ECOLOGIA BALKANICA

2020, Vol. 12, Issue 2 December 2020 pp. 131-139

Heavy Metals and Metabolite Profiling - A Case Study of Achillea millefolium L.

Vladimir M. Ilinkin[1, 2*], *Milena T. Nikolova*[2], *Violeta G. Dimitrova*[3], *Sonya B. Damyanova* [3], *Dimitar N. Dimitrov*[4]

1 – Institute of Soil Science, Agrotechnologies and Plant Protection "Nikola Poushkarov", 7 Shosse Bankya Str., Sofia 1331, BULGARIA
2 - Institute of Biodiversity and Ecosystem Research - Bulgarian Academy of Sciences 23, Georgi Bonchev Str. Sofia, 1113, BULGARIA
3 - University of Forestry, Faculty of Forestry, Dendrology Department, 10 Kliment Ohridski blvd., Sofia 1756, BULGARIA
4 - Forest Research Institute, Kliment Ohridski 132 blvd., Sofia 1756, BULGARIA
[*]Corresponding author: ilinkin@mail.bg

Abstract. Mining is one of the industries that has had the greatest impact on natural resources. Ore extraction is a mining process that has a significant impact on the environment. Heavy metals at concentrations higher than the normal levels inhibit plant growth. Studies by different authors show that various plants can be used for phytoremediation. The aim of this study was to investigate the relations between heavy metal content in soils and plants and metabolite content in *Achillea millefolium* L. in a heavy metal contaminated environment due to ore mining. The results of our investigation lead to the conclusion that heavy metal contamination does not have a negative influence on the normal growth and development of *Achillea millefolium* L., and the species is suitable for phytoremediation use because it is able to produce sustainable communities.

Key words: heavy metals, metabolites, *Achillea millefolium*.

Introduction

The sustainable management of natural resources is a particularly relevant issue both in Bulgaria and worldwide (LeDuc & Terry, 2005; Bogdanov, 2014; Teoharov & Hristov, 2016; Glogov & Pavlova, 2016). Mining is one of the industries that has had the greatest impact on the natural resources. Its effects can be either direct or indirect and people's health can be adversely affected (Samecka-Cymerman & Kempers 2004; Alexander et al., 2006; Bogdanova et al., 2016; Fiket et al., 2019). The mining activities can result in the loss of topsoil, habitat destruction, landscape changes, etc. (Donov et al., 1978; Nenova et al., 2018). The territories around mined areas or areas, where technological processes related to mining, are carried out are often subjected to very strong anthropogenic pressure (Kumpiene et al., 2007; Tsolova et al., 2014; Nenova et al., 2015). Ore extraction is a mining process that has a significant impact on the environment (Samecka-Cymerman & Kempers 2004; Kumpiene et al., 2007).

Heavy metals (some of which at certain concentrations can be nutrients) adversely

Union of Scientists in Bulgaria – Plovdiv
University of Plovdiv Publishing House

affect the normal growth of plants at higher concentrations (Gorbanov et al., 2005). The toxicity of heavy metals varies with different species and different concentrations and can inhibit the function of certain organs or of the whole plant, can cause changes in pigment content and ratios, can inhibit photosynthesis and respiration or cause other changes in plant growth and development (Kuboi et al., 1986; Gorbanov et al., 2005; Alexander et al., 2006; Monterroso et al., 2014).

Studies by different authors show that various plants can be used for phytoremediation (Chaney, 1997; Salt et al., 1998; Van der Ent, 2012; Monterroso et al., 2014). Most studies focus on phytoaccumulation and phytostabilization (Sekara et al., 2005, Yoon et al., 2006; Singh, 2012).

The aim of this study was to investigate the relations between heavy metal content in soils and plants and metabolite content in *Achillea millefolium* L. in a heavy metal contaminated environment due to ore mining.

Materials and Methods

The objects of study are:

- a population of *Achillea millefolium* L. and soils of the *Luvisols – Chromic Luvisols* groups (WRB, 2014) located to the north of the village of Petarch (Sofia Region, Bulgaria) – control group (SP1);
- a population of *Achillea millefolium* L. and soils of the *Technosols* group (WRB, 2014) located in the vicinity of the village of Lokorsko (SP2), Sofia Region, Bulgaria. The soils have been formed as a result of the ore mining activities of Kremikovtsi Metallurgical Plant and are characterized by high concentrations of heavy metals.

According to Bulgarian forest vegetation zoning (Zahariev et al., 1979), the studied sites are located in the Moesian forest vegetation area, Lower forest vegetation zone.

Methods of study. Five soil and five plant samples were collected from each site, accounting to a total of ten soil and ten plant samples. The samples were taken using the systematic sampling technique according to Petersen & Calvin (1996). The taxonomy of the plant species is presented according to Delipavlov & Cheshmedjiev (2003). Plant samples were collected during their flowering period. The above-ground part of the plants was used for analysis. Each sample was formed by combining of parts of five different plants. The samples were air-dried, ground to a fine powder (in an agate mortar to prevent their contamination with metals when using grinding machines) and dry matter content was determined (Sparks et al., 1996; ISO 638:2008). All results were recalculated based on the absolutely dry weight.

The soil samples were taken at depths of 0-20 cm. The analysis of the soil characteristics listed below was made by utilizing the following methods:

- Preparation of samples by Sparks et al. (1996);
- Soil Organic Matter (SOM, %), oxidation with a solution $K_2Cr_2O_7/H_2SO_4$ according to Donov et al. (1974);
- Total Kjeldahl Nitrogen (TKN, %) according to ISO 11261:2002;
- P_2O_5 (mg.100g^{-1}) and K_2O (mg.100g^{-1}) according to Ivanov (1984);
- Plant available metals (Fe, Pb, Cu, Cd, Mg; mg.kg^{-1}) using a 1 mol.L^{-1} NH_4NO_3 (ISO 19730:2008).

Plant samples. The metals were determined by atomic absorption analysis (ISO 5961:1994). The Kjeldahl method was used to determined total nitrogen (Brashnarova & Stanchev, 1981). Phosphorous contents was determined by Ammonium molybdate spectrometric method (ISO 6878:2004).

Extraction procedure. The dry, ground plant material (100 mg) and internal standards of 50 µg of 3,4 dichloro-4-hidroxy benzoic acid were extracted with 1 mL methanol by classical maceration for 24 h. An aliquot of 300 µL from the extract was placed in glass vial and evaporated. The dry extract was silylated with 50 µL of N,O-bis-(trimethylsilyl)trifluoro-acetamide (BSTFA) in 50 µL of pyridine for 2 h at 50°C.

GC/MS analysis. The GC/MS spectra were recorded on a Termo Scientific Focus GC coupled with Termo Scientific DSQ mass detector as described by Nikolova et al. (2018).

Spectrophotometric analysis. Total phenolic content of the studied samples was determined by Folin-Ciocalteu reagent and gallic acid as standard (Nikolova et al., 2013) Total flavonoid content was determined according to Miliauskasa et al. (2004), using rutin as a reference compound.

Data analysis. The relationships among the soil characteristics, heavy metal content in the aboveground portion of the plants and the metabolites under investigation were analysed using Pearson's product-moment correlation. SPSS for MacOS was used to generate pairwise correlation coefficients. A significance level of $\alpha=0.05$ was chosen. The statistical significance of the differences in the soil characteristics between SP1 and SP2 was tested at $\alpha=0.05$ c t-Test (Excel for MacOS).

Results and Discussion

The sample plots are laid out in two grass communities. A sample plot (control SP1) was set up in a grassland composed of 27 species belonging to 14 families and 27 genera, with the most representative families being Poaceae and Asteraceae, co-dominant being *Poa pratensis* L. and *Festuca valesiaca* Schleich, ex Gaud., with greater abundance were the species: *Agrimonia eupatoria* L. and *Fragaria vesca* L., with the single participation were the species such as: *Bromus mollis* L., *Sambucus ebulus* L., *Eryngium campestre* L., etc. Perennial herbaceous plants predominated - 85%, annual herbaceous were 7%, biennial herbaceous were absent, representatives of the shrubs - *Rosa canina* L. and *Crataegus monogyna* Jacq. were about 7%.

The second sample plot of heavy metal contamination (SP2) was set in a grass community consisting of 18 species belonging to 10 families and 18 genera. The families Asteraceae and Fabaceae had the most representatives. Dominant was *Poa pratensis* L., with a greater share was the species *Achillea millefolium* L., single participation had species such as: *Plantago media* L., *Potentila argentea* L., *Euphorbia cyparissias* L. and others. Perennial herbaceous plants predominated - 78%, biennial herbaceous plants were 11%, annual herbaceous plants were 6% and there was a single share of shrubs - *Rosa canina* L.

Under natural conditions (SP1) correlations were found among the chemical elements studied and SOM in the soil on the one hand and the metabolites studied in the aboveground portion of the plants on the other hand, where the group of the phenolic acids (4 correlations) showed the highest number of correlations, followed by the group of the saccharides and saccharide derivatives (2 correlations) and the total phenols (1 correlation). The group of the organic acids didn't show any correlations.

Under the influence of heavy metal contamination, the highest number of correlations among the chemical elements studied and SOM in the soil on the one hand and the metabolites studied in the aboveground portion of the plants on the other hand were found in the group of the saccharides and saccharide derivatives (8 correlations) (where these data are consistent with the results from other studies (Fryzova et al., 2017), followed by the group of the phenolic acids (2 correlations), organic acids (1 correlation) and total phenols (1 correlation).

Under the influence of heavy metal contamination, Inositol 1 was the metabolite with the highest number of correlations (4 correlations), where only the correlation with available phosphorous was positive, and the others were negative. Sucrose had three correlations with the soil characteristics studied, where only the correlation with potassium was positive. Salicylic acid had two negative correlations (with Cu and Cd).

Table 1. Table of the Pearson correlation coefficients among SOM and the chemical elements studied in the soil and the metabolites.

	Metabolites	Sample Plot	N SSD, %	P, mg.kg^{-1}	K SSD, mg.kg^{-1}	SOM %	Fe SSD, mg.kg^{-1}	Pb SSD, mg.kg^{-1}	Cu SSD, mg.kg^{-1}	Cd SSD, mg.kg^{-1}	Mg SSD, mg.kg^{-1}
Phenolic acids	Salicylic Acid	SP1	0.444	0.986**	0.638	-0.826	0.232	-0.399	-0.418	0.560	0.504
		SP2	-0.250	0.795	0.252	-0.813	-0.684	0.073	-0.940*	-0.883*	-0.714
	Protocatechuic acid	SP1	-0.130	0.689	0.802	-0.884*	0.189	0.045	-0.136	-0.251	0.116
		SP2	0.130	0.087	-0.340	0.055	-0.031	-0.128	-0.219	-0.060	0.063
	Quinic Acid	SP1	0.354	0.635	0.281	-0.615	-0.282	-0.818	-0.722	0.811	0.057
		SP2	0.170	0.668	0.735	-0.684	-0.561	0.034	-0.579	-0.497	-0.684
	Caffeic Acid	SP1	-0.329	-0.017	-0.283	-0.312	-0.460	-0.566	-0.403	0.424	-0.676
		SP2	-0.333	0.020	-0.192	-0.152	0.024	0.522	-0.220	-0.289	-0.029
	Chlorgenic acid cis	SP1	-0.243	-0.608	-0.263	0.271	-0.904*	-0.530	-0.487	-0.004	-0.735
		SP2	0.497	-0.346	0.431	0.358	0.302	-0.110	0.639	0.645	0.223
	Chlorgenic acid trans	SP1	0.469	0.182	-0.357	0.082	-0.136	-0.617	-0.384	0.948*	0.143
		SP2	0.597	-0.118	0.556	0.131	0.164	0.109	0.343	0.435	0.031
Organic acids	Phosphoric Acid	SP1	-0.289	-0.566	-0.311	0.211	-0.865	-0.540	-0.471	0.055	-0.776
		SP2	0.294	0.210	0.225	-0.240	-0.041	0.597	-0.322	-0.166	-0.180
	Succinic Acid	SP1	-0.608	0.290	-0.099	-0.681	0.090	-0.074	0.025	0.109	-0.570
		SP2	-0.842	0.195	-0.386	-0.270	-0.315	-0.302	-0.327	-0.553	-0.180
	Malic Acid	SP1	-0.583	0.402	0.095	-0.800	0.123	-0.041	0.002	0.018	-0.488
		SP2	-0.385	0.709	0.690	-0.707	-0.843	-0.948*	-0.418	-0.593	-0.785
	Pyroglutamic Acid	SP1	-0.171	0.287	-0.044	-0.549	-0.364	-0.645	-0.508	0.544	-0.471
		SP2	0.073	-0.010	0.696	-0.038	-0.127	-0.514	0.408	0.244	-0.176
Saccharides and saccharide derivatives	Fructose 1	SP1	-0.851	-0.391	-0.264	-0.196	-0.385	-0.012	0.017	-0.365	-0.972**
		SP2	-0.294	0.202	0.485	-0.352	-0.266	-0.037	-0.057	-0.247	-0.348
	Fructose2	SP1	-0.355	0.014	-0.644	0.077	0.800	0.606	0.790	0.075	-0.010
		SP2	-0.186	0.147	0.725	-0.252	-0.298	-0.499	0.216	-0.022	-0.349
	Monosaccharide 1	SP1	-0.474	-0.778	-0.622	0.434	-0.648	-0.232	-0.115	-0.079	-0.847
		SP2	0.159	-0.333	-0.144	0.427	0.238	-0.429	0.471	0.462	0.328
	Glucose	SP1	-0.214	-0.353	0.392	-0.021	-0.774	-0.272	-0.457	-0.504	-0.462
		SP2	0.443	0.144	0.538	-0.024	-0.174	-0.563	0.154	0.230	-0.175
	Inositol 1	SP1	-0.086	0.152	-0.507	-0.178	0.006	-0.383	-0.123	0.717	-0.285
		SP2	0.026	0.939*	0.664	-0.909*	-0.824	-0.163	-0.911*	-0.805	-0.888*
	Monosaccharide 2	SP1	-0.327	-0.329	-0.340	-0.032	-0.713	-0.593	-0.463	0.264	-0.780
		SP2	0.235	0.676	0.974**	-0.657	-0.644	-0.383	-0.398	-0.362	-0.741
	Inositol 2	SP1	-0.173	-0.121	-0.151	-0.196	-0.736	-0.742	-0.630	0.396	-0.649
		SP2	-0.600	-0.182	0.149	-0.008	-0.014	-0.219	0.351	-0.005	-0.015
	Disaccharide	SP1	-0.577	-0.119	0.441	-0.370	-0.294	0.157	-0.049	-0.754	-0.502
		SP2	0.021	0.310	0.870	-0.372	-0.396	-0.455	0.051	-0.086	-0.476
	Sucrose	SP1	-0.123	-0.721	-0.670	0.599	-0.646	-0.388	-0.227	0.218	-0.576
		SP2	-0.291	0.814	0.919*	-0.860	-0.891*	-0.695	-0.522	-0.672	-0.918*
	Trisaccharide	SP1	0.498	0.965**	0.824	-0.774	0.266	-0.255	-0.363	0.328	0.642
		SP2	0.062	0.064	0.747	-0.120	-0.187	-0.478	0.324	0.165	-0.249
	Total flavonoids	SP1	-0.243	-0.404	-0.809	0.584	0.626	0.678	0.840	-0.086	0.020
		SP2	-0.811	0.220	-0.287	-0.361	-0.284	0.054	-0.403	-0.618	-0.229
	Total phenols	SP1	0.940*	0.328	0.088	0.209	-0.029	-0.503	-0.424	0.790	0.734
		SP2	-0.271	-0.727	-0.896*	0.641	0.702	0.590	0.448	0.371	0.745

Legend: SSD indicate that there is a statistically significant difference between the content of the chemical elements/chemical compounds in SP1 and that of their corresponding counterparts in SP2 ($p<0.05$); * indicate statistically significant correlation at p≤0,05; ** indicate statistically significant correlation at p≤0,01 .
indicate statistically signifint correlation at SP1; indicate statistically significant correlation at SP2

There were number of correlations among the content of the chemical elements studied (in the aboveground portion of the plants) and the metabolites in the control group plants and in the plants exposed to heavy metal contamination (Table 2).

The groups of the saccharides and saccharide derivatives and of the phenolic acids showed the highest number of correlations with the elements studied (6 each with the control group and 8 and 4 respectively with the plants exposed to heavy metal contamination). The group of the organic acid had 4 correlations with the control group plants and one correlation with the plants exposed to heavy metal contamination. The group of the total phenols and flavonoids had only one correlation with the plants exposed to heavy metal contamination.

Under natural conditions without anthropogenic pressure, the elements studied had a positive influence on the synthesis of metabolites, where only the salicylic acid and Mg showed the negative correlations. Copper was the element that had a positive effect on the largest number of metabolites in 3 out of 4 metabolic groups (which confirmed the results of studies conducted by other authors (Kumar et al., 2004), whereas iron did not show any statistically significant correlations.

Under the influence of heavy metal contamination, the main nutrients (N, P, K) showed positive correlations with the metabolites. All other elements showed negative correlations, with the exception of iron and cadmium, which showed both positive and negative correlations. The plants exposed to heavy metal contamination had the highest number of correlations (negative) among Mg and the metabolites.

The correlations found among the soil characteristics and the metabolites in this study confirmed data found by other authors (Akula & Ravishankar, 2011; Fahimirad & Hatami, 2017) on the influence of the environment on the synthesis of metabolites. The different heavy metals both in the soil and in the aboveground portion of the plants had a different effect (positive and/or negative) on the different metabolites, which was consistent with studies carried out by other authors (Misra, 1992; Macnair, 1993; Tumova & Blazkova, 2002; Tumova et al., 2001).

Low concentrations of some heavy metals could be used (as nutrients) to increase the synthesis of a certain metabolite or a group of metabolites. Such data have also been presented by other authors (Kumar et al., 2004).

Conclusion

The heavy metals studied (both in the soil and in the aboveground portion of the plants) have the strongest influence on the group of the saccharides and saccharide derivatives, whereas the group of the organic acid has remained relatively stable under the influence of the soil characteristics.

The heavy metal contamination does not have an adverse effect on the successful growth and development of *Achillea millefolium* L, and the species is able to create sustainable communities, which makes it suitable for the purposes of phytoremediation.

Acknowledgements

This work was supported by the University of Forestry, Sofia, Bulgaria under project "Metabolic profile of plants species of phytocoenosis located on Technogenic soils" approved by SRS – B – 1011/27.03.2019.

References

Akula, R., & Ravishankar, G. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior*, *6*(11), 1720–1731. doi:10.4161/psb.6.11.17613.

Alexander, P. D., Alloway, B. J., & Dourado, A. M. (2006). Genotypic variations in the accumulation of Cd, Cu, Pb and Zn

Table 2. Table of the Pearson correlation coefficients among the chemical elements in the plants and the metabolites.

	Metabolites	Sample Plot	N, %	P, mg.kg⁻¹	K, mg.kg⁻¹	Fe, mg.kg⁻¹	Pb, mg.kg⁻¹	Cu, mg.kg⁻¹	Cd, mg.kg⁻¹	Mg, mg.kg⁻¹
Phenolic acids	Salicylic Acid	SP1	0,932*	0,249	0,493	-0,659	-0,063	0,123	-0,063	-0,933*
		SP2	0,662	0,445	-0,207	-0,016	0,689	0,127	-0,898*	0,087
	Protocatechuic acid	SP1	0,656	0,039	0,427	-0,252	-0,322	-0,027	-0,204	-0,331
		SP2	-0,309	-0,216	0,900*	0,261	0,237	-0,497	0,444	0,789
	Quinic Acid	SP1	0,584	0,739	0,601	-0,326	0,534	0,685	0,386	-0,831
		SP2	0,959**	0,871	-0,515	-0,654	0,159	-0,069	-0,866	-0,673
	Caffeic Acid	SP1	-0,163	0,991**	0,73	0,472	0,961**	0,984**	0,880*	-0,229
		SP2	0,223	-0,064	-0,857	0,357	0,556	0,865	-0,719	-0,105
	Chlorgenic acid cis	SP1	-0,517	0,546	-0,005	0,521	0,638	0,714	0,414	0,410
		SP2	0,093	0,302	-0,189	-0,658	-0,771	-0,292	0,333	-0,779
	Chlorgenic acid trans	SP1	0,122	0,509	0,230	-0,209	0,596	0,46	0,439	-0,604
		SP2	0,471	0,59	-0,456	-0,767	-0,476	-0,178	-0,093	-0,906*
Organic acids	Phosphoric Acid	SP1	-0,514	0,642	0,113	0,563	0,731	0,789	0,525	0,347
		SP2	0,723	0,545	-0,811	-0,257	0,468	0,410	-0,824	-0,482
	Succinic Acid	SP1	0,019	0,802	0,981**	0,481	0,662	0,688	0,809	-0,291
		SP2	-0,442	-0,579	0,298	0,708	0,302	0,262	-0,004	0,708
	Malic Acid	SP1	0,156	0,711	0,961**	0,380	0,506	0,596	0,666	-0,314
		SP2	0,227	0,343	0,524	-0,45	-0,451	-0,677	-0,023	-0,145
	Pyroglutamic Acid	SP1	0,141	0,983**	0,817	0,230	0,853	0,941*	0,777	-0,487
		SP2	0,177	0,355	-0,171	-0,709	-0,894*	-0,332	0,157	-0,858
Saccharides and saccharide derivatives	Fructose 1	SP1	-0,523	0,654	0,548	0,863	0,647	0,696	0,702	0,413
		SP2	0,453	0,328	-0,834	-0,300	-0,161	0,421	-0,625	-0,755
	Fructose 2	SP1	-0,277	0,016	0,373	0,314	0,179	-0,165	0,457	-0,092
		SP2	0,286	0,361	-0,401	-0,607	-0,733	-0,094	-0,141	-0,884*
	Monosaccharide 1	SP1	-0,792	0,526	0,068	0,773	0,733	0,647	0,616	0,542
		SP2	-0,591	-0,332	0,818	-0,011	-0,553	-0,599	0,924*	0,301
	Glucose	SP1	-0,15	0,068	-0,224	0,223	-0,033	0,257	-0,194	0,513
		SP2	0,110	0,404	0,633	-0,702	-0,677	-0,932*	0,487	-0,29
	Inositol 1	SP1	-0,072	0,803	0,692	0,245	0,861	0,699	0,854	-0,496
		SP2	0,901*	0,814	-0,068	-0,507	0,352	-0,304	-0,823	-0,252
	Monosaccharide 2	SP1	-0,38	0,874	0,441	0,56	0,915*	0,950*	0,758	0,070
		SP2	0,855	0,929*	-0,151	-0,932*	-0,313	-0,515	-0,499	-0,791
	Inositol 2	SP1	-0,151	0,913*	0,492	0,372	0,878*	0,980**	0,691	-0,129
		SP2	0,806	0,709	0,251	0,909	0,477	0,353	0,835	0,399
	Disaccharide	SP1	-0,074	0,044	0,131	0,366	-0,139	0,125	-0,082	0,460
		SP2	0,523	0,614	-0,357	-0,801	-0,65	-0,263	-0,262	-0,949*
	Sucrose	SP1	-0,689	0,442	-0,109	0,514	0,698	0,562	0,505	0,365
		SP2	0,646	0,668	-0,039	-0,667	-0,333	-0,412	-0,5	-0,578
	Trisaccharide	SP1	0,985**	-0,049	0,252	-0,776	-0,384	-0,146	-0,376	-0,790
		SP2	0,281	0,431	-0,264	-0,739	-0,829	-0,285	0,026	-0,911*
	Total flavonoids	SP1	-0,571	-0,299	-0,129	0,340	0,012	-0,39	0,210	0,282
		SP2	-0,095	-0,366	-0,353	0,634	0,513	0,687	-0,505	0,344
	Total phenols	SP1	0,456	-0,152	-0,352	-0,767	-0,118	-0,160	-0,321	-0,587
		SP2	-0,674	-0,834	-0,359	0,883*	0,357	0,859	0,195	0,470

Legend: * indicate statistically significant correlation at p≤0,05; ** indicate statistically significant correlation at p≤0,01 .
(green) indicate statistically significant correlation at SP1; (orange) indicate statistically significant correlation at SP2.

exhibited by six commonly grown vegetables. *Environmental Pollution*, *144*(3), 736–745. doi:10.1016/j.envpol.2006.03.001.

Bogdanov, S. (2014). Results from fertilization of Scot Pine stand on Brown Forest soils, *Ecological Engineering and Environment Protection*, *3-4*:99-104.

Bogdanova, K., Slavkova, T., & Blagoeva, D. (2016). Necessity of conducting osteoporosis prophylaxis in school. In Varna Medical Forum, *5*,441-444. (In Bulgarian).

Brashnarova, A. & Stanchev, L. (1981). Methods for analyses microelements in plants. Microelements and microfertilieses. In: L. Stanchev (Ed.) *Microelements and microfertilizers* (pp. 158-186), Sofia, Bulgaria. (In Bulgarian).

Chaney, R.L., Malik, M., Li, Y.M., Brown, S.L., Brewer, E.P., Angle, J.S., & Baker, A.J. (1997). Phytoremediation of soil metals. *Current Opinion in Biotechnology*, *8*(3), 279–284. doi:10.1016/s0958-1669(97)80004-3.

Delipavlov, D. & Cheshmedzhiev, I. (2003). Key to the Plants of Bulgaria. *Agrarian Univ. Acad. Press*, Plovdiv, p. 591. (In Bulgarian).

Donov, V., Gencheva, S. & Yorova, K. (1974). *A manual for practical seminars in Forest soil science*. Sofia. Zemizdat. (In Bulgarian).

Donov, V., Gencheva, S., Zheleva, E., Delkov, N., Pavlov, D., & Milanov, R. (1978). *Recultivation of industrial embankments*. Sofia, Bulgaria. (In Bulgarian).

Glogov, P., & Pavlova, D. (2016) Medicinal plants on the territory of Lozenska mountain. *Annuaire de l'Université de Sofia "St. Kliment Ohridski" Faculte de Biologie*, *103*(4), 152-163.

Gorbanov, S., Stanev, L., Matev, I., Tomov & Rachovski, G. (2005). *Agrochemia*. Bulgaria: Dionis. (In Bulgarian).

Fahimirad, S. & Hatami, M. (2017). Heavy Metal-Mediated Changes in Growth and Phytochemicals of Edible and Medicinal Plants. In M. Ghorbanpour & Varma. A. (Eds.), *Medicinal Plants and Environmental Challenges*, (pp. 189–214), Springer. doi:10.1007/978-3-319-68717-9_11.

Fiket, Ž., Medunić, G., Vidaković-Cifrek, Ž., Jezidžić, P., & Cvjetko, P. (2019). Effect of coal mining activities and related industry on composition, cytotoxicity and genotoxicity of surrounding soils. *Environmental Science and Pollution Research*, *27*, 6613–6627. doi:10.1007/s11356-019-07396-w.

Fryzova, R., Pohanka, M., Martinkova, P., Cihlarova, H., Brtnicky, M., Hladky, J., & Kynicky, J. (2017). Oxidative Stress and Heavy Metals in Plants. *Reviews of Environmental Contamination and Toxicology*, *245*, 129–156. doi:10.1007/398_2017_7.

IUSS Working Group WRB. (2014). *World reference base for soil resources 2014. International soil classification system for naming soils and creating legends for soil maps*. World Soil Resources Reports No. 106. FAO, Rome, Italy.

Ivanov, P. (1984). New AL method to determined the plants available phosphorus and potassium in soil. *Soil and agrochemistry*, *4*(1), 88-98 (In Bulgarian).

IBM Co. (2016). SPSS (Statistical Package for the Social Sciences - Data analysis software system), Vers. 24 (for MacOS). Retrieved from ibm.com.

ISO 20483:2013 Cereals and pulses — Determination of the nitrogen content and calculation of the crude protein content — Kjeldahl method.

ISO 6878:2004 Water quality — Determination of phosphorus — Ammonium molybdate spectrometric method.

ISO 638:2008 Paper, board and pulps — Determination of dry matter content — Oven-drying method.

ISO 11261:2002 Soil quality – Determination of total nitrogen - Modified Kjeldahl method.

Kuboi, T., Noguchi, A., & Yazaki, J. (1986). Family-dependent cadmium accumulation characteristics in higher plants. *Plant and soil*, *92*(3), 405-415.

Kumar, S., Narula, A., Sharma, M.P. & Srivastava, P.S. (2004). In vitro propagation of *Pluchea lanceolata*, a medicinal plant, and effect of heavy metals and different aminopurines on quercetin content. *In Vitro Cellular & Developmental Biology - Plant*, *40*(2), 171–176. doi:10.1079/ivp2003490.

Kumpiene, J., Lagerkvist, A., & Maurice, C. (2007). Stabilization of Pb- and Cu-contaminated soil using coal fly ash and peat. *Environmental Pollution*, *145*(1), 365–373. doi:10.1016/j.envpol.2006.01.037.

LeDuc, D. L., & Terry, N. (2005). Phytoremediation of toxic trace elements in soil and water. *Journal of Industrial Microbiology & Biotechnology*, 32(11-12), 514–520. doi:10.1007/s10295-005-0227-0.

Macnair, M. R. (1993). The genetics of metal tolerance in vascular plants. *New Phytologist*, *124*(4), 541–559. doi:10.1111/j.1469-8137.1993.tb03846.x.

Miliauskasa, G., Venskutonisa, P. R. & Van Beek, T. A. (2004). Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry, 85*, 231–237.

Misra, A. (1992). Effect of zinc stress in Japanese mint as related to growth, photosynthesis, chlorophyll content and secondary plant products - the monoterpenes. *Photosynthetica*, *26,* 225–234.

Monterroso, C., Rodríguez, F., Chaves, R., Diez, J., Becerra-Castro, C., Kidd, P. S., & Macías, F. (2014). Heavy metal distribution in mine-soils and plants growing in a Pb/Zn-mining area in NW Spain. *Applied Geochemistry*, *44*, 3–11. doi:10.1016/j.apgeochem.2013.09.001.

Nenova, L., Atanassova, I., Simeonova, T., Atanasova, E. & Teoharov, M. (2015). Mobility and bioavailability of heavy metals on the background of high levels of added copper in technogenically affected soils. - Soil Science, *Agrochemistry and Ecology*, *48*: 43-54. (In Bulgarian).

Nenova, L., Zgorelec, Z., Benkova, M., Simeomova, C., Velichkova, N., & Atanassova, I. (2018). Solubility and availability of copper, zinc lead and iron in technosols under the effect of increasing copper levels. *International Journal of Hydrology*, *2*(3), 379-386.

Nikolova, M., Petrova, M., Zayova, Vitkova, A. & Evstatieva, L. (2013). Comparative study of *in vitro, ex vitro* and *in vivo* grown plants of *Arnica montana* - polyphenols and free radical scavenging activity. *Acta Botanica Croatica*, *72*(1), 13-22.

Nikolova, M., Aneva, I. & Berkov, S. (2016). GC-MS metabolic profiling and free radical scavenging activity of *Micromeria dalmatica. Biologica Nyssana*, 7, 159-165.

Petersen, R. & Calvin, L. (1996). Sampling. In J. Birgham (Ed.) *Methods of soil analysis, Part 3. Chemical methods*, No. 5. Madison, WI: Soil Science Society of America.

Salt, D. E., Smith, R. D., & Raskin, I. (1998). Phytoremediation. Annual Review of *Plant Physiology and Plant Molecular Biology*, *49*(1), 643–668. doi:10.1146/annurev.arplant.49.1.643.

Samecka-Cymerman, A., & Kempers, A. (2004). Toxic metals in aquatic plants surviving in surface water polluted by copper mining industry. *Ecotoxicology and Environmental Safety*, *59*(1), 64–69. doi:10.1016/j.ecoenv.2003.12.002.

Sekara, A., Poniedzialeek, M., Ciura, J., & Jedrszczyk, E. (2005). Cadmium and lead accumulation and distribution in the organs of nine crops: implications for phytoremediation. *Polish Journal of Environmental Studies*, *14*(4), 509-516.

Singh, S. (2012). Phytoremediation: a sustainable alternative for environmental challenges. *International Journal of Green and Herbal Chemistry*, *1*, 133-139.

Sparks, D. L., Page, A. L., Helmke, P. A., Loeppert, R. H., & Hossner, L. R. (1996). Dissolution for Total Elemental Analysis. In D.L. Sparks, A.L. Page, P.A. Helmke, R.H. Loeppert, P.N. Soltanpour, M.A. Tabatabai, C.T. Johnston, M.E. Sumner. (Eds.), *Methods of Soil Analysis Part 3 –*

Chemical Methods. 5.3. SSSA Book Series, Wiley. doi:10.2136/sssabookser5.3.c3.

Teoharov, M. & Hristov, B. (2016). Soils of Zlatiza-Pirdop valley and surrounding area. In *Geochemical and agroecological problems in Zlatiza-Pirdop field and surrounding area*. (pp. 9-27). (In Bulgarian).

Tsolova, V., Hristova, M., Bech Borras, J., Roca Pascual, N., & Dimitrov Banov, M. (2014). Pb, Cu and Zn geochemistry in reclaimed soils (Technosols) of Bulgaria. *Journal of Geochemical Exploration*, *144*, 337–344. doi:10.1016/j.gexplo.2014.02.019.

Tumova, V. & Blazkova, R. (2002). Effect on the formation of flavonoids in the culture of *Ononis arvensis* L. in vitro by the action of $CrCl_3$. *Ceska Slov Farm*, *51*, 44–46.

Tumova, L., Poustkova, J., Tuma, V. (2001). $CoCl_2$ and $NiCl_2$ elicitation and flavonoid production in *Ononis arvensis* L. culture in vitro. *Acta Pharmaceutica*, *51*, 159–162.

Van der Ent, A., Baker, A. J. M., Reeves, R. D., Pollard, A. J., & Schat, H. (2012). Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. *Plant and Soil*, 362(1-2), 319–334. doi:10.1007/s11104-012-1287-3.

Yoon, J., Cao, X., Zhou, Q., & Ma, L.Q. (2006). Accumulation of Pb, Cu, and Zn in native plants growing on a contaminated Florida site. *Science of the total environment*, *368*(2-3), 456-464.

Zahariev, B., Donov, V., Petrunov, K. & Massurov, S. (1979). *Forest vegetation zoning of the People's Republic of Bulgaria*. Bulgaria: Zemizdat, Sofia. (In Bulgarian).

Received: 04.09.2020
Accepted: 08.12.2020