaccumulating plants is especially dependent on environmental factors, as they are only capable of absorbing readily available metal fractions (Mcgrath, 1998). Hyperaccumulating plants are characterised by a shoot heavy metal concentration exceeding the threshold value for the given metal

and by the ability to absorb less soluble metal fractions.

The heavy metal content of plants depends on the state of development, the plant organ and the sex (in dioecious plants). The element contents in young plants may be an order of magnitude higher than in flowering, and especially in mature plants. In general the major part of the metals absorbed remains in the roots, and only a small proportion is transferred to the aboveground parts (Huang et Cunningham, 1996; Pichtel et al., 2000). In the same way, the vegetative parts, particularly the foliage, generally have higher element content than the seeds or berries (Csathó, 1994; Kádár, 1995).

Heavy metal toxicity and plant tolerance may be influenced by many factors. According to Pais (1991), if an element becomes toxic above a given limit concentration, it may cause damage to a particular plant organ, or to the growth or metabolism of the plant. Below a critical soil concentration, non-essential elements do not exhibit any effect, but above this value they are potentially toxic.

## 2. MATERIALS AND METHODS

Table 1. The physical and chemical parameters of the test soil.

Measured component	Brown forest soil with alternate layers of clay, formed on sandy bedrock; (Inke) horizon A
Mechanical composition	
Sand %	77.9
Silt %	15.1
Clay %	7.0
humus %	2.02
Upper level of plasticity ( $K_A$ )	25
$pH_{DV}$	5.7
$pH_{KCl}$	4.89
Hydrolytic acidity ( $y_1$ )	8.27
Exchangeable acidity ( $y_2$ )	0.33
Dry matter content (%)	94
Metal content (extracted with 2M $HNO_3$ ) mg/kg dry matter	
As	0.25
Cd	0.102
Cr	0.92
Cu	5.99
Hg	<0.001
Ni	0.40
Pb	6.13

Smooth plastic pots (volume: 500 ml, height: 40 mm, diameter: 120 mm) were each filled to a thickness of approx. 2 cm with 200 g soil (Tab. 1.), previously passed through a 2 mm sieve, and five levels of Cd, Pb and Cu pollution were adjusted, each in three replications. In each case the 1× treatment was equivalent to the limit concentration for soil pollution (B), as follows (Tab. 2.).

Table 2. Level of heavy metal pollution

Element	mg/kg soil				
	0 x	0.75x	1 x	2 x	4 x
Cd	0	0.75	1	2	4
Pb	0	75	100	200	400
Cu	0	56.2	75	150	300

To achieve these levels of pollution, stock solutions were prepared containing 0, 0.75, 1, 2 or 4 mg/ml Cd in the form of cadmium acetate [ $\text{Cd}(\text{CH}_3\text{COO})_2 \times 2 \text{H}_2\text{O}$ ], 0, 75, 100, 200 or 400 mg/ml Pb in the form of lead acetate [ $\text{Pb}(\text{CH}_3\text{COO})_2 \times 3 \text{H}_2\text{O}$ ] or 0, 56.2, 75, 150 or 300 mg/ml Cu in the form of copper sulphate ( $\text{CuSO}_4 \times 5 \text{H}_2\text{O}$ ). Fifty ml distilled water containing 0.2 ml of the relevant stock solution was then mixed into the 200 g soil samples.

In the greenhouse, 2 g cotton-wool was placed in each of 45 plastic pots (volume: 500 ml, height: 40 mm, diameter: 120 mm) and moistened with approx. 50 ml distilled water. Each pot was planted with 2 g English ryegrass (*Lolium perenne*), spreading the seeds evenly over the cotton-wool, and the seedlings were grown for 14 days. The cotton-wool pads containing the seedlings were then placed on the polluted test soil in the bowls, which were kept in a random block design at 20°C with a relative humidity of 60%, 10 h/day illumination, and ad libitum irrigation for 10 days.

When placed on the test soil, plants grown in a nutrient-deficient environment exhibit rapid nutrient uptake, including that of heavy metals present in available form. Depending on the mobility of the heavy metals within the plant, they are accumulated to varying extents in the root system and shoots.

On the 10<sup>th</sup> day, after an adequate period of soil-plant contact (which depends on the plant species), the cotton-wool containing the test plants was removed from the soil and the mean shoot height (mm) was recorded. The shoots were then cut from the cotton-wool, and the fresh and dry mass (g/pot) and the shoot moisture content (%) were determined.

Air-dry shoot samples (0.5 g) were then hydrolysed in 10 ml 2 M HCl for 3 h at 105°C. After cooling, the solution was made up to 10 ml with distilled water, homogenised and filtered.

After determining the root mass, 1 g samples of dried, chopped roots were hydrolysed in 15 ml 2 M HCl for 3 h at 105°C. After cooling, the solution was made up to 20 ml with distilled water, and filtered after shaking.

The Cd, Pb and Cu contents of the hydrolysed shoot and root samples were then determined using an ICP spectrometer (type ICAP 61E).

### 3. RESULT AND DISCUSSION

At 0.75× Cd pollution level a 10% reduction was recorded in the fresh mass of English ryegrass (Tab. 3., 4., 5., 6.), while the soil limit value led to a decline of over 20% in this parameter. Higher pollution levels led to a further reduction. The decrease was statistically significant (b value: -0.64; R<sup>2</sup> value: 0.73).

Table 3. Quantities of cadmium accumulated by the ryegrass test plant, as a function of the pollution level

Measured parameter	Level of Cd pollution, mg/kg air-dry soil					
	0	0.75	1	2	4	LSD <sub>5%</sub>
Cd content absorbed by shoot samples in terms of dry matter (mg/200 g soil)	0.30	0.56	1.29	1.92	4.48	0.28
Shoot Cd content (mg/kg D.M.)	0.46	0.93	2.22	3.29	7.91	0.45
Cd content absorbed by root samples in terms of dry matter (mg/200 g soil)	0.23	2.10	1.90	6.00	12.6	0.30
Root Cd content (mg/kg D.M.)	0.20	1.91	2.01	6.50	14.7	0.20

Table 4. Effect of cadmium pollution on the shoot and root mass of the test plant

Measured parameter	Level of Cd pollution (mg/kg air-dry soil)					
	0	0.75	1	2	4	LSD <sub>5%</sub>
Fresh shoot mass (g/200 g soil)	6.33	5.58	4.27	3.99	3.54	0.11
Damaging effect (%)*		11.8	32.5	37.0	44.7	
Dry shoot mass (g/200 g soil)	0.65	0.60	0.58	0.58	0.57	0.02
Damaging effect (%)*		7.7	10.8	10.8	12.3	
Shoot moisture content (%)	89.7	89.3	86.4	85.4	84.0	0.10
Damaging effect (%)*		0.45	3.7	4.8	6.4	
Shoot height (mm)	106	100	96.3	85.7	74.7	3.30
Damaging effect (%)*		5.7	10.1	19.1	29.5	
Root mass (g/200 g soil)	1.15	1.10	0.95	0.92	0.86	0.02
Damaging effect (%)*		4.4	17.4	20.0	25.2	

Note: \*Damaging effect: % reduction compared with the control

Table 5. Estimated linear correlations for cadmium pollution in plant biotest on ryegrass

Measured parameter	Evaluated parameter	
	Regression coefficient (b value)	Coefficient of determination (R <sup>2</sup> value)
Fresh shoot mass	-0.64	0.73
Dry shoot mass	-0.02	0.58
Shoot moisture content	-1.44	0.80
Shoot height	-7.83	0.96
Absorbed Cd content in the shoot	1.07	0.97
Shoot Cd content	1.90	0.97
Root mass	-0.07	0.75
Absorbed Cd content in the root	3.18	0.99
Root Cd content	3.76	0.98

A reduction of over 10% in the shoot height of English ryegrass (EC<sub>10</sub>) was not observed until the soil limit value was reached, while a reduction of over 20% (EC<sub>20</sub>) was recorded when Cd pollution was increased to 2 mg/kg D.M. The decline in the shoot height was significant at all levels, and the coefficient of determination (0.96) also indicated that the reduction in the shoot height of English ryegrass could be associated at a probability level of 96% with the increasing level of Cd pollution in the

soil. The negative slope of the trend line representing the toxic effect was also the greatest, compared with the b values of the other plant physiological parameters tested.

As the Cd level in the soil increased, the root mass of the test plants responded very sensitively to the toxic effect. The soil limit value caused acute toxicity at the EC<sub>10</sub> level, while a value of EC<sub>20</sub> was observed at a Cd pollution level of 2 mg/kg D.M.

However, the applicability of this plant physiological parameter is questionable, as the reduction in root mass did not reach the significant level as the Cd pollution level rose, and the b value was very low.

Table 6. Toxicological evaluation of cadmium pollution in plant biotest on ryegrass

Parameter tested	EC values		
	EC <sub>10</sub>	EC <sub>20</sub>	EC <sub>50</sub>
Fresh shoot mass	0,75x pollution	1x pollution	(-)
Dry shoot mass	1x pollution	(-)	(-)
Shoot moisture content	(-)	(-)	(-)
Shoot height	1x pollution	2x pollution	(-)
Root mass	1x pollution	2x pollution	(-)

Legend: (-): A 10, 20 or 50% reduction in this parameter compared with the control was not observed.

The results suggest that measurements on reductions in the shoot height and fresh mass of English ryegrass could be a suitable plant biotest for obtaining rapid information on the toxic effect of soils polluted with cadmium.

The intensity and quantity of Pb uptake by the test plants (Tab. 7.) were much lower than the values recorded for Cd in the shoots. Nevertheless, the coefficient of determination indicated an 80–90% probability that the rising trend of Pb accumulation could be attributed to Pb pollution there were considerable differences in the Pb quantities accumulated in the shoots of English ryegrass at the various pollution levels.

Table 7. Quantities of lead accumulated by the ryegrass test plant, as a function of the pollution level

Measured parameter	Level of Pb pollution, mg/kg air-dry soil					
	0	75	100	200	400	LSD <sub>5%</sub>
Pb content absorbed by shoot samples in terms of dry matter (mg/200 g soil)	1.14	11.1	17.6	28.6	32.5	1.2
Shoot Pb content (mg/kg D.M.)	1.8	16.8	26.1	42.3	51.3	1.8
Pb content absorbed by root samples in terms of dry matter (mg/200 g soil)	5.83	107	120	135	281	2.2
Root Pb content (mg/kg D.M.)	5.0	100	117	142	329	1.3

As the level of soil Pb pollution rose, the tendency for Pb to be accumulated in the roots was far greater than for the shoots (Tab. 8, 9, 10.). This intensity of Pb uptake is demonstrated by the values of the coefficients (b value: 0.62; R<sup>2</sup> value: 0.93).

As in the case of Cd pollution, the soil Pb contamination could not be adequately detected from the statistical and/or ecotoxicological point of view on the basis of the dry matter or moisture content of the shoots. It should be noted, however,

that when the mean dry mass of English ryegrass samples originating from unpolluted soil was compared with that of test plants exposed to Pb pollution, data from the literature (Csathó, 1994) indicating that the dry matter content of the plants increased in response to heavy metal pollution were confirmed up to a soil pollution level of 200 mg/kg D.M. At the highest Pb pollution rate applied (4×), however, severe toxic symptoms were observed on the test plants.

Table 8. Effect of lead pollution on the shoot and root mass of the test plant

Measured parameter	Level of Pb pollution (mg/kg air-dry soil)					
	0	75	100	200	400	SzD <sub>5%</sub>
Fresh shoot mass (g/200 g soil)	6.36	5.65	5.33	5.20	4.32	0.12
Damaging effect (%)*		11.2	16.2	18.2	32.1	
Dry shoot mass (g/200 g soil)	0.65	0.66	0.67	0.68	0.63	0.02
Damaging effect (%)*		-1.5	-3.1	-4.6	3.1	
Shoot moisture content (%)	89.8	88.3	87.4	87.0	85.4	0.1
Damaging effect (%)*		1.7	2.7	3.1	4.9	
Shoot height (mm)	106	96.0	89.0	79.3	67.0	2.3
Damaging effect (%)*		9.4	16.0	25.5	36.8	
Root mass (g/200 g soil)	1.17	1.08	1.03	0.96	0.85	0.02
Damaging effect (%)*		7.7	12.0	18.0	27.4	

Table 9. Estimated linear correlations for lead pollution in plant biotests on ryegrass

Measured parameter	Evaluated parameter	
	Regression coefficient (b value)	Coefficient of determination (R <sup>2</sup> value)
Fresh shoot mass	-0.005	0.92
Dry shoot mass	-0.000005	0.19
Shoot moisture content	-0.01	0.90
Shoot height	-0.09	0.94
Absorbed Pb content in the shoot	0.076	0.83
Shoot Pb content	0.12	0.87
Root mass	-0.0007	0.94
Absorbed Pb content in the root	0.62	0.93
Root Pb content	0.75	0.96

A reduction of more than 10% in the shoot height of ryegrass (EC<sub>10</sub>) was first observed in the ecotoxicological evaluation when the pollution level reached the soil limit value, while the decrease averaged more than 20% (EC<sub>20</sub>) when the soil was polluted with 200 mg/kg Pb and reached the EC<sub>30</sub> value at the 4× soil pollution rate. A biometric evaluation of the results indicated that the reduction in shoot height was consistently significant, while the R<sup>2</sup> value of this plant physiological parameter demonstrated that more than 90% of the reduction in shoot height in ryegrass in this soil-plant system could be attributed to the toxic effect of Pb pollution. At the same time, the steepness of the trend line (b value) depicting changes in the shoot height of ryegrass in response to Pb pollution was smaller than that obtained for Cd pollution, probably due to the limited mobility of Pb.

A rise in the soil Pb content had an extremely toxic effect on the root mass of the test plants, demonstrating the damaging effect of Pb accumulation in the root system. The ecotoxicological analysis revealed that the soil limit value led to acute toxicity at the EC<sub>10</sub> level in the root mass of ryegrass, as in the case of Cd pollution.

Table 10. Toxicological evaluation of lead pollution in plant biotest on ryegrass

Parameter tested	EC values		
	EC <sub>10</sub>	EC <sub>20</sub>	EC <sub>50</sub>
Fresh shoot mass	0,75x pollution	4x pollution	(-)
Dry shoot mass	(-)	(-)	(-)
Shoot moisture content	(-)	(-)	(-)
Shoot height	1x pollution	2x pollution	(-)
Root mass	1x pollution	4x pollution	(-)

The results indicate that the reduction in the shoot height of English ryegrass could be a useful parameter in mapping Pb pollution in the soil.

After the 10-day experimental period the Cu quantity absorbed by the shoots and roots of the test plants from the natural Cu content of the soil was clearly seen in the control. In response to increasing rates of Cu pollution, the Cu uptake in the shoots and roots also exhibited a rising tendency, and hyperaccumulation was observed at the highest 300 mg/kg soil pollution level, resulting in lethal toxicity (Tab. 11, 12, 13, 14).

Table 11. Quantities of copper accumulated by the test plants, as a function of the soil pollution level

Measured parameter	Level of Cu pollution, mg/kg air-dry soil					
	0	56.25	75	150	300	LSD <sub>5%</sub>
Cu content absorbed by shoot samples in terms of dry matter (mg/200 g soil)	8.61	20.8	29.4	43.1	315	1.9
Shoot Cu content (mg/kg D.M.)	13.2	29.8	39.5	67.4	610	8.9
Cu content absorbed by root samples in terms of dry matter (mg/200 g soil)	27.4	197	254	631	704	6.1
Root Cu content (mg/kg D.M.)	23.8	188	259	699	1124	2.0

Table 12. Effect of copper pollution on the shoot and root mass of the test plant

Measured parameter	Level of Cu pollution (mg/kg air-dry soil)					
	0	56.25	75	150	300	LSD <sub>5%</sub>
Fresh shoot mass (g/200 g soil)	6.34	6.00	5.83	4.07	2.83	0.14
Damaging effect (%)*		5.4	8.0	35.8	55.4	
Dry shoot mass (g/200 g soil)	0.65	0.70	0.74	0.64	0.52	0.02
Damaging effect (%)*		-7.7	-13.8	1.5	20.0	
Shoot moisture content (%)	89.7	88.4	87.3	84.3	81.7	0.1
Damaging effect (%)*		1.5	2.7	6.0	8.9	
Shoot height (mm)	106	94.0	88.0	71.0	58.0	3.4
Damaging effect (%)*		11.3	17.0	33.0	45.3	
Root mass (g/200 g soil)	1.15	1.05	0.98	0.90	0.63	0.02
Damaging effect (%)*		8.7	14.8	21.7	45.2	

As the level of soil Cu pollution increased, a considerably greater accumulation of Cu was observed in the roots than in the shoots, as also seen for Pb, with high values for the coefficients of regression and determination (b value: 2.33, R<sup>2</sup> value 0.86).

From the ecotoxicological point of view, a valuable level of damage in the fresh mass of ryegrass (35.8% reduction, equivalent to the EC<sub>20</sub> level) was only observed at a soil pollution level of twice the limit value, while the EC<sub>50</sub> level was reached at the highest pollution rate, due to the death of the plants.

Table 13. Estimated linear correlations for copper pollution in plant biotest on ryegrass

Measured parameter	Evaluated parameter	
	Regression coefficient (b value)	Coefficient of determination (R <sup>2</sup> value)
Fresh shoot mass	-0.01	0.95
Dry shoot mass	-0.0006	0.63
Shoot moisture content	-0.03	0.96
Shoot height	-0.16	0.93
Absorbed Pb content in the shoot	1.04	0.86
Shoot Pb content	2.04	0.84
Root mass	-0.002	0.99
Absorbed Pb content in the root	2.33	0.86
Root Pb content	3.82	0.98

The effect of increasing rates of Cu pollution could be clearly traced in the shoot height of ryegrass, which exhibited a significant inhibitory tendency. The extent and intensity of the reduction were also representative, since the regression coefficient (b value: - 0.16) was higher than that of the other plant physiological parameters, while the reduction could be attributed to the toxicity of Cu pollution with a probability of 93%.

The biometric analysis revealed that a Cu pollution level of 0.75× caused a reduction of more than 10% in the shoot height, while the 2× rate of 150 mg/kg D.M. Cu pollution resulted in a shoot height depression of over 30% compared with the control plants (EC<sub>50</sub> value). This tendency should be given particular attention when determining the ecotoxicological level of Cu pollution on a given area.

In the case of root mass the EC<sub>10</sub> level of acute toxicity was recorded at the soil limit value for Cu pollution.

Table 14. Toxicological evaluation of copper pollution in plant biotests on English ryegrass

Parameter tested	EC values		
	EC <sub>10</sub>	EC <sub>20</sub>	EC <sub>50</sub>
Fresh shoot mass	2x pollution	2x pollution	4x pollution
Dry shoot mass	4x pollution	4x pollution	(-)
Shoot moisture content	(-)	(-)	(-)
Shoot height	0,75x pollution	2x pollution	(-)
Root mass	1x pollution	2x pollution	(-)

#### 4. CONCLUSIONS

In the test plants used, the increase of the heavy metal content could be undoubtedly correlated to heavy metal loading levels.

The greatest impacts of increased heavy metal loading were observed in the heavy metal content taken up by the shoots and the roots. Heavy metal contents taken up by the shoots can be much more sensitive indicators of heavy metal pollution of soils, than the numerical values of soil limit levels.

Our results with Cd, Pb and Cu loading of test soils have shown that shoot height reduction of perennial rye-grass (*Lolium perenne*) can be a well treatable and sensitive indicator of heavy metal pollution level of soils, as it is characteristic for the inhibiting effect of heavy metal pollution and for the acute toxicity level as well. Acute toxicities of heavy metal loading could be easily monitored by the decreasing of green mass.

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