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AGRICULTURE AND ENVIRONMENTAL BIOTECHNOLOGY

# ECOTOXICOLOGICAL ASSESSMENT MODEL OF CULTURAL PLANT-SOIL COMPLEX TREATED WITH WASTE WATER

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## ABSTRACT

*The industrial environmental "hot spots" create significant ecological hazards for terrestrial and aquatic ecosystems. Guidelines and legislation often refer to the total amount of contamination without estimating the complex relationship between the environmental factors and the toxicant. In cases of suspicion for adverse effect on the environment bio-assessment can be used as a tool to detect the presence of hazardous chemicals. Bioassays with vascular plants are considered to be universal tools of identifying the combined effects of pollutants in ecotoxicology. The purpose of this article was to evaluate the toxicological effect of plant-soil complex treated with waste water from Radomir Metal Industries, Bulgaria. The conclusion is that the sewage from the metallurgical plant Radomir Metal is used properly for irrigation of arable land. The question can be which kind of plants are suitable to be cultivated there. The effluent seems toxic for the aquatic systems and has a slight negative impact on the soil breathing and germination of treated plants. Nevertheless, in the bioassay for all examined plants a stimulating effect on the weight of roots and stems was registered under the treatment with soil extract.*

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**Keywords:** ecotoxicology, soil pollution, soil respiration, energy of germination, germination, early development, culture plant

## Introduction

Human activities all over the world have increased environmental pollution by heavy metals in agricultural soil. Contamination with heavy metals is a major problem for crop quality, human health, and environmental quality. Most of the heavy metals are persistent in soil because of their immobile nature (4, 11). Chemical analyses are often insufficient to provide insight into the potential ecological risk, since they do not allow an evaluation of possible combined effects of the different contaminants mixed together, as well as to their bioavailabilty. Guidelines and legislation often refer to the total metal content in the soil without taking into consideration what proportion of that total amount may be biologically available to the organisms. Actually, the toxic effects are related to the bioavailability of metals in soils. Therefore, bioassays which can mitigate these constraints are recommended for the assessment of ecological risks in soils or other matrices (5).

Contaminated areas create serious environmental hazards for terrestrial and aquatic ecosystems. They are sources of pollution and may bring about ecotoxicological effects. At severely contaminated spots acute effects occur, but the core problem lies in possible long-term chronic effects (7). Effects of toxicants at high concentrations that induce a high rate of acute mortality are observed easily, even in complex communities. The effects of toxicants at low concentrations that do not immediately result in acute mortality are much more difficultly detectable. In such cases, communities are shaped not only by the effects of the toxicants but also to a great extent by other environmental factors. As a result, the complex relationship between a multitude of environmental factors and the composition of the community obscures the effects of the toxicants (13).

Ecotoxicological effects occur at all levels of the biological organization, from the molecular to the ecosystem level. Not only may certain organisms be affected, but the ecosystems as a whole in their functions and structure (6, 7). In ecotoxicology on the basis of conducted bioassays with sensitive test objects or test objects from treated ecosystems the toxic effects on the whole ecosystem are evaluated. The purpose of this study was to make an ecotoxical assessment of the culture plant-soil complex in agroecosystems treated with waste water from Radomir Metal Industries, Bulgaria, by using a set of bioassays with cultivated plants in the region.

## Materials and Methods

### Contamination of the study region

According to the World Bank report (34), Pernik/Radomir with ferrous metallurgy and cement manufacturing are included in the acute industrial environmental "hot spots" in Bulgaria. In these areas, high airborne levels of dust, sulfur dioxide, and lead are common as well as hydrogen sulfide, mercaptans, ammonia, hydrochloric acid, hydrogen fluoride, and other substances according to the particular size.

**Radomir Metal Industries**

The Bulgarian metal casting plant Radomir Metals, previously Leko Ko, is based in the south-western town of Radomir, Pernik District, Bulgaria. The climate is temperate-continental (33). The region is related to the Sofia-Kraishte Province, which is part of the Mediterranean soil region. The soils are Vertisols – Gleyic and Eutric (22). The plant is erected on 1 800 000 $m^2$ area and situated at about 50 km south-west of the capital of the Republic of Bulgaria - Sofia, on road E79 (Coordinates: 42°31'10"N 22°59'12"E). It has excellent infrastructure, connected with railway network and natural gas supplying. The plant has its own transformer electric power station directly connected to the National high voltage electric-power network. Environmental protection is one of the priorities of the company. The purification facilities fully meet the requirements of the European Standards for the quality of the air and water (24).

Overall, the environment and biodiversity in the municipality of Radomir are preserved, as there are no large industrial and agricultural pollutants, and no cross contamination. Indicative of relatively clean natural environment are the data of air soil and water samples (25).

Radomir Metal Industries has a complex permit № 145-NO/2008 within the scope of Paragraph 2.2. Annex 4 of the Law on Environmental Protection. The company has developed a policy for environment protection aimed at limiting the negative effects on the environment and prevents pollution of groundwater. Under the complex permit Radomir Metal Industries has established annual emission standards.

For the good environmental status, of importance is the functioning of the purifying station for faecal-bit and effluents from industrial plants (mainly Radomir Metal Industries, which owns the treatment plant). Purification is a mechanical and biological with drying and stabilization of sludge. Receiver of purified water is the Struma River (II - Second category receiving water). Along the outlet wastewater to the water treatment point, canal water is intensively used for irrigation. Industrial waste water is discharged to the water purification plant through a channel running through arable land. In this regard, the research in this work was done to establish whether there is compliance with the established standards.

**Test objects and parameters**

In general, the aim of bioassays is to determine the substance concentration or dilution at which 50% mortality or change in the relevant indicator occurs in the test-organisms over a determined time ($LC_{50}$, $EC_{50}$) (21). For the purpose of the study tests were conducted with different vegetable, leguminous forage and cereal crops, earthed-up and unearthed-up – *Lepidium sativum* L., *Raphanus sativus var. radicula, Medicago sativa* L. (variety Pleven), *Zea mays* L. (variety Kneja 509- hybrid, 3-th fraction) and *Triticum vulgare* Host. (variety Sadovo), which are cultivated in the region. The tests were conducted according to the relevant standards (16). In assessing the toxicity of the effluent of Radomir Metal Industries the following indicators were used: soil respiration; energy of germination and germination (*Eg* and *G*); also for early development of culture plants – length of root and stem (Lr, Ls), absolute dry weight of root and stem (Wr, Ws), and bioaccumulation of Fe and Zn in plant biomass.

**Description of the conducted tests**

Generally bioassays are used to evaluate the toxicity of wastewater, polluted air, soil, sediment, etc. or a particular pollutant using standard test organisms. The latter are exposed to different concentrations of the substance and report mortality or change in behavior or morphology and physiology of organisms. To determine the toxicity, standard protocols are followed in order to have comparability of results (16).

The conducted tests were performed with sewage and soil extract in various dilutions, as well as solutions of $ZnSO_4$ in some cases because of perceived zinc content in soil above the maximum allowable concentration (MAC).

**Soil respiration**

Soil contamination negatively affects the number and activity of populations of soil microorganisms. Contaminated soils respire more intensively during the first 30-40 min at relatively low concentrations that do not cause mass extinction, but only stress in populations. For a long time (2 to 4 hours) and at higher concentrations the respiration decreases and completely stops. Thus, for a fixed period of time – the first 40 min after contamination with the toxic substance, the degree of variation in the intensity of contaminated soils respiration from the same soil type under the same other conditions depends on the type and concentration of the contaminant.

For the correctness of the results of the bioassay (real reflection of the soil condition), it is held several times in different seasons and times of the day.

The reduced soil respiration, reflecting deterioration of the populations of soil organisms, affects the functioning of the whole community. Exposure time of the conducted test in the research was 1 hour.

**Energy of germination (*Eg*), germination (*G*), length of root (Lr), and length of stem (Ls)**

The seeds of each of the test plants (garden cress, radishes, wheat, maize and alfalfa) were flooded with water and left to stay for 24 hours. They were arranged in 5 rows with 10 seeds each at equal distances in large plates on two-layer filter paper moistened with 10 ml of distilled water (control); or soil extracts in different concentrations.

On the lid of the dish one-ply paper was placed and was wetted with 5 ml of distilled water or appropriate solutions. The filter paper and Petri dishes were pre-sterilized. Each variant and control was set at 6 repetitions. After exposure for 48 hours the length and weight of sprouts in each dish were measured. The results for each variant and control were averaged and assessed statistically as shown.

For the tests, in advance, the soil extracts were prepared by dissolving 5 g of soil in 200 ml of distilled water or effluent in the appropriate dilution. They were left on a shaker for 16 hours.

The variants of the experiments were as follows: control – distilled water, version 1 – 30% effluent, version 2 – 50% waste water; version 3 – 70% effluent, and version 4 – 100% effluent.

### Absolute dry weight of root (Wr) and stem (Ws)

The absolute dry weight of samples was measured by drying for 48 hours at 85 °C and weighing (to 0.001 g) on an analytical balance.

### Assessment of water and soil contamination

Biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were determined for the test sewage (29). BOD is most commonly expressed in milligrams of oxygen consumed per liter of sample during 5 days of incubation at 20 °C. It is often used as a robust measure of the degree of organic water pollution. However BOD does not take into account the total mass of organic substances present in the water. Therefore, to obtain a more accurate and more complete assessment of the quality of organic matter, it is necessary to determine COD. A common application of the COD test in environmental chemistry is as an indirect measure of the amount of organic compounds in water. In most cases COD is used to determine the amount of organic pollutants in surface water (e.g. lakes and rivers), which makes COD a useful measure of water quality. COD indicates the mass of oxygen consumed per liter of solution and is expressed in milligrams per liter ($mg \cdot l^{-1}$), or in parts per million (ppm) as may be seen is some older references.

Other indicators of water and soil, e.g. mechanical composition, content of elements in soil and wastewater, etc. were examined.

The oxidation was determined in the Executive Environment Agency (EEA), Lab - Water and in the Faculty of Biology - Department of Hydrobiology, Sofia University “St. Kliment Ohridski”, Bulgaria.

Other methods such as Kaczynski’s method were used for determining the mechanical composition of soil, acidity of the soil solution, humus content, etc. Soil samples were processed in the Research Institute of Soil Science and Agroecology “N. Pushkarov” - Laboratory of Soil Genesis, Bulgaria. The nitrogen content was studied in the Faculty of Biology, Sofia University “St. Kliment Ohridski”, Laboratory for Analysing Amino Acids. The study of bioaccumulation of heavy metals and others was carried on by atomic-adsorption analysis.

Soil samples were taken from the humus horizon at 50 points of the region. The mixed average sample were processed and measured in the Faculty of Chemistry, Sofia University “St. Kliment Ohridski”. The bioaccumulation of Fe and Zn was scored for the period of germination by BSS (Bulgarian State Standard) in plant biomass.

### Statistics

All obtained results were assessed statistically. It has been shown that the resulting averages are representative of the performance using t-test. The discussed values are average of 6 repetitions of each version and for 300 seeds or plants of option (50 pcs per repetition × 6 repetitions). The statistical significance level in this study was defined at $P < 0.05$.

## Results and Discussion

### Samples from wastewater

**Table 1** presents the results of some additional analysis of samples of waste water and limit concentrations of metals under Regulation № 6.

The marginal limit rates are defined in the particular circumstances of production taken into account for the manufacture of iron and steel, production of iron and steel castings, cast cars and other non-ferrous metals.

The data obtained show that the resulting concentrations are within the limits of regulation.

### Soil analysis

The results of the analysis of soil used in the tests are presented in **Table. 2**. The study of the mechanical structure showed that the soil is sandy – loam average (4.5) and secured with average humus. The humus content is 5%.

The comparison with the limit concentrations (down in the Ordinance on Soil Protection for use of sludge from waste water for agricultural purposes, Table 3), shows that they are in compliance. The only exception was the level of zinc, which exceed the maximum concentration even at the highest levels of pH.

### Soil respiration

Soil respiration consists of autotrophic root respiration and heterotrophic respiration which is associated with decomposition of litter, roots and soil organic matter (SOM). Soil respiration ($CO_2$ efflux) responds to contaminants and is a common index of soil health (10), but it is also affected by temporal changes in temperature, moisture, light conditions, and spatial variation in soil fertility (2, 3). Positive exponential relationships between soil respiration and soil temperature, as well as positive linear relationships between soil respiration and soil moisture have been found in some warm and moist forests (18). It has been accepted that under the same hydrothermal conditions soil respiration would be influenced by the degree of contamination and can be used as a test for assessment of soil contamination.

The results of the bioassay showed that all tested concentrations led to a change in the intensity of breathing of soil and affected the functioning of the communities. The impact with the lowest test concentration (30% effluent) led to a strong reduction of soil respiration by 66.70% compared to control breathing. Changes in soil respiration by 50% compared to control ($LC_{50}$) occurred between 40-50% dilutions of the effluent. Increasing the concentration caused stress reactions in

**TABLE 1**

Values of the analyzed parameters of effluent samples

| Elements | Marginal limit concentrations*, µg·l$^{-1}$ | Acidified sample, unfiltered, µg·l$^{-1}$ | Not acidified sample, unfiltered, µg·l$^{-1}$ |
|---|---|---|---|
| **Cd** | 500 | 3.2±0.2 | 2.3±0.2 |
| **Cu** | 500 | 12±2 | 8±1 |
| **Cr** | 500 | 2.8±0.2 | 2.1±0.1 |
| **Fe** | 5000 | 189±14 | 156±12 |
| **Ni** | 500 | 4±1 | 3±1 |
| **Pb** | 200 | 3.8±0.1 | 2.1±0.1 |
| **Zn** | 2000 | 230±15 | 190±12 |
| **Hg** | 10 | <1 | <1 |
| **Other indicators** | pH – 7.92<br>Chemical oxygen demand (COD) (BBM 0208:2001; t = 20$^{0}$±3) - 17.2±1.63<br>COD (oximetric)<br>20.48 mg·l$^{-1}$ – filtered sample;<br>73.10 mg·l$^{-1}$ – unfiltered sample | $NH_4^+$ - 0.2 mg·l$^{-1}$<br>$NO_3^-$ - 5.7 mg·l$^{-1}$ | $SO_4^{2-}$ - 19.5 mg·l$^{-1}$<br>Cl – 10 mg·l$^{-1}$<br>$PO_4^{3-}$ - < 0.1 mg·l$^{-1}$ |

**TABLE 2**

Values of the analyzed indicators of soil samples

| **Elements** | **Samples** | **Other characteristics** | | | | |
|---|---|---|---|---|---|---|
| **Cd, µg·$^{g}$**-1 | 1.2±0.2 | Common nitrogen Keldal, gr % | pH | Bulk density, Q | Marginal field humid capacity, % | Humidity, W, % |
| **Cu, µg·g$^{-1}$** | 32±2 | 1.208 | pH $_{H2O}$ 7.43 | 1.52 | 24 | 60.72 |
| **Cr, µg·g$^{-1}$** | 48±3 | 1. 569 | pH$_{CaCl2}$ 6.57 | | | |
| **Ni, µg·g$^{-1}$** | 11.5±1.3 | 1.500 | | | | |
| **Pb, µg·g$^{-1}$** | 28±2 | 1.444 | | | | |
| ***Zn, µg·g$^{-1}$*** | *147±25* | Average | | | | |
| **As, µg·g$^{-1}$** | 12±3 | 1.430 | | | | |

**TABLE 3**

Limit concentrations of heavy metals in the soil*

| **№** | **pH*1** | **Limits (MAC) mg·kg$^{-1}$ dry matter** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Pb** | **Cu*** | **Zn** | **Cd** | **Ni*** | **Cr** | **Hg** | **As** |
| 1. | 4 | 25 | 20 | 30 | 0.4 | 25 | 150 | 1 | 25 |
| 2. | 5 | 40 | 40 | 60 | 0.8 | 35 | 170 | 1 | 25 |
| 3. | 5.5 | 50 | 60 | 60 | 1 | 50 | 180 | 1 | 25 |
| 4. | 6 | 70 | 120 | 200 | 1.5 | 60 | 190 | 1 | 25 |
| 5. | 7 and >7 | 80 | 140 | 300 | 3 | 70 | 200 | 1 | 25 |

* The competent authorities may allow exceeding of these values with soil pH, lasting more than 7. The maximum limit concentration of these heavy metals must not exceed the values for pH = 7 with more than 50%.

populations of soil microorganisms associated with increased intensity of respiration (**Fig. 1**).

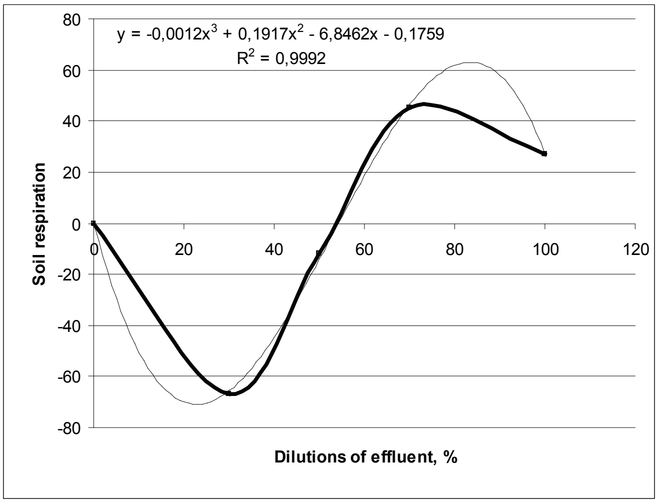


**Fig. 1.** Alteration of soil respiration at soil moisture with tested wastewater concentrations.

It has been reported by Fliessbach et al. (8) that similar increasing soil respiration, and especially respiration per unit biomass ($qCO_2$), took place with increasing amounts of heavy metals from contaminated sludge. A moderately contaminated sludge was applied as received from the sewage treatment plant and after additional metal contamination. The ratio of biomass C to soil organic C (Cmic/Corg) even decreased when low metal sludge was applied (8, 9, 10).

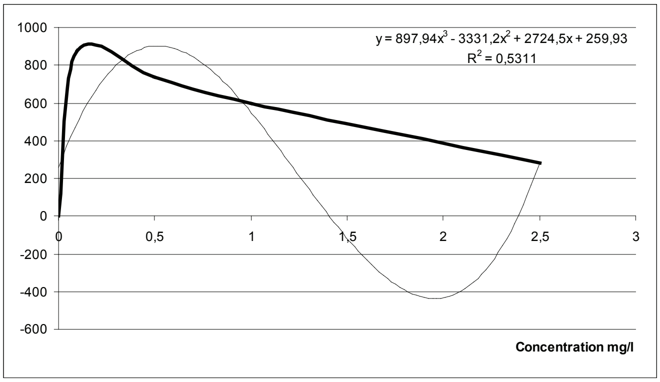


**Fig. 2.** Alteration of soil respiration under the effect of zinc ions.

The toxicity of heavy metals depends on soil acidity and organic matter because these factors strongly influence metal/metalloid bioavailability (14). Most contaminants interact with the soil physiochemical structure to a different extent which modifies their influence on soil respiration. As a result of these complex interactions, bivariate scattergrams of soil respiration values versus contaminant concentrations often display a characteristic ‘wedge-shape’ pattern that suggests contaminants act to limit maximum respiration values (32). The same dose-response curve was obtained when the influence of Ni-solutions with different concentration was evaluated (16).

The results from a study characterizing the modifications in the genetic structures of soil bacterial communities induced by different doses of Cu, Cd, and Hg added independently or in combination (Cu + Cd + Hg), allowed Ranjard et al. (27) to deduce the following order of impact: (Cu + Cd + Hg) >> Hg > Cd >Cu. The obtained results demonstrated that there was a cumulative effect of metal toxicity. The trend of modifications on soil bacterial communities was consistent with the “hump-backed” relationships between biological diversity and disturbance (9, 27).

According to the analysis of contaminated soil and wastewater zinc exceeded the allowed limits of content, which was the reason to evaluate the changes in soil respiration under treatment with solutions of different concentrations of Zn ($ZnSO_4$). The registered tendency was that, for the incubation period, soil respiration increased in comparison to control (**Fig. 2**).

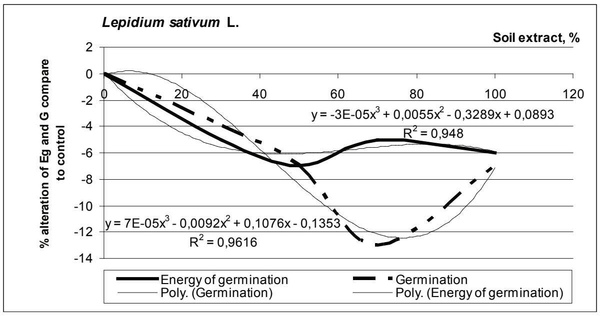
The most intensive respiration (30, 80 mg $CO_2$) was observed at the lowest concentration (0.1 $mg \cdot l^{-1}$) and, compared to the control (3, 15 mg $CO_2$), it is 879.02% higher. With increasing the concentration of zinc ions the intensity of soil breathing was reduced, but it remained much higher than the control level, at least 279.72% over the control in the highest concentration (2.5 $mg \cdot l^{-1}$).

In a short-term laboratory experiment to evaluate the impact on soil microorganisms of different metals (Cu, Zn, and Cd) added singly or in combination it was found that soil respiration and microbial biomass were significantly affected by Zn and Cu, respectively. Generally, in all study cases, polymetal contamination had a greater impact on soil microorganisms than single-metal contamination, leading to the conclusion that there are additive effects of metal toxicity (27, 28).

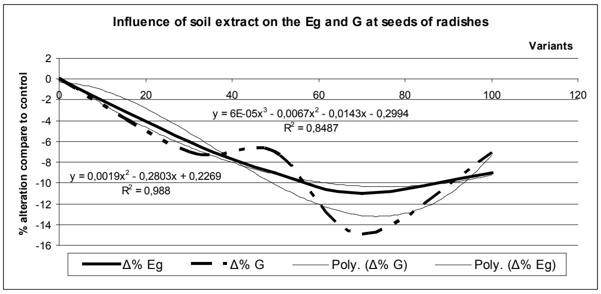
### Energy of germination (*Eg*), germination (*G*)

A widespread tool for identifying the effects of pollutants present in soils are considered to be the bioassays with vascular plants. Bio-assessment is used as a tool to detect the combined effects of hazardous chemicals in the environment that can be expressed as synergism, additivity and antagonism (12, 15, 16, 23). In environmental bio-monitoring the seed germination and early-seedling growth have been widely used as important and most sensitive stages in the whole plant growth process. In order to make an ecotoxicological assessment of soil complex treated with sewage from the heavy metal plant we examined the alteration of energy of germination (*Eg*) and germination (*G*) under the influence of soil extract on the seeds of different plant species (**Fig. 3**).

The alteration of energy of germination (*Eg*) and germination (*G*) of different plant species seeds under the influence of soil extracts are given in **Fig. 3**. The results of the conducted bioassay showed that the energy of germination (*Eg*) of the treated seeds of *Lepidium sativum* L. was 5 to 7% lower compared to the control values ($P < 0.05$). The energy of germination for garden cress seeds was most strongly inhibited at the impact of 50% soil extract (**Fig. 3a**). The germination (*G*) of *Lepidium sativum* L. under treatment with soil extract was 4 to 13% lower compared to that of control seeds ($P < 0.05$). The strongest suppression occurred with 70% extract (**Fig. 3a**).



a



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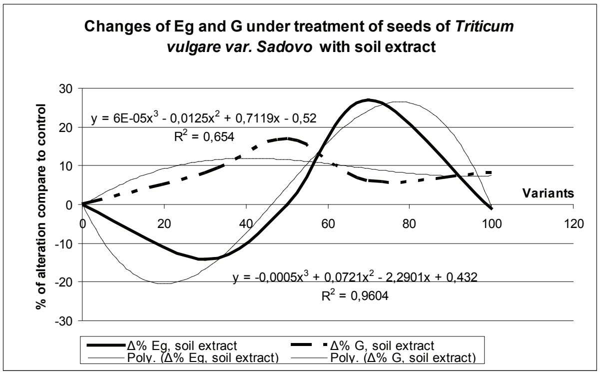

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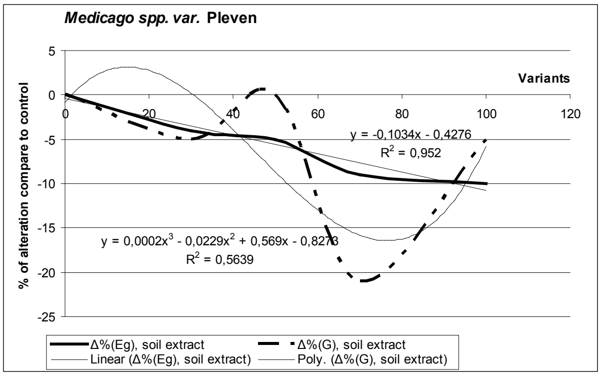

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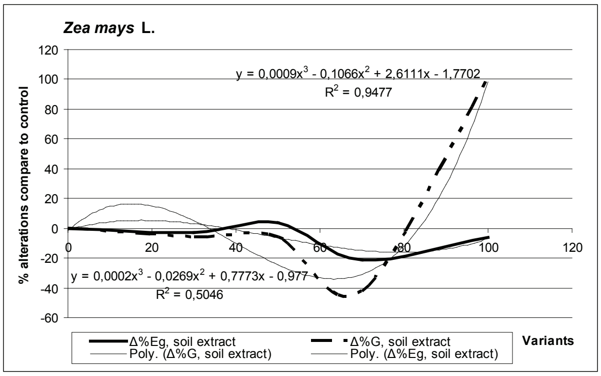


e

**Fig. 3.** Alteration of energy of generation (*Eg*) and germination (*G*) of the seeds under the influence of soil extract in different dilutions: seeds of *Lepidium sativum* L. (**a**), *Raphanus sativus* var. *Radicula* (**b**), *Triticum vulgare* var.*Sadovo* (**c**), *Medicago* spp. L. var. *Pleven* (**d**), *Zea mays* L. (**e**).

The process of reducing the energy of germination (*Eg*) for the seeds of *Raphanus sativus* var. *radicula* was by 6 to 11% compared to the control respectively treated with 30% and 70% soil extract ($P < 0.01$). The reduction of *Eg* was by 9% in the other two variants – 50% and 100% soil extract from the contaminated soil (**Fig. 3b**).

Germination decreased from 7 to 15% compared to the control at 70% soil extract ($P < 0.01$) and at 100% solutions germination of sprouting was relatively uniform (**Fig. 3b**). Both observed patterns for alteration of energy of germination (*Eg*) and germination (*G*) under the influence of soil extract on the seeds of *Lepidium sativum* L. and *Raphanus sativus* var. *radicula* were very similar (**Fig. 3a** and **Fig. 3b**). It has been reported by Rahul et al. (26) that the germination rate and seedling growth of different crops were reduced due to heavy metals toxicity. The germination and early seedling development assay is commonly adopted as a basic test for evaluating the toxic effect of metals or chemicals on plants (1). Germination inhibition is one of the best-known effects induced by toxic heavy metals. Under heavy metal stress, both the processes of germination and embryo growth are suppressed (1).

For the wheat seeds (*Triticum vulgare* var. *Sadovo*) 14% reduction of *Eg* in the 30% variant compared to control and a stimulating effect by 27% in the 70% variant were observed ($P < 0.01$) (**Fig. 3c**).

The influence of the undiluted extract and the 50% variant was weak. On impact with the soil extract, stimulation of germination of seeds was observed, mostly pronounced at a dilution of 50% (**Fig. 3c**). Plants expressed different reactions to soil metal contamination (20, 30, 31). Heavy metals such as Co, Cu, Fe, Mn, Mo, Ni, and Zn are essential metals and when present in the soil, most plants exhibit a stimulation effect. Zinc is one of the necessary trace elements for plants and rural soils have optimum Zn content for plant growth. It has been indicated that the application of Zn can efficiently increase the yield of the *Thlaspi caerulescens* (30). In the sewage, Zn is the metal that exceeded the allowed limits of regulation (**Table 3**), and also in the soil it had higher content compared to other metals (**Table 4**). It has been reported that the application of Zn in the normal area and Zn deficient areas was effective in reducing the Cd concentration in the wheat grain. When growing in nutrient solution containing low concentration of Cd, a strong antagonistic effect of Zn on Cd accumulation was found in young leaves of lettuce or spinach (17).

A clear and strong negative impact of soil extracts on *Eg* was observed for alfalfa (*Medicago* spp. L. *var. Pleven*) seeds. The values of the energy of germination were from 4 to 10% lower compared to the control ones ($P < 0.01$). With increasing the concentration, the negative deviation from the control sample increased (**Fig. 3d**). A linear regression with correlation $R^2 = 0.952$ was obtained. There was also a negative influence on the germination of alfalfa seeds (*Medicago* spp. L. *var. Pleven*) from 5 to 21% compared to control at impact with the soil extract ($P < 0.01$) (**Fig. 3d**). The most negative impact was observed with 70% extract.

Regarding the germination of *Zea mays* L. seeds, soil extracts were associated with reduced energy of germination in treated seeds. The energy of germination decreased by 3 to 21% in treated seeds compared to control. Weak stimulation in *Eg* was observed under the influence of 50% soil extract (**Fig. 3e**). There were reductions of germination from 6 to 42% on impact with the soil extract, version-3 (70% soil extract). Both indicators were most strongly affected in variant-3 (70% soil extract).

### Length of root (Lr), and length of stem (Ls)

The toxicological effects of soil extract in different concentrations on the early development of the garden cress (*Lepidium sativum* L.) were studied by measuring the lengths of stems and roots of the treated plants. The resulting data showed that the soil extract stimulated the roots with 3 to 21% compared to control (**Fig. 4**). This trend was less pronounced in the undiluted extract and most prominent at 50% dilution. The reaction of the stems showed slight retention of growth with 2 to 8% versus the control, respectively undiluted extract and that with 70% soil extract. At 50% dilution stimulation of growth was observed by 4% over the control (**Fig. 4**).

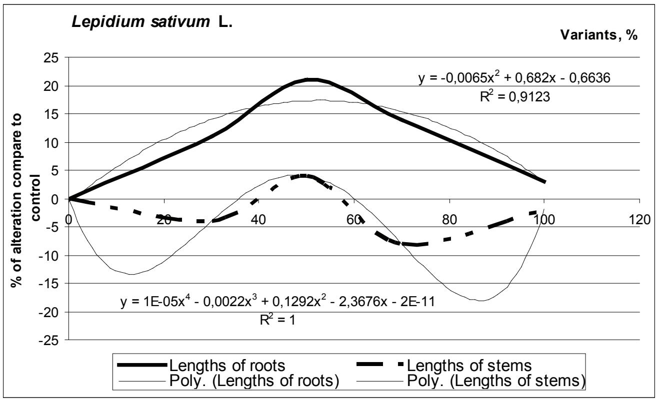


**Fig. 4.** Influence of soil extract on the lengths of roots (Lr) and stems (Ls) of *Lepidium sativum* L.

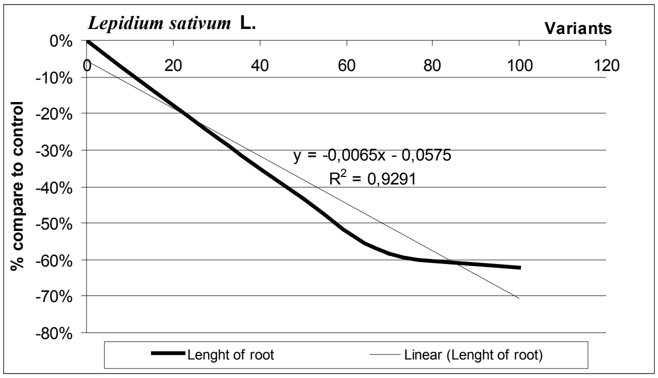


**Fig. 5.** Influence of waste water on the length of root (Lr) of *Lepidium sativum* L.

Clear and strong negative effect on the growth of garden cress roots was registered for the treatments with wastewater (**Fig. 5**). The obtained results generated a linear regression with correlation coefficient $R^2 = 0.9291$. In this case retardation in the development of root could be seen from 26 to 62% compared to control, which increased with decreasing the dilution of sewage ($P < 0.001$).

**TABLE 4**

Heavy elements bioaccumulation coefficient (BA) in biomass of treated juvenile plants of investigated species and control plants

| **Element/ Coefficient** | **Root** | | | |
|---|---|---|---|---|
| | $BA_1$, min-max | Species, min-max | $BA_2$ min-max | Species, min-max |
| Cd | 0.4-1.2 | A-M,W | 0.4-1.1 | A-M |
| Fe | 0.2-11 | M-A | 0.3-1.9 | R-W |
| Pb | 0.4-1.5 | R-A | 0.3-1.1 | R-A |
| Zn | 0.6-1.9 | M-W | 0.6-1.0 | R-A |
| **Element/ Coefficient** | **Stem** | | | |
| | $BA_3$, min-max | Species, min-max | $BA_4$, min-max | Species, min-max |
| Cd | 0.5-1.6 | R-M,W | 0.5-1.6 | R-A |
| Fe | 0.3-0.8 | A-W | 0.1-2.4 | R-A |
| Pb | 1.1-2.2 | A,M-R | 0.9 | All sp. |
| Zn | 0.2-1.4 | M-W | 0.2-1.4 | R-W |

$BA_1$ and $BA_3$ – for plants treated with 100% waste water and control; $BA_2$ and $BA_4$ – for plants treated with 100% soil extract and control; A – Alfalfa; M – Maize; W – Wheat; R – Radish

The toxicity of Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, and Zn ions were examined in blank, nitrate ($N–NO_3$)-, phosphate ($KH_2PO_4$)-, and saline (NaCl)-contaminated media. The acute toxicity of the tested metal ions in the blank media according to their $IC_{50}$ (50% inhibitory concentration) values increased in the order of Pb<Fe<Co<Zn<Mn<Cr<Cu<Cd<Ni (20). The toxicity of single metals according to the relative growth of seedlings' root of *Lepidium sativum* L. decreased

in the following order: Cr(VI) > Cu(II) > Ni(II) > Zn(II) (35). It has been found that the impact of metals on the terrestrial plant *L. sativum* and the interactions of metals within their mixtures differed. Therefore, in order to forecast the changes in the functioning of the ecosystem under exposure of various contaminants, it is necessary not only to determine the biological impact caused by multicomponent mixtures determined in accordance with naturally found concentrations, but also to assess the joint effects in these mixtures (19).

The results from samples of waste water showed that the resulting concentrations of metals were within the limits of Regulation № 6 (**Table 4**). Many chemical mixtures, where concentrations of individual chemicals commonly exist at levels not considered toxic, are often present in aquatic systems. However, it is reckoned that chemical mixtures where individual constituents are present at low, non-toxic concentrations may trigger toxicity due to additive or synergistic effects among the constituents. The antagonistic or additive interactive effects found in almost all metal ion mixture combinations confirms the presumption that the interaction between ions can be caused by competition for the same reaction center on cell membranes if these ions belong to the same group of Lewis acids (19).

In the case of combinations of Ni(II) + Cr(VI) and Cu(II) + Ni(II) at equal concentrations of each metal (5; 10 and 20 $mg\cdot l^{-1}$) a suppression of the inhibitory effects of Cr(VI) and Cu(II) was revealed. This allowed the authors to make a presumption that Ni may suppress the inhibitory effects of the above-mentioned metals to the root growth of *L. sativum*. Sresty and Madhava Rao (31) stated that the inhibitory effects of Cu(II) and Cr(VI) may decrease due to Zn(II) and Ni(II), whose relatively low concentrations may induce a greater degree of plant cell vacuolization, increasing cell ability to reduce the cytotoxic effects of the metals. However, some authors have proposed that copper could inhibit the binding and cellular uptake of zinc, resulting in decreased toxicity of these metal mixtures to plants (6, 11).

**Weight of root and stem**

The results obtained for the influence of soil extract on the weight of root and stem of wheat sprouts are presented in **Fig. 6a**. Overall, the impact of soil extracts on wheat (*Triticum vulgare* L. var. *Sadovo*) stimulated the gain of weight of roots and stems. The linear regression described the trend for the weight of stems with correlation coefficient $R^2 = 0.8288$. Increasing the weight of roots had a polynomial trend with $R^2 = 0.8426$. The stimulating effect of metals on the development of plants in appropriate concentrations has been reported by many authors (35).

The influence of soil extract on the weight of root and stem of the radish *(Raphanus sativus* var. *radicula)* is presented in **Fig. 6b**. The soil extract did not affect the weight of the root, there was no significant difference between the controls and the treated plants. The average weight of root in the control plants was 0.911 ± 0.11 g, while in those treated with 100% soil extract it was 0.963 ± 0.06 g. The stems gained weight under the treatment with the soil extract and a linear regression was obtained. The correlation index of the linear regression was $R^2 = 0.878$. The weights of stems in the 100% variant were 3.261 ± 0.11 g, while in control plants just 2.89 ± 0.11 g ($P < 0.05$).

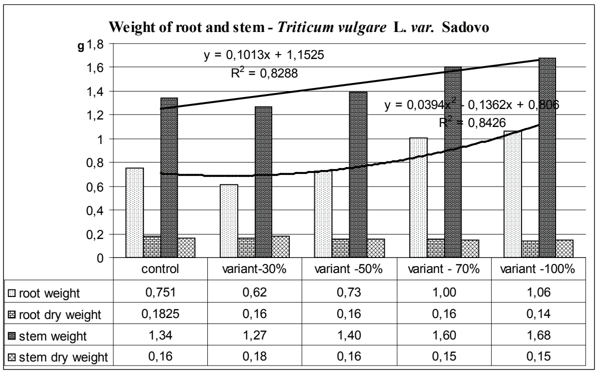
**TABLE 5**

Heavy elements bioaccumulation coefficient (BA) in the biomass of treated juvenile plants of the investigated species and the content in waste water and soil

| **Element/ Coefficient** | **Root** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $BA_5$, min-max | Species, min-max | $BA_6$, min-max | Species, min-max | $BA_7$, min-max | Species, min-max | $BA_8$, min-max | Species, min-max |
| Cd | 6.5-9.6 | A-W | 6.5 - 10.4 | A-R | 0.01-0.02 | W | 0.01-0.02 | A,M-R,W |
| Fe | 9.0-80.0 | M-R | 3.2 - 46.8 | A-W | No data | | | |
| Pb | 42.9-76.2 | R-W | 33.3 - 61.9 | R-M | 0.03-0.06 | R-W | 0.03-0.05 | A,R-M,W |
| Zn | 3.7-18.9 | A-W | 2.6 - 9.5 | A-M,W | 0.00-0.02 | A-W | 0.00-0.01 | A-M,W, R |
| **Element/ Coefficient** | **Stem** | | | | | | | |
| | $BA_9$, min-max | Species, min-max | $BA_{10}$, min-max | Species, min-max | $BA_{11}$, min-max | Species, min-max | $BA_{12}$, min-max | Species, min-max |
| Cd | 7.0-13.5 | A-M | 7.0 - 18.3 | M-A | 0.01-0.03 | A-M | 0.01-0.04 | A |
| Fe | 5.8-22.4 | A-R | 2.6 – 50.0 | R-A | No data | | | |
| Pb | 42.9-95.2 | A-R | 38.1 - 57.1 | R,A-M | 0.03-0.07 | A-R | 0.03-0.04 | M |
| Zn | 3.7-7.9 | A-R | 2.6 – 20.0 | R-M | 0.00-0.01 | A | 0.01-0.03 | M |

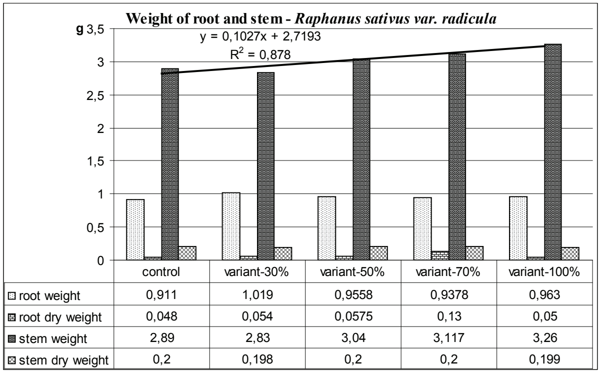
$BA_5$, $BA_6$, $BA_7$ and $BA_8$ – for plants treated with 100% waste water and waste water content; $BA_9$, $BA_{10}$, $BA_{11}$ and $BA_{12}$ – for plants treated with 100% soil extract and soil content; A – Alfalfa; M – Maize; W – Wheat; R – Radish

In the conducted bioassay *Zea mays* L. showed a pronounced stimulation effect from the influence of soil extract. The plant is tolerant to high metal content in the soil (35). With increasing concentration of soil extract a gradual increasing of the weight of roots was observed, respectively, from 1.69 ± 0.29 g in the control to 2.5 ± 0.3 g for the 100% variant ($P < 0.05$). A linear regression with high correlation coefficient was obtained ($R^2 = 0.892$). A linear regression was also determined for the weight of stems. The correlation index was $R^2 = 0.7355$, the weight of stems increased from 1 ± 0.2 g in the control to 1.4 ± 0.2 g (**Fig. 6c**).



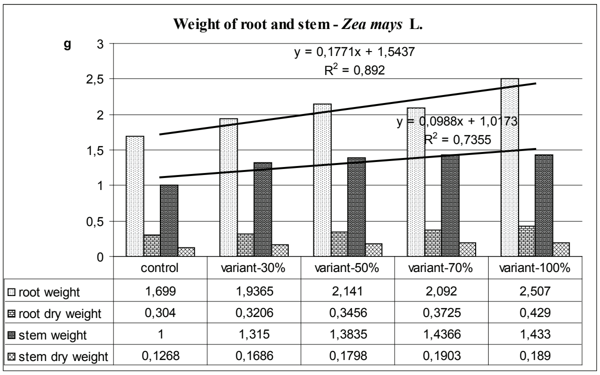
| | control | variant-30% | variant -50% | variant - 70% | variant -100% |
|---|---|---|---|---|---|
| ☐ root weight | 0,751 | 0,62 | 0,73 | 1,00 | 1,06 |
| ▨ root dry weight | 0,1825 | 0,16 | 0,16 | 0,16 | 0,14 |
| ■ stem weight | 1,34 | 1,27 | 1,40 | 1,60 | 1,68 |
| ▨ stem dry weight | 0,16 | 0,18 | 0,16 | 0,15 | 0,15 |

a



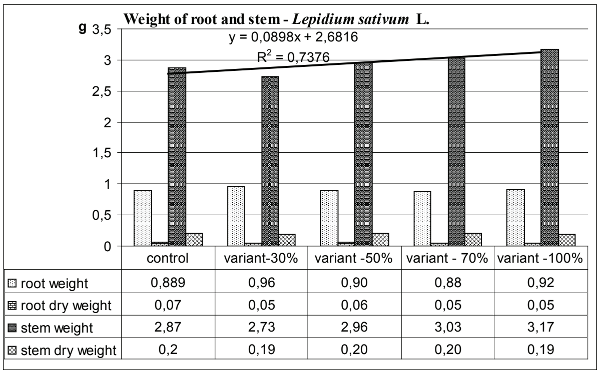
| | control | variant-30% | variant-50% | variant-70% | variant-100% |
|---|---|---|---|---|---|
| ☐ root weight | 0,911 | 1,019 | 0,9558 | 0,9378 | 0,963 |
| ▨ root dry weight | 0,048 | 0,054 | 0,0575 | 0,13 | 0,05 |
| ■ stem weight | 2,89 | 2,83 | 3,04 | 3,117 | 3,26 |
| ▨ stem dry weight | 0,2 | 0,198 | 0,2 | 0,2 | 0,199 |

b



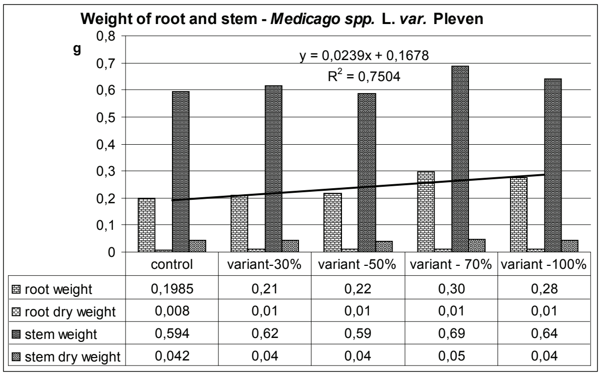
| | control | variant-30% | variant-50% | variant-70% | variant-100% |
|---|---|---|---|---|---|
| ☐ root weight | 1,699 | 1,9365 | 2,141 | 2,092 | 2,507 |
| ▨ root dry weight | 0,304 | 0,3206 | 0,3456 | 0,3725 | 0,429 |
| ■ stem weight | 1 | 1,315 | 1,3835 | 1,4366 | 1,433 |
| ▨ stem dry weight | 0,1268 | 0,1686 | 0,1798 | 0,1903 | 0,189 |

c



| | control | variant-30% | variant -50% | variant - 70% | variant -100% |
|---|---|---|---|---|---|
| ☐ root weight | 0,889 | 0,96 | 0,90 | 0,88 | 0,92 |
| ▨ root dry weight | 0,07 | 0,05 | 0,06 | 0,05 | 0,05 |
| ■ stem weight | 2,87 | 2,73 | 2,96 | 3,03 | 3,17 |
| ▨ stem dry weight | 0,2 | 0,19 | 0,20 | 0,20 | 0,19 |

d



| | control | variant-30% | variant -50% | variant - 70% | variant -100% |
|---|---|---|---|---|---|
| ▨ root weight | 0,1985 | 0,21 | 0,22 | 0,30 | 0,28 |
| ▨ root dry weight | 0,008 | 0,01 | 0,01 | 0,01 | 0,01 |
| ■ stem weight | 0,594 | 0,62 | 0,59 | 0,69 | 0,64 |
| ▨ stem dry weight | 0,042 | 0,04 | 0,04 | 0,05 | 0,04 |

e

**Fig. 6** Influence of soil extract on the weight of stems and roots of wheat sprouts (**a**), radish sprouts (**b**), maize sprouts (**c**), *Lepidium sativum* L. sprouts (**d**), *Medicago spp.* L. var. *Pleven* (**e**).

It has been reported that maize (*Zea mays* L.) can be used for phytoextraction and remediation of soils contaminated with heavy metals (35). A plant for this purpose needs be heavy-metal tolerant, grow rapidly with a high biomass yield per hectare, have high metal accumulating ability in the foliar parts, have a profuse root system, and a high bioaccumulation factor. Certain metals (e.g. Cd and Pb) have been reported to accumulate in *Zea mays* L. above the level defining metal hyperaccumulation. Maize (*Zea mays* L.) has been evaluated as a widely grown staple cereal with promising attributes of a heavy metal accumulator (35).

The weight of roots in control and treated plants of *Lepidium sativum* L. did not differ statistically significantly. The stems under treatment gained weight; linear regression with $R^2 = 0.7376$ extrapolated the results (**Fig. 6d**).

The same trend was observed for the weight of roots and stems of *Medicago spp*. L. var. *Pleven*. There were no significant difference for the weight of stems between treated and control plants. Linear regression with correlation $R^2 = 0.7504$ was found for the roots of pants.

Generally, in all examined plants, a stimulating effect under the treatment with soil extract on the weight of roots and stems was registered.

### Bioaccumulation

The calculated bioaccumulation coefficients of the studied elements in the biomass of 10-day juvenile plants treated with 100% waste water or 100% soil extract in relation to the 10-day juvenile control plants, watered with distilled water, and showed accumulation of elements in the biomass in most cases (**Table 4**).

The coefficients were about 1 and below 1, respectively for: Cd – in the stems and roots of the treated wheat and maize and alfalfa roots; Fe – in the stems and roots of wheat and alfalfa; Pb – in alfalfa roots and stems of the studied species; and Zn – in the roots of wheat and alfalfa and wheat stems. The coefficients of bioaccumulation in the roots and stems of plants towards the content of elements in the soil were insignificant, which probably is associated with a high content of the elements in the soil, but a low content of their mobile forms. Bioaccumulation of elements to the content in the waste water was high (**Table 5**). The coefficient of bioaccumulation for Cd reached 18.3 (in alfalfa stems), for Fe – 80 (in the roots of radishes), for Pb – 76.2 (in wheat roots), and for Zn the maximum ratio was 20 in maize stems.

## Conclusions

Negative toxic effect of wastewater on the soil respiration was registered. The results of the bioassay showed that all tested concentrations led to a change in the intensity of respiration of soil ($P < 0.05$).

The influence of soil extracts on the germination of seeds was associated with reduced energy of germination in treated seeds for all tested plants. Strong negative impact of soil extracts on the energy of germination was registered for alfalfa (*Medicago* spp. L. var. *Pleven*) seeds. A linear regression with correlation $R^2 = 0.952$ was obtained. The greatest negative impact was observed in the 70% soil extract ($P < 0.01$).

Clear negative effect on the growth of garden cress (*Lepidium sativum* L.) roots was registered for the treatments with wastewater. The obtained results generated a linear regression with correlation coefficient $R^2 = 0.9291$. Retardation in the development of roots were 26 up to 62% compared to control, which increased with decreasing the dilution of sewage ($P < 0.01$).

In the conducted bioassay *Zea mays* L. showed a pronounced stimulation effect from the influence of the soil extract. Maize is more tolerant to the metal contamination.

The effluent is toxic for the aquatic systems and has a slight negative impact on the soil respiration and germination of the treated plants. Nevertheless, in the bioassay for all examined plants a stimulating effect on the weight of roots and stems was observed under the treatment with soil extract.

The stimulation effect and bioaccumulation potential of cultivated plants for the investigated heavy metals in waste water generate a significant risk for the agroecosystems and the human population in the region. Usage of the sewage from the metallurgical plant Radomir Metal for irrigation of arable land is not recommended.

The shown model may be applied for the ecotoxicological investigations of the cultural plants-soil-water complex in agroecology.

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