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Original Research

Response of Oxidative Stress Variables, Proteins, and Chlorophyll in Three Plant Species Caused by Moderate Soil Pollution with Toxic Elements

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Abstract

The ecotoxicological effects in the field can be directly assessed by measuring the concentration of the pollutant in soil or plant samples, and also by measuring response variables such as biochemical ones. However, there are few such studies integrating data on pollutants and plant biochemical variables and there is a knowledge gap about how dominant species in various ecological contexts respond in all their plant parts to heavy metal stress by changing biochemical variables. In this context, the objective of the research reported here is to describe how select biochemical variables varied in three plant parts of three plant species sampled from two areas with different levels of pollution. It was also of interest to identify to what extent they could be used in the non-destructive routine monitoring of pollution in industrial areas. We found a systematic decrease of chlorophylls and carotenoids in the aboveground parts of all species, and an increase of protein concentrations in all species and plant parts coupled with a decrease of superoxide dismutase and peroxidase activity. Although these patterns were correlated with a decrease of toxic element concentrations, both as pseudo-total and available forms in all plant parts, we cannot conclude that only a change in toxic elements pollution led to the observed patterns, because P nutrition also differed between plants. A further key direction of research is to clarify how the available major nutrients (N, P) modulate bioaccumulation of toxic elements and what effects they might have on biochemical variables of plants, in particular on oxidative stress.

Keywords: *Taraxacum officinale*, *Lotus corniculatus*, *Plantago major*, polymetallic pollution, oxidative stress

Introduction

Exposure of plants to metal concentration in excess could cause a number of toxic symptoms, for instance growth retardation, inhibition of photosynthesis, and induction or inhibition of enzymes that generate the oxidative stress [1-4]. When plants are subjected to stress caused by the existence of one or several pollutants, cell membranes are the first to be affected, leading to an increase of their permeability [2]. A variety of reactive oxygen species (ROS) are simultaneously induced, such as superoxyde (O_2^-), hydroxyl radical ($OH^\cdot$), hydrogen peroxyde (H_2O_2),

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peroxides and their decomposition products. Another known ROS is atmospheric ozone (O_3), which is capable of producing alterations characteristic of hypersensitivity in plants [3]. Therefore, the free radicals of oxygen may appear in plants either as a consequence of external stress factors (such as exposure to atmospheric pollution or any other type of pollution), or as a result of chemical reactions. These reactions take place in plants after treatment with reagents capable of producing free radicals, or during some physiological processes such as photosynthesis. In fact, all organisms are exposed to various stress factors and they generally have the ability to adapt to unfavorable conditions. This adaptation can be possible:

1) By inducing an osmotic adjustment
2) By producing antioxidant compounds such as vitamins E and C, uric acid, and beta carotene
3) By induction or inhibition of antioxidant enzymes (superoxide dismutase – SOD, catalases – CAT, and peroxidases – POD)
4) By induction or inhibition of some metal chelation agents (such as transferrin, lactoferrin, and ceruloplasmin)

These agents function as an antioxidant system by binding the potentially harmful metallic ions [4]. Ions of elements such as Fe, Cu, Zn, Co, or Ni are essential micronutrients that are involved in functional activities of a large number of proteins whose role is to sustain the growth and development of living organisms. On the other hand, plants also can be exposed to highly toxic ions such as Cd, Pb, Hg, and other metals that are generally considered non-essential [5]. However, micronutrients may also become toxic if they accumulate higher concentrations in the organelles of plant cells. For many elements, the concentration ranges of deficiency, optimal supply, and toxicity are very close. Moreover, the phytotoxic effect occurs differentially on the toxic metals uptake in different plant species, being strongly influenced by the composition of different soil components such as carbonates, hydroxides, organic matter, and silica. It is also important to note that the determination of total or pseudo-total concentrations of toxic elements does not provide enough useful information about the risk of their bioavailability, their ability to remobilize, their toxicity in the environment, and about the chemical form in which they are available. That is why it is very often advisable to evaluate the speciation of elements with toxic potential, thus allowing their bioavailability assessment [6].

In order to get an idea of the ecotoxicological effects on plants in the field, not only is it important to measure the concentration of different forms of the pollutant in soil or plant samples, but also to measure response variables such as biochemical ones [7]. However, there are few such studies integrating data on pollutants and plant biochemical variables in the field. Dazy et al. [8] reported the existence of alterations in biochemical variables (oxidative stress, protein concentrations and photosynthetic pigments) in plant communities, but working only on leaves on strong gradients of soil heavy metal pollution. Al Sayegh Petkovšek [9] also worked only on spruce needles and demonstrated that as the concentrations of pollutants decrease, tree vitality increases. This vitality was correlated with a higher concentration of total ($a + b$) chlorophyll and a stronger defense capability that was demonstrated by the high concentration of ascorbic acid. There is insufficient information about how dominant species in various ecological contexts respond in all their plant parts to metal stress by changing biochemical variables. Do these changes have the same pattern in all plant parts, are these changes identifiable only on strong pollution gradients, or on small pollution gradients as well? Such questions are important both from the point of view of basic science and for the design of monitoring programs.

In this context, the objective of the research reported here is to describe how select biochemical variables varied in three plant parts of three plant species sampled from two areas with different levels of pollution. We believe that an early detection of plant metabolic changes may help to assess whether they can be used as bioindicators of metals in soil. Thus, the results of this study will allow researchers to focus on establishing and implementing a coherent program of remediating metal-polluted industrial areas, a program supported by a permanent monitoring of oxidative stress variables. We were interested in finding out the extent to which the patterns of biochemical variables (as result of pollution) differ depending on the plant species and the plant parts analyzed.

Materials and Methods

Plant Selection and Polluted Area Description

Three species of plants from spontaneous flora have been studied in a polluted industrial area. Two of them were herbaceous plants, the dandelion (*Taraxacum officinale*) and the plantain (*Plantago major*). The third one, the clover, was leguminous (*Lotus corniculatus*). These species were chosen because they have a large spread in the studied area, allowing their harvesting on soils with different degrees of pollution in order to perform a comparative analysis to highlight changes that may serve as biomarkers to assess the impact anthropic area. On the other hand, specialized literature has often stated that these species have a relatively high potential of tolerance to metal pollution [10, 11]. Both soil and plant samples were sampled from two different areas located near the pollution source: an area located at approximately 250 m away, coded with A, and an area situated on the same side as the pollution source, 2 km from it, in a supposedly unpolluted meadow, coded with B. The pollution source was the largest aluminum manufacturing company in Central and Eastern Europe (except Russia), which is located in Romania 120 kilometers west of Bucharest (geographical coordinates: 44º26′ 13″ N, 24º22′ 12″ E in WGS84 system) and has polluted an area of more than 100 ha. The manufacturing of aluminium started in 1966, with a capacity of 55,000 tons annually. The current production capacity of the S.C. ALRO S.A. plant is of 260,000 tons annually. This ferrous metallurgical enterprise (primarily aluminium and

Table 1. Physicochemical characterization of soils from investigated area.

	Variable unit	pH	EC	H	$N\text{-}NH_4^+$	$N\text{-}NO_3^-$	$N\text{-}NO_2^-$	$P\text{-}PO_4^{3-}$
		H_2O	µS/cm	%	µg/g d.w.			
A1	$\bar{x}$	5.84	35.33	14.38	6.156	3.587	BDL	30.16
(n = 6)	S	0.06	8.164	1.920	0.229	1.228	-	13.08
A2	$\bar{x}$	6.63	54.66	15.94	6.826	4.661	BDL	50.58
(n = 6)	S	0.27	19.32	5.48	0.244	3.01	-	30.40
B1	$\bar{x}$	5.27	16.74	9.678	9.908	36.32	0.560	53.27
(n = 50)	S	0.34	9.448	1.023	1.807	22.86	0.405	26.94

n – number of replicates, BDL – below detection limit Detection limit (<2 µg/NO_2-N/l)

Table 2. Pseudo-total of elements content.

	Variable unit	As	Cu	Cr	Mn	Ni	P	Pb	Zn
		µg/g d.w.							
A_1	$\bar{x}$	13.77	16.90	130.9	841.3	72.67	520.7	22.89	76.65
(n = 6)	S	0.42	1.38	12.14	71.5	46.96	34.38	0.82	2.29
A_2	$\bar{x}$	12.95	16.33	109.9	998.2	52.88	605.0	20.52	72.63
(n = 6)	S	1.291	0.501	2.02	2.069	6.26	19.88	11.19	6.59
B_1	$\bar{x}$	7.880	18.52	28.31	440.2	50.98	854.4	26.43	77.22
(n = 50)	S	2.228	3.43	6.935	105.0	25.66	111.8	4.99	29.63

n – number of replicates

Table 3. Element content of bioavailable fraction.

	Variable unit	As	Cr	Cu	Mn	Ni	P	Pb	Zn
		µg/g d.w.							
A_1	$\bar{x}$	0.022	0.946	2.449	22.18	0.852	6.509	0.104	1.683
(n = 6)	S	0.003	0.166	0.158	0.117	0.117	0.430	0.029	0.360
A_2	$\bar{x}$	0.016	0.752	1.690	18.37	0.689	9.980	0.077	1.820
(n = 6)	S	0.000	0.070	0.138	1.987	0.075	0.777	0.003	0.171
B_1	$\bar{x}$	0.010	0.415	2.630	13.71	0.514	10.68	0.047	1.440
(n = 6)	S	0.002	0.036	0.579	3.018	0.113	1.397	0.019	0.264

n – number of replicates

alloys) releases waste gases from central heating, sulphur dioxide (SO_2), particulates, petroleum coke, etc. from the anode section, and aerosols, carbon monoxide (CO), sulphur dioxide (SO_2), and particulate matter containing fluorine from the electrolysis sections. Moreover, an average volume of 700 cubic meters/day of sewage wastes are discharged into the sewage system of Slatina city [12]. Therefore, as a direct consequence of continuing the above-mentioned industrial activities, it was found that the fluoride and metals pollution has affected the animal and human population. Fluorides such as NaF and AlF_3 are obtained through electrolysis of the cryolite-alumina fusion with coal anode and aluminum cathode melted at temperatures of 950-970ºC. Also, the metals resulted from the activities of obtaining and processing aluminum have polluted the crops, pastures, and spontaneous vegetation in the vicinity of the aluminum plant [13], also contaminating the surface and underground waters [14].

Soil Characterization and Sampling

The soil from this polluted area belongs to the podzoluvisols class of soils according to the FAO/UNESCO classification [15], presenting a high quantity of clay, calcareous concretions and a 5 to 8 cm layer of cemented organic matter on the surface [16]. Also, the soil nearby the

Table 4. Soil variables and element content from the three plant rhizospheres.

Soil variable	Units	Area (A)			Area (B)		
		Dandelion	Clover	Plantain	Dandelion	Clover	Plantain
pH		5.24±0.154	7.60±0.193	7.50±0.186	5.03±0.100	6.05±0.127	5.74±0.106
EC	$\mu S \cdot cm^{-1}$	30.71±3.302	102.5±15.11	125.9±14.18	92.00±13.12	110.7±22.23	102.2±13.45
H	%	16.23±6.106	13.37±3.458	12.12±3.821	4.424±0.679	7.877±1.770	7.223±0.803
$N\text{-}NH_4^+$	$\mu g \cdot g^{-1}$ d.w.	1.360±0.762	1.674±0.489	1.931±0.413	10.70±5.802	12.05±4.373	8.711±2.014
$N\text{-}NO_3^-$		10.56±5.462	9.480±8.071	5.702±6.452	48.28±36.19	97.34±79.50	77.02±41.79
$N\text{-}NO_2^-$		0.110±0.039	0.295±0.095	0.274±0.076	0.439±0.133	0.789±0.339	0.645±0.252
$P\text{-}PO_4^{3-}$		14.75±4.679	18.77±3.984	16.42±8.680	18.39±2.407	35.53±5.820	22.54±2.027
As[a]		8.652±1.850	8.683±1.533	10.13±1.548	4.387±0.478	4.278±0.779	3.931±0.111
As[b]		0.010±0.004	0.007±0.002	0.008±0.001	0.008±0.002	0.004±0.001	0.003±0.001
Cr[a]		95.88±6.238	102.7±9.410	119.5±8.701	25.83±3.035	22.66±2.992	24.68±4.507
Cr[b]		0.798±0.112	0.538±0.050	0.571±0.114	0.269±0.036	0.175±0.026	0.213±0.109
Cu[a]		30.29±7.824	26.30±5.893	25.26±5.186	27.52±8.766	27.44±5.675	29.52±3.487
Cu[b]		2.098±0.605	1.161±0.246	1.058±0.210	1.777±0.479	1.200±0.294	1.427±0.203
Mn[a]		851.3±147.1	655.7±123.9	735.6±131.4	352.8±64.29	323.9±44.01	321.9±61.45
Mn[b]		17.15±2.577	11.48±2.007	13.78±2.489	15.06±2.565	11.17±1.585	12.17±2.432
Ni[a]		69.80±15.50	89.27±20.54	98.71±16.91	29.17±4.637	36.73±6.020	29.79±4.507
Ni[b]		1.760±0.320	1.470±0.326	1.552±0.233	1.266±0.201	1.103±0.181	1.171±0.204
P[a]		655.0±77.40	744.5±51.26	699.1±58.69	681.9±40.92	798.6±72.48	741.0±45.77
P[b]		10.94±0.727	13.01±0.781	11.68±0.809	12.01±1.201	13.50±1.382	12.99±0.716
Pb[a]		16.18±3.346	11.57±3.739	11.84±2.467	19.73±7.001	11.88±4.011	11.13±2.867
Pb[b]		0.084±0.015	0.043±0.027	0.053±0.022	0.068±0.024	0.044±0.008	0.058±0.010
Zn[a]		67.31±13.26	76.93±8.335	73.24±8.231	101.4±33.28	113.2±48.84	69.71±5.352
Zn[b]		2.12±0.360	1.51±0.322	1.82±0.254	2.533±0.831	1.883±0.813	2.074±0.098

Underlined values exceed the acceptable level in soil for the plants (see section *Plant Selection and Polluted Area Description*)
a – *aqua regia*, b – ammonium nitrate.

pollution source (area A) and the soil sampled from an area approximately 2 km away from the pollution source (area B) were characterized by the physicochemical and chemical variables listed in Tables 1-3. All soil samples were sampled at a depth of 0-10 cm, from two different locations (with six replicates each) in area A (coded with A_1 and A_2), and from one location (with 50 replicates) in area B (coded with B_1).

Furthermore, other soil samples were sampled from the rhizosphere of all three selected plant species (n = 54 soil samples, of which 27 are from area A and 27 from area B), by shaking down the soil from the roots of the plants directly into labeled polyethylene bags. After sampling, the soil was placed in airtight plastic bags and transported to the laboratory in freezer, bags, where they were kept for 24 hours at 4ºC. Before determining any of the variables, the soil was well homogenized manually.

Variables Measured on Soil Samples

Using moist, unsieved soil, after removing any stones and/or plant debris, we determined the pH, conductivity (EC), soil humidity, and mineral nitrogen and phosphate. The soil mixed with distilled water (1:2.5 m/v) was analyzed after stirring for 15 minutes and allowed to stand for one hour, in order to measure the pH and EC by means of a glass electrode and a conductivity probe from a multi-parameter kit WTW 340i (according to DIN ISO 11260 1997-05). Soil humidity (H) was determined by working with 10 g of wet soil that was brought to a constant weight at 105ºC. The determination of the pseudototal content of metals/metalloids and phosphorus was conducted according to the Hoffmann [17] method, after digesting the samples in *aqua regia* (suprapure acids from Merck, $HCl:HNO_3$=3:1 (v/v)) by means of a pressure-assisted

Table 5. p-values of the three plant species, of the polluted areas, and of the interaction factor for the soil variables.

Adjusted values of the plant	p-values			Adjusted values of the plant	AR[a] p-values			Adjusted values of the plant	AN[b] p-values		
Variable	Species	Area	Interaction factor	Variable	Species	Area	Interaction factor	Variable	Species	Area	Interaction factor
log(pH)	**<0.001**	**<0.001**	**<0.001**	As	0.297	**<0.001**	**0.031**	As	**<0.001**	**<0.001**	0.167
EC	**<0.001**	**0.001**	**<0.001**	log(Cu)	0.778	0.557	0.232	log(Cu)	**<0.001**	0.362	**0.013**
log(U)	0.091	**<0.001**	**<0.001**	Mn	**0.011**	**<0.001**	0.072	Mn	**<0.001**	**0.038**	0.488
log(N-NH_4^+)	0.165	**<0.001**	**0.027**	Ni	**0.005**	**<0.001**	**0.012**	log(Ni)	**0.030**	**<0.001**	0.917
log(N-NO_3^-)	0.650	**<0.001**	0.095	log(Pb)	**<0.001**	0.550	0.433	Pb	**<0.001**	0.538	0.249
log(N-NO_2^-)	**<0.001**	**<0.001**	0.066	1/Zn	**0.003**	**<0.001**	**0.002**	log(Zn)	**<0.001**	**0.022**	0.982
log(P-PO_4^{3-})	**<0.001**	**<0.001**	0.116	1/Cr	0.213	**<0.001**	0.163	Cr	**<0.001**	**<0.001**	**0.008**
				P	**<0.001**	**0.015**	0.804	P	**<0.001**	**0.001**	0.399

Bold – p-values indicate significant differences (p<0.05). [a]AR – *aqua regia*, [b]AN – ammonium nitrate

Table 6. p-values of the three plant species and polluted areas, as well as the 95% Confidence Intervals for the means.

	p-values		95% Confidence Intervals		
Adjusted values of the soil variables	Species	Polluted areas	1 2	1 3	2 3
log(N-NO_3^-)	0.666	**<0.001**	(-1.116, 1.559)	(-1.469, 1.178)	(-1.583, 0.850)
log(N-NO_2^-)	**<0.001**	**<0.001**	(0.2451, 1.3204)*	(0.1134, 1.1887)*	(-0.6197, 0.3562)
log(P-PO_4^{3-})	**<0.001**	**<0.001**	(0.1601, 0.7624)*	(-0.1756, 0.4266)	(-0.6091, -0.0625)*
Aqua regia					
log(Cu)	0.782	0.401	(-0.2446, 0.1340)	(-0.2199, 0.1588)	(-0.1471, 0.1965)
Mn	**0.014**	**<0.001**	(-308.3, 83.8)	(-269.4, 122.8)	(-138.9, 216.9)
log(Pb)	**<0.001**	0.681	(-0.6609, -0.2134)*	(-0.6588, -0.2113)*	(-0.2009, 0.2052)
1/Cr	0.224	**<0.001**	(-0.0122, 0.0172)	(-0.0145, 0.0149)	(-0.0156, 0.0110)
P	**<0.001**	**0.010**	(51.45, 154.75)*	(-0.05, 103.25)	(-98.37, -4.62)*
Ammonium nitrate					
As	**<0.001**	**<0.001**	(-0.0059, -0.0014)*	(-0.0057, -0.0012)*	(-0.0017, 0.0022)
Mn	**<0.001**	**0.047**	(-6.740, -2.831)*	(-5.086, -1.178)*	(-0.120, 3.427)
log(Ni)	**0.026**	**<0.001**	(-0.3547, 0.0240)	(-0.2902, 0.0885)	(-0.1073, 0.2363)
Pb	**<0.001**	0.732	(-0.0483, -0.0167)*	(-0.0360, -0.0044)*	(-0.0020, 0.0266)
log(Zn)	**<0.001**	**0.018**	(-0.5411, -0.1403)*	(-0.3565, 0.0444)	(0.0027, 0.3665)*
P	**<0.001**	**0.001**	(0.878, 2.681)*	(-0.041, 1.762)	(-1.738, -0.101)*

Bold – p-values indicate significant differences (p<0.05), *indicates significant differences between plant species

digestion system (Microwave 3000 Anton Paar), using a three-step program with progressive increase of IR up to 210ºC and of pressure up to 40 bar (0.3 bar/s) for 65 min., without filtering the samples before measuring. Every digestion batch had one blank and two analytical replicates. Single chemical extraction in 1M NH_4NO_3 p.a. Merck solution was used for the estimation of potentially available element content by working with 20 g of air-dried and sieved (<2 mm) soil and 50 ml ammonium nitrate, and using an overhead shaker (GFL 3040), at 10 rpm [18]. For the second step of this method, all samples were centrifuged at 6000 rpm for 20 minutes and the supernatant was filtered using

0.45 µm cellulose acetate filters and preserved with suprapure 65% HNO_3. The analysis of the ions of solution elements was done using mass spectrometry with inductively coupled plasma ICP-MS, Perkin-Elmer ELAN DRC-e with axial field technology for trace and rare earth element analyses (the DRC-e is a dynamic reaction cell placed before the traditional quadrupole chamber of the ICP-MS device for the purpose of eliminating isobaric interference; this chamber is filled with reaction methane gas, which reacts with the introduced sample, eliminating some of the interference). Standard solutions were prepared by diluting a 10-µg/ml multielement solution (multielement ICP calibration standard 3, matrix 5% HNO_3, Perkin Elmer Pure Plus).

Other variables measured during the two sampling campaigns were mineral nitrogen ($N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and $N\text{-}NO_2^-$) and available phosphorus ($P\text{-}PO_4^{3-}$). The procedure of extracting mineral nitrogen was performed by using 20 g of fresh soil with potassium chloride (100 ml 0.2M KCl) for one hour, whereas for the available phosphorus ($P\text{-}PO_4^{3-}$), 5 g of soil were used with sodium hydrogen carbonate (100 ml 0.5M $NaHCO_3$) for half an hour. Afterward, the extract was filtered using a glass filter (Whatmann GF/C) and analyzed by colorimetric methods (according to the methods described by Neagoe et al. [19].

Variables Measured on Plant Samples

Seven dandelion replicates (*Taraxacum officinale*) and 10 replicates of clover (*Lotus corniculatus*) and plantain (*Plantago major*) were sampled from each site and transported to the laboratory in a cooler bag. These will be further referred to as species 1, species 2, and species 3, respectively. Then, they were separated in roots and aboveground part of plants (the stem together with the leaves, and the flowers separately) in order to assess the variables of oxidative stress and the content of metals/metalloids and phosphorus. After separating the underground part from the aboveground one, the roots were quickly washed with lots of tap water, rinsed several times with distilled water, and finally with ultrapure water. The entire plant material was weighed, dried through lyophillization, ground by means of a cooler stainless steel mill (IKA, 156 A11basic) to a very fine powder, and frozen at -45ºC until further processing. In all plant parts, the content of metals/metalloids and phosphorus was determined after microwave digestion with suprapure nitric acid (65%) using a three-step program with progressive increase of IR up to 140ºC and pressure up to 40 bar (0.3 bar/s), for 45 min. Every digestion batch had one blank and two analytical replicates. The quality assurance and quality control criteria were satisfied by checking the standard reference material CRM 281 of ryegrass. The differences were of no more than 5%. The element analysis was performed using ICP-MS (the same instrument as described in the soil section). For protein and enzyme assays, dry plant material (50 or 100 mg) was homogenized in 4 ml cold 100 mM potassium phosphate buffer (pH 7.2) containing 2% polyvinylpyrolidone 2 mM EDTA and 2 mM dithioerithrol, and centrifuged at 6,000 rpm for 20 minutes at 4ºC. The supernatant was dialyzed overnight at 4ºC in 5 mM potassium phosphate buffer (pH 7.2). Protein concentrations were determined spectrophotometrically with alkaline copper reagent and Folin-Ciocâlteu reagent against a BSA standard curve (according to Lowry et al. [20]). Superoxide dismutase was measured through the inhibition of the reduction rate of Cytochrome c by the superoxide radical, observed at 550 nm (according to McCord and Fridovich [21]). Peroxidase activity was determined spectrophotometrically by measuring the transformation of guajacol to tetraguajacol in the presence of H_2O_2. The reaction mixture contained 33 mM guajacol and 0.3 mM H_2O_2 in 50 mM citrate/phosphate buffer (pH 5) (according to Lagrimini [22]; more details can be found in Neagoe et al. [19]). The estimation of lipid peroxides involves the determination of malondialdehyde, resulting from the decomposition of peroxides of polyunsaturated fatty acids, by using thiobarbituric acid and the colorimetric method at room temperature. Tests were performed in the following way: 20 mg of dry biomass were homogenized with 4 ml TBA solution containing 10% trichloroacetic acid and 0.25% thiobarbituric acid in ultrapure water, heated for 30 min at 95ºC, cooled for 15 min at room temperature, and centrifuged and measured spectrophotometrically at 440, 532, and 600 nm using an equation described by Hodges et al. [23]. In addition, the photosynthetic pigments chlorophyll *a* and *b* and the carotenoids were assayed using 50 mg of dry aboveground plant matter. Plant samples were homogenized with a v:v solution of 80% acetone, 15% water, and 5% solution of NH_3 (25% concentration). Samples were then centrifuged to remove solids, and to obtain a clear supernatant without being filtered before being spectrophotometrically measured at 480, 645, 647, 663, and 664 nm, in order to determine the photosynthetic pigments chlorophyll *a*, chlorophyll *b,* and carotenoids, according to the Schopfer method [24].

Statistical Analysis

Data were analyzed using the Minitab statistical software package (version 15.0). The test used was 2-way ANOVA, which performs an analysis for testing the equality of population means when classification is by two variables. Since one of the two-way ANOVA assumptions is normality of the data, a transformation (either log (response), or 1/response) was applied to the response variable when required. Normality of the data is required as this statistical test cannot be performed if there is concern that there is too much skewness in the data or potential for outliers. The assumption of normality was checked by plotting the data on a Q-Q Plot and checking whether the points followed the equality line. If they did, then the assumption of normality was satisfied. For all the cases when the data were adjusted, the p-values and the 95% Confidence Intervals were calculated using the adjusted values. If the p-value of the interaction factor is greater than 0.05 ($p>0.05$), it means that there is no interaction factor, in which case this is eliminated. The Confidence Intervals allow us to assess the practical significance of differences among means, in addition to statistical significance. If the

Table 7. Element concentration in plants.

Area	Roots	Units	As	Cr	Cu	Mn	Ni	P	Pb	Zn
A	Dandelion	μg·g^{-1} d.w.	0.593±0.063	7.643±2.299	13.31±1.605	131.1±64.86	13.86±3.118	937.5±166.8	4.43±1.754	59.74±27.48
	Clover		0.517±0.11	7.204±2.705	11.18±3.421	31.68± 24.6	9.113±5.066	733.0±207.8	3.896±2.828	38.41±18.44
	Plantain		0.689±0.218	7.684±2.69	12.7± 1.012	37.5± 11.94	5.221± 1.05	1290±328.7	4.498±1.571	80.0±23.62
B	Dandelion		0.423±0.053	1.34± 0.577	13.33±2.234	40.09±10.17	1.973±1.206	1684±621.5	8.441±5.275	72.63±11.86
	Clover		0.422±0.059	1.039±0.589	15.25±3.381	44.76±11.78	2.028±1.149	623.1±565.7	4.482±4.653	64.26±26.38
	Plantain		0.378±0.099	1.507±0.741	16.99±3.543	47.89±13.09	3.364±1.661	1501.6±301.6	6.803±2.375	92.29±19.81
	Shoots*									
A	Dandelion	μg·g^{-1} d.w.	0.446±0.109	5.634±2.466	4.122±0.839	80.45±16.4	7.636±4.524	923.2±108.9	2.916±0.947	14.79±4.705
	Clover		0.449±0.078	3.864±1.454	7.582±1.276	34.2±7.503	4.196±1.523	1315±246.9	3.305±1.265	20.46±5.13
	Plantain		0.445±0.058	6.929±5.328	5.417±1.126	37.97±27.85	4.57±4.025	1363±525.5	4.271±2.19	24.58±11.26
B	Dandelion		0.399±0.042	0.798±0.249	6.597±2.585	38±10.06	0.682±0.712	2018±865.8	6.796±1.059	27.52±13.17
	Clover		0.359±0.02	0.863±0.521	7.205±0.085	41.47±9.562	0.822±0.465	1331±501.8	3.996±1.196	28.74±27.58
	Plantain		0.329±0.139	0.835±0.363	6.269±0.623	45.09±12.4	1.057±0.358	1952±411	5.901±1.116	32.2±24.19
	Flowers**									
A	Dandelion	μg·g^{-1} d.w.	0.419±0.037	4.20±0.965	9.479±1.326	108.0±17.97	11.63±1.379	2610±210	3.971±1.603	33.56±4.44
	Clover		0.449±0.116	6.856±4.597	9.368±1276	25.99±5.136	9.51±2.078	2686±212.2	3.706±1.613	34.04±5.615
	Plantain		0.362±0.057	2.446±1.443	6.452±1.687	24.38±12.92	2.309±0.957	1845±712.3	2.645±1.494	28.56±13.35
B	Dandelion		0.247±0.019	0.63±0.218	5.094±0.997	51.88±15.56	0.354±0.357	3199±996.2	6.034±4.108	44.46±11.95
	Clover		0.242±0.03	0.572±0.289	5.993±0.963	85.33±61.19	0.671±0.146	3077±501.7	7.039±4.054	48.05±22.12
	Plantain		0.239±0.026	0.557±0.221	5.12±1.451	66.55±48.03	0.596±0.486	2826±540.6	5.996±2.521	31.43±11.91

*Aboveground part of plants, **Inflorescence

Confidence Interval does not contain the zero value, it means that there is a difference between means. The results of the ANOVA test and the Tukey comparison can conflict. For example, it is possible for the ANOVA to reject the hypothesis of no differences among the level means, and yet for all the Tukey-pairwise Confidence Intervals to contain zero value [25]. A correlation analysis (Pearson's Correlation) was performed between the concentrations in soil and their concentrations in different plant parts.

Results and Discussion

In areas A_1 and B_1, the soils showed low acidic content (Table 1), whereas in area A_2 there was neutral pH, according to the INRA classification [26]. Concerning the nitrogen (ammonia, nitrate, and nitrite) content, in A_1 and A_2, there was a very low level in all the measured samples due to a lack of mineral N, whereas in B1 the concentration was higher, which could be considered sufficient value for the development of many plants, according to Griffin [27]. The available phosphorus presented higher concentrations in all soil samples. These concentrations are considered optimum values for the development of many plant species according to Howard [28], who reported that agronomic thresholds are less than 60 mg·kg^{-1}. The EC registered low values, corresponding to the unsalinated soil class (0-2 mS/cm) according to Arshad and Martin [29]. Regarding the pseudo-total content of As, Cr, Mn, and Ni, due to the anthropogenic inputs, we recorded concentrations above the acceptable values for plant growth in soil (Table 2), as found by Effroymson et al. [30] and Kabata-Pendias and Pendias [31], which were of 2-10 μg·g^{-1} for As, 50-100 μg·g^{-1} for Cr, 30-35 μg·g^{-1} for Ni, and 100-500 μg·g^{-1} for Mn, whereas Cu was below the acceptable limit of 30-100 μg·g^{-1}. On the other hand, Pb and Zn were found in concentrations acceptable for plant growth in soil (2-60 μg·g^{-1} for Pb and 17-125 μg·g^{-1} for Zn). If we compare the contents of the pseudo-total and available forms of these elements (Table 3), we can observe that the pseudo-total content of investigated elements is linearly positively correlated with extractable forms. This positive correlation was also found by Senila [32], who used 1M HCl instead of 1M NH_4NO_3 (as in our measurements). This author's explanation is that both *aqua regia* and diluted HCl can attack the potentially mobile elements from soil, but not the elements bound in the silicate matrix, which was confirmed by our results using diluted NH_4NO_3.

Physicochemical Characteristics of Soils Sampled from Rhizospheres

Physicochemical variables show that the soils from the rizosphere of the three plant species (A area) are acidic (pH between 5 and 6.5 according to INRA, [26]) while in the case of area B an acidic pH was found only in the rhizosphere of dandelion, and in the clover and plantain rhizospheres the pH was within the upper limit of the class of neutral soils (6.5-7.7) (Table 4). These findings are according to our own results presented above (for areas A_1, A_2 and B_1), and also to the study performed by Becherescu et al. [33] on a surface of approximately 100 ha near the same polluted area. EC was lower in the A dandelion rhizosphere as compared to other A and B rhizospheres. However, both A and B areas belong to the unsalinated soils category (0-2 mS/cm according to Arshad and Martin [29]). If we look at humidity values, we can see that the soil of plants collected from the B rizospheres was drier than that of A. In area A the concentrations of mineral N were statistically significantly lower in the soil from the rhizosphere of all three plant species. Moreover, in all three rhizospheres in area A, mineral N had values below 20 $\mu g \cdot g^{-1}$ d.w., which means that it belongs to the class of soils with a low level of supply for wild plants, whereas for area B the concentrations fluctuated between 61-100 $\mu g \cdot g^{-1}$ d.w., which is characteristic for the soils with a high level of supply according to Griffin [27].

The concentration of bioavailable P using the $NaHCO_3$ extractant showed, as in the case of N, higher values in area B, ranging between the 18.1-36 $\mu g \cdot g^{-1}$ d.w. These values are specific to the soils with an average level of supply, and were compared to the values registered in area A, where the concentration of P ranged between 8.1-18 μg/g d.w., values specific to the soils with low supply, according to Howard [28]. Moreover, if we compare the concentrations of available P obtained by extraction with $NaHCO_3$ with those obtained using NH_4NO_3, we observe that the latter had much lower values. Comparing the pseudo-total concentrations of elements such as As, Cr, Mn, Ni, Cu, Pb, and Zn determined in the soil from the rhizosphere of the three plant species, it was observed that in area A elements such as As, Cr, Mn, and Ni were found in concentrations exceeding the acceptable level in soil for plants which can be used by the human population (according to Effroymson et al. [30] and Kabata-Pendias and Pendias [31]). In the case of Cu, a deficiency could be observed, while for Pb and Zn, acceptable concentrations were registered that do not affect the growth of plants from spontaneous flora.

The presence of metals in soils from this polluted area was also mentioned in the research project conducted by the City of Slatina [16]. Many authors have demonstrated that there is a necessity to identify the chemical forms in which the elements exist in soils. These forms can strongly influence their speciation and are used for estimating their bioavailability, their physicochemical reactivity and their mobility into the food chain [34]. In general, mobile forms are more toxic to plant growth than strongly complex forms [35]. In our study, the bioavailable form of these elements determined in the rhizosphere soils shows a pattern of variation similar to that found in the case of the soils sampled from areas A_1, A_2, and B_1, as described above.

Metals must be in soluble form to be absorbed by the plant root system. Hydroxides and carbonates of metals are in general quite insoluble, and the possibility of forming insoluble hydroxides and carbonates increases when the pH is higher. To minimize toxic metal availability, soil pH should be maintained around 6.5 by amendment. As expected, in our study the mobilization of elements, with the exception of P, increased as a result of alleviating soil pH acidity as can be seen in Table 4. Metals bound to the bioavailable fraction were also found by the Zheng et al. [36] to be very sensitive to pH changes. Also, Smith [27] found that very low transfers of metals to plant tissues occur at high pH.

The pH value below which the mobility and biological availability and toxic metals increase was approximated (in μg/g) by Martinez and Motto [37] for some elements such as for Pb (5.2), Zn (6.2), and Cu (5.5). Another factor that can strongly influence the ability of some elements to be phytotoxic in soil is organic matter [38, 39]. Because this depends to an extent on soil type [40], respectively, on the presence of soluble organo-metal complexes in soil, we can clearly state that organic matter played an important role in our studied soils, which showed a 5-8 cm layer of cemented organic matter [16]. On the other hand, the formation of humic substances could transform Zn from a potentially existing sulfide fraction (which could come from such waste gases as the sulphur dioxide SO_2 [12]). To an organic fraction, which is a form not easily accessible to plants. The oxido-reduction states of the mineral components as well as the redox potential of the system also influence the mobility of the elements. For instance, Cr is commonly present in soils as Cr(III) and Cr(VI). These forms present distinctive chemical properties and toxicities [41]. Cr (VI) is 10 up to 100 times more toxic than Cr(III), thus being a strong oxidizing agent, while non-hazardous Cr(III) is insoluble in water and plays a beneficial role as a micronutrient [6]. For the Pb in soil the most common form is sulfate ($PbSO_4$), the organic phase often being related to the soil, in combination with Mn and Fe oxides or carbonates. Pb^{2+} has a stronger affinity to the adsorption sites on the clay materials. Zn in soil forms complexes with chlorides, phosphates, nitrates, and sulfates. In the case of Zn, based on the redox potential, Zn^{2+} can be expected to stay in ionic form in solution. It seems that the $ZnSO_4$ and $ZnHPO_4$ forms are the most important as they contribute significantly to the concentration of Zn in soils, thus increasing Zn solubility and mobility. Zn forms soluble complexes with the fulvic acids, leading to an increase in its mobility [6, 41].

From a statistical point of view, p-values for three plant species for polluted areas A and B, and for the interaction factor for the related rhizosphere soils were calculated. Also, a comparison between every two species of plants (1-2, 1-3, 2-3) was made using the Tukey – all pairwise

Table 8. p-values of the species, polluted areas, and interaction factor for elements from the plant part.

Adjusted values of the plant variable	Roots p-values			Adjusted values of the plant variable	Aboveground p-values			Adjusted values of the plant variable	Inflorescence p-values		
	Species	Area	Interaction factor		Species	Area	Interaction factor		Species	Area	Interaction factor
log(As)	0.487	**<0.001**	**0.012**	As	0.499	**0.001**	0.519	log(As)	0.077	**<0.001**	0.146
log(Cr)	0.295	**<0.001**	0.603	log(Cr)	0.565	**<0.001**	0.202	log(Cr)	**0.007**	**<0.001**	**0.013**
Cu	0.108	**0.001**	0.071	Cu	**<0.001**	**0.007**	**0.009**	log(Cu)	**<0.001**	**<0.001**	**0.023**
log(Mn)	**<0.001**	0.269	**<0.001**	log(Mn)	**0.002**	0.316	**<0.001**	log(Mn)	**<0.001**	**0.002**	**<0.001**
log(Ni)	0.563	**<0.00**1	**<0.001**	log(Ni)	0.977	**<0.001**	**0.008**	log(Ni)	**<0.001**	**<0.001**	**<0.001**
Pb	**0.022**	0.492	0.202	log(Pb)	**0.022**	**<0.001**	**0.028**	log(Pb)	0.339	**<0.001**	**0.269**
Zn	**<0.001**	**0.001**	0.182	log(Zn)	0.188	**0.010**	0.511	log(Zn)	**0.004**	**0.016**	0.680
P	**<0.001**	**0.013**	**0.014**	log (P)	0.158	**<0.001**	**0.013**	log(P)	**0.006**	**<0.001**	0.280

Bold – p-values indicate significant differences ($p<0.05$)

approach, and the results are presented in Tables 5 and 6. Given the issues outlined in the statistical analysis section, we could observe that there are statistically significant differences between species in the case of the following soil variables: pH, EC, $N\text{-}NO_2^-$, $P\text{-}PO_4^{3-}$, pseudo-total concentrations of Mn, Ni, Pb, Zn, and P, and all easily extractable elements: As, Cu, Cr, Mn, Ni, P, Pb, and Zn. Moreover, for most of the variables (except for Cu and Pb in the two forms: pseudo-total and easily extractable) there were statistically significant differences between the polluted areas. Every two species were then compared by calculating 95% Confidence Intervals for the means of the soil variables. We could see that in the case of $N\text{-}NO_2^-$, Pb in both forms, easily extractable As and easily extractable Mn, there was a difference between species 1 and 2, and 1 and 3. In addition, the content of $P\text{-}PO_4^{3-}$, easily extractable Zn and P in the two forms was different between species 1 and 2, and 2 and 3. For all the others, there was no sufficient evidence of a difference between species, since the 95% Confidence Interval did not contain the zero value. We could also observe that while the 2-way ANOVA gave a difference between species for Mn ($p=0.014$), the Tukey comparison showed that there was no evidence of a difference, which once again proved that these two can sometimes conflict. What is more, for example, the rizosphere soil of clover species has a content of $N\text{-}NO_2^-$ higher than that of dandelion species by a value between exp(0.2451) and exp(1.3204), hence between 1.278 and 3.745 $\mu g \cdot g^{-1}$. The content of $N\text{-}NO_2^-$ in the rizosphere soil of plantain species is higher than that of the dandelion species by a value between exp(0.1134) and exp(1.1887), hence between 1.120 and 3.283 $\mu g \cdot g^{-1}$. The difference in the $N\text{-}NO_2^-$ content between the rhizosphere soil of the plantain species and clover species lies somewhere between exp(-0.6197) and exp(0.3562), that is between 0.5138 and 1.428 $\mu g \cdot g^{-1}$. This variation of nitrite content in rhizospheres might be due to differential oxygen release by the roots of the three plant species. By releasing oxygen into the rhizosphere, a protective oxidative film is formed around the surface of the roots. Systems without plants are totally different from those with plants, which have a significant influence on the redox potential that fluctuates strongly in the rhizosphere [42]. The nitrite evolution could also suggest an accumulation of NO and N_2O in soil. Moreover, the nitrite could be reduced using sulphides (probably as electron donors), which might be generated into soils as result of the presence of sulphate in the polluted areas. We can conclude that there are statistically significant differences, both between the rhizosphere of plant species and the two polluted areas A and B.

Element Concentration in Plants

In the present study, we tried to get a response regarding the pollution level after analyzing the element content in different parts of selected plants grown in both areas A and B. Although in terms of the elements analyzed in different plant parts, a level of pollution was registered, they developed just as vigorously in both investigated areas. But it is well documented that terrestrial plants have developed complex strategies for the efficient acquisition of essential metal micronutrients and for resistance to highly accumulated concentrations [43]. Many other authors also investigated the heavy metal concentration in different plant parts. Thus they used a non-destructive method without harvesting the whole plant individual, in order to conclude that these plant species can be bioindicators. They described the evolution of a pollutant over a longer period of time [44, 45]. However, many previous studies have revealed the phytotoxic effects of metals translocated from soil to roots and their aboveground parts. The phytotoxic effects are manifested on plant growth and also on the physiological and molecular plant processes [44, 46]. They adopt adaptive strategies to survive at high concentrations of metals and complete their life cycle [47]. The translocation of elements from soil into root and then to aboveground parts of plants is dependent on the mobility of elements in the rhizosphere, but especially on the bioavailability of each plant species [48].

Table 9. p-values of the species, polluted areas as well as the 95% Confidence Intervals for the means for the plant parts.

Adjusted values of the soil variables	p-values		95% Confidence Intervals		
Roots	Species	Polluted areas	1 2	1 3	2 3
log(Cr)	0.290	**<0.001**	(-1.195, 0.646)	(-0.967, 0.874)	(-0.589, 1.045)
log(Pb)	**0.024**	0.587	(-1.2214, 0.0835)	(-0.5877, 0.7172)	(0.0546, 1.2128)*
log(Zn)	**<0.001**	**0.001**	(-0.6252, 0.0208)	(-0.0380, 0.6080)	(0.3004, 0.8739)*
log(Cu)	0.129	**<0.001**	(-0.2446, 0.1634)	(-0.1093, 0.2986)	(-0.0458, 0.3163)
Aboveground					
As	0.494	**<0.001**	(-0.0983, 0.0617)	(-0.1150, 0.0450)	(-0.0893, 0.0559)
log(Zn)	0.183	**0.014**	(-0.220, 0.601)	(-0.108, 0.704)	(-0.266,0.481)
log(Cr)	0.538	**<0.001**	(-0.971, 0.792)	(-0.842, 0.903)	(-0.682,0.922)
Inflorescence					
log(As)	0.089	**<0.001**	(-0.2329, 0.2863)	(-0.3646, 0.1547)	(-0.3708, 0.1075)
log(Pb)	0.366	**<0.001**	(-0.4308, 0.5499)	(-0.6415, 0.3498)	(-0.6562, 0.2454)
log(Zn)	**0.003**	**<0.001**	(-0.2514, 0.3011)	(-0.578, -0.020)*	(-0.578, -0.070)*
log(P)	**0.007**	**<0.001**	(-583.6, 537.1)	(-1109.8, 230.0)	(-1035.3, -5.0)*

Bold – p-values indicate significant differences ($p<0.05$), *indicates significant differences between species.

Bothe [49] presents details about the mechanisms used by plants to tolerate metals, while providing a vast literature. As it is known [45, 49] in general, and also in our studies, roots have a higher metal content than the aboveground part of plants, which can be seen in Tables 8, and 9. It was also found that in area A, elements such As, Cr, Mn, and Ni presented higher concentrations in all three plant parts as compared to area B (Tables 8 and 9). However, when working with soils there is an obvious risk that microscopical soil particles may adhere to the root surface and there is also a high probability of finding nanoparticles of biominerals on the root surface, too. These particles are extremely difficult to remove from the root, and if they contain any pollutant metals they will be considered part of the root biomass.

In the case of clover and plantain, Mn concentrations were even higher in their inflorescence in area B as a result of increased mobility probably due to decreased pH. This is confirmed in the case of the dandelion, for which pH had almost similar values. In this case, the Mn concentration did not increase in the plants collected from area B. It was actually lower in all three plant parts as a consequence of concentration alleviation in soil. We mentioned that both Cr and Ni registered excessive or toxic values (>0.1-0.5 $\mu g \cdot g^{-1}$ Cr and >0.5-1 $\mu g \cdot g^{-1}$ Ni according to Kabata-Pendias and Pendias [31]) in almost all plant parts for both areas. The other elements presented here did not exceed the acceptable level in plants. Moreover, as we can see in Tables 8 and 9, the content of both P and toxic elements in plant parts was significantly different in the two polluted areas. In inflorescence, there were differences between areas A and B for all elements. In roots, there seemed to be no evidence of a difference for Mn and Pb between areas, whereas in the aboveground parts there was no evidence of a difference in the Mn content between the two areas. In general, there is no correlation between the soil metal content and the plant [50]. In the case of our studies, the plots from Fig. 1 also show that not all elements and pseudo-total concentrations of P in soil are correlated with the concentrations found in the three parts of the plants (roots, aboveground, and inflorescence) from the A and B areas. Also, the diagrams from Fig. 2 show the distribution of toxic elements and P in plant parts and soil. This was done by considering each polluted area separately, in order to spot the differences between the two areas. Thus it could be observed that the As, Cr, Mn, and Ni content in soil was higher in area A than in area B. The higher concentrations in the case of the clover and the plantain in area A could also be caused by the fact that the pH in the rhizospheres was higher, and it is known that neutral pH increases As solubilization and the potential bioaccumulation of this element in the food chain, depending of course, of their speciation which exist in soils [31, 51]. Similar patterns of variation to those shown in Figs. 1 and 2 were found in the case of element concentration in bioavailable form (data not shown).

Oxidative Stress

It is documented that in the environments where concentrations of toxic metals are over the accepted limit, if the enzymatic and non-enzymatic activities in plants increase, they can better tolerate the stress induced by metals [52].

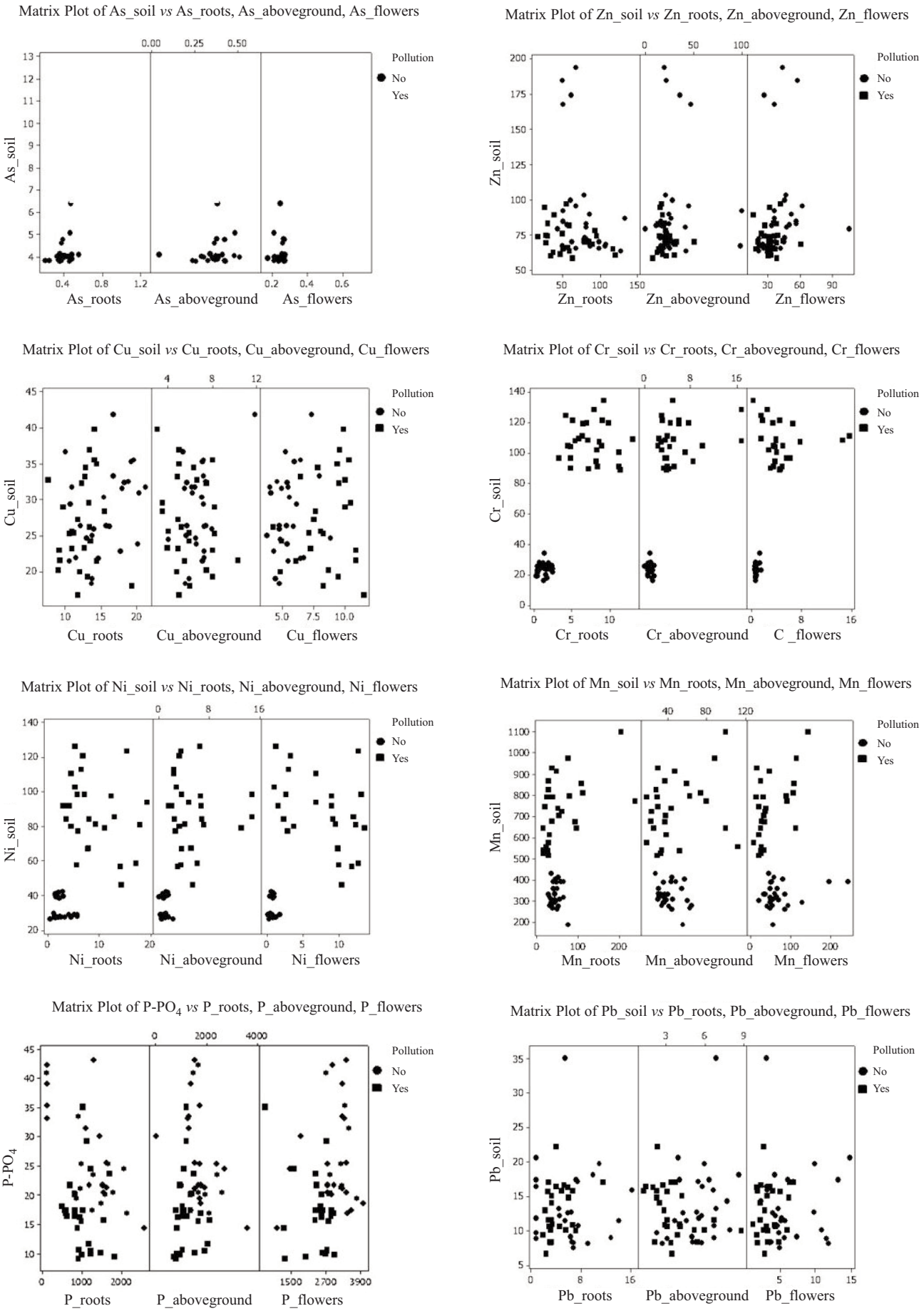
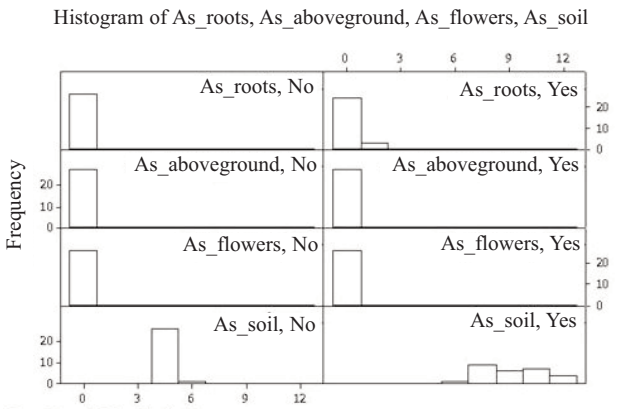
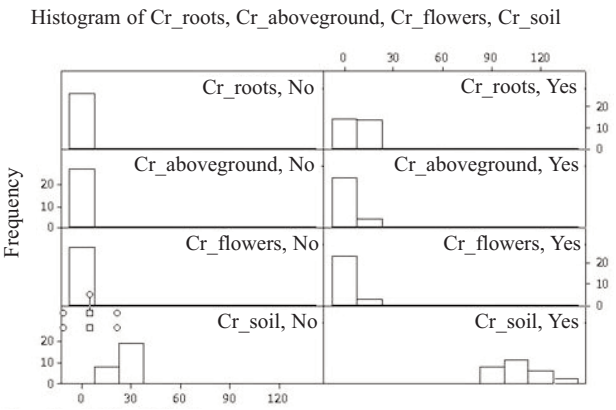
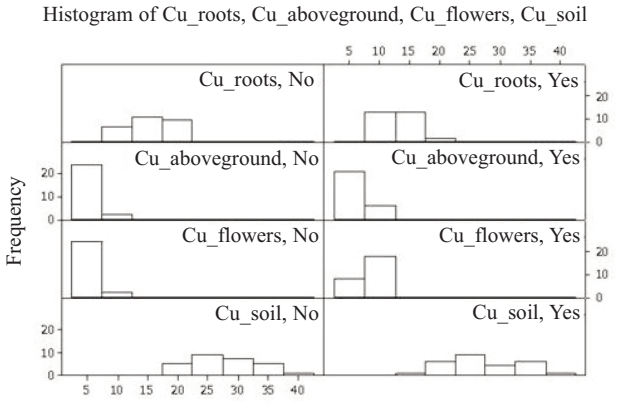
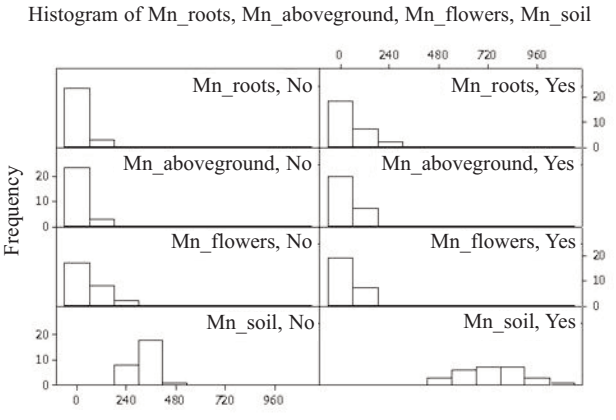


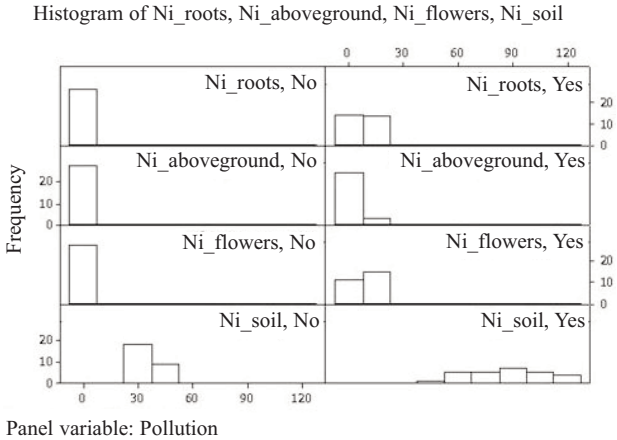
Fig. 1. Correlations between the concentrations of heavy metals and P in soil and plant parts.

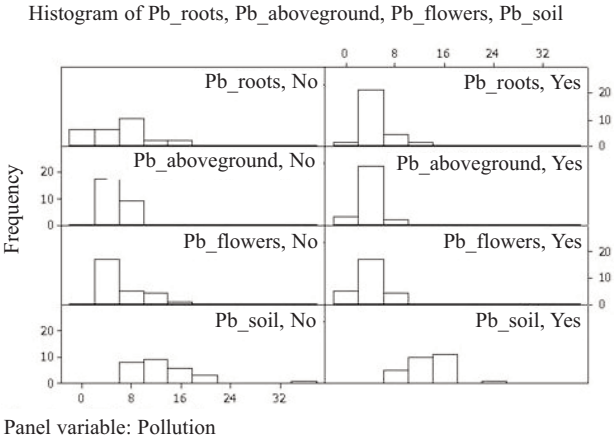


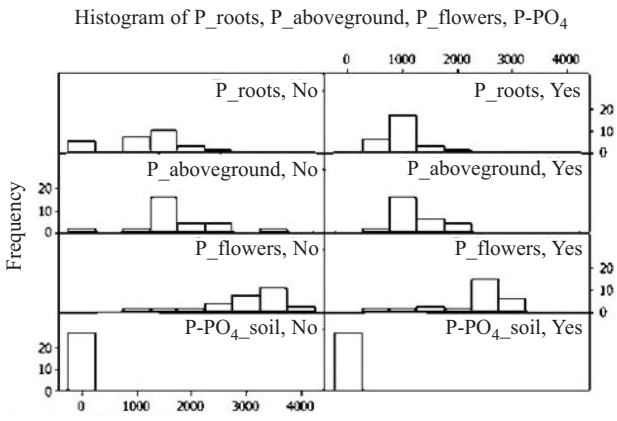












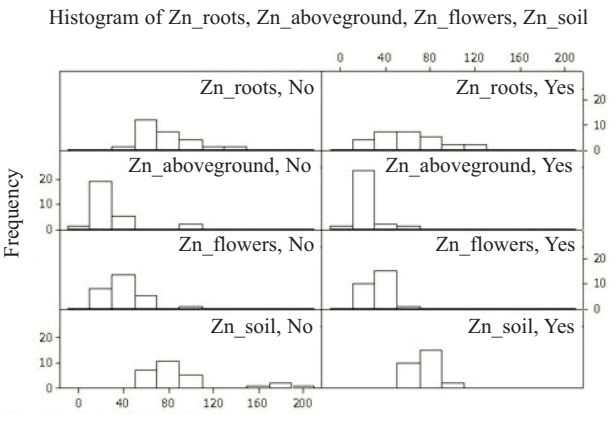


Fig. 2. Histograms showing the distribution of heavy metals and P in plant parts and soil in the polluted area.

Table 10. Biochemical variables of the three species of plants.

Area	Roots d.w.	Proteins mg·g^{-1}	SOD mU·mg^{-1}·prot.	POD µU·mg^{-1}·prot.	Chl *a* mg·g^{-1}	Chl *b* mg·g^{-1}	Carotenoids mg·g^{-1}	LP µM MDA·g^{-1}
A	Dandelion	1.788±0.319	9.809±1.839	2.235±0.418	na	na	na	17.45±7.041
	Clover	2.057±0.300	13.60±2.650	3.745±0.999	na	na	na	22.03 ±3.744
	Plantain	1.616±0.067	15.04±3.475	2.015±0.359	na	na	na	BDL
B	Dandelion	32.87±8.449	0.769±0.187	0.020±0.013	na	na	na	0.215 ±0.047
	Clover	33.30±13.39	0.892±0.328	0.029±0.013	na	na	na	0.318 ±0.089
	Plantain	28.56±3.068	0.963±0.228	0.039±0.009	na	na	na	BDL
Area	Aboveground d.w.	Proteins mg·g^{-1}	SOD mU·mg^{-1}·prot.	POD µU·mg^{-1}·prot.	Chl *a* mg·g^{-1}	Chl *b* mg·g^{-1}	Carotenoids mg·g^{-1}	LP µM MDA·g^{-1}
A	Dandelion	5.138±9.350	3.276±0.413	0.563±0.098	1.329±0.248	0.470±0.092	0.071±0.010	15.26±2.271
	Clover	6.096±0.496	3.516±0.996	0.577±0.124	2.747±0.941	0.938±0.295	0.144±0.046	18.61±2.717
	Plantain	2.184±0.139	2.840±0.703	1.166±0.306	1.419±0.463	0.620±0.176	0.087±0.023	BDL
B	Dandelion	33.05±3.785	0.576±0.094	0.018 ±0.009	1.043±0.274	0.347±0.087	0.064±0.016	0.245±0.034
	Clover	26.76±5.278	0.733±0.038	0.019±0.008	2.612±0.478	0.797±0.140	0.132±0.016	0.283±0.113
	Plantain	27.71±3.731	0.733±0.204	0.034±0.007	0.648±0.084	0.215±0.033	0.050±0.005	BDL
Area	Inflorescence d.w.	Proteins mg·g^{-1}	SOD mU·mg^{-1}·prot.	POD µU·mg^{-1}·prot.	Chl *a* mg·g^{-1}	Chl *b* mg·g^{-1}	Carotenoids mg·g^{-1}	LP µM MDA·g^{-1}
A	Dandelion	0.881±213.9	7.554±2.657	1.446±0.522	na	na	na	21.37±6.240
	Clover	1.180±0.153	1.513±0.663	1.236±0.243	na	na	na	59.82±3.351
	Plantain	0.578±0.163	10.98±9.263	2.754±7.358	na	na	na	BDL
B	Dandelion	34.46±3.928	0.526±0.148	0.017±0.009	na	na	na	0.247±0.030
	Clover	32.48±8.816	0.507±0.151	0.013±0.005	na	na	na	0.795±0.074
	Plantain	32.48±8.761	0.614±0.167	0.023±0.006	na	na	na	BDL

na – not analyzed (not relevant for the part of plant), BDL – below detection limit (< 0.02 µM MDA)

Antioxidants and antioxidative enzymes such as superoxide dismutases (SOD), catalases (CAT), and peroxidases (POD) function by interrupting the negative effect of the ROS. Plants produce several forms of SOD, which contain Cu, Zn, Fe, and Mn in their active centers. Thus, when bigger quantities of metals such as Ni, Zn, and Mn are added, the SOD activities increase with up to 20% more than the means obtained on the control area [53].

In our studies we also assessed the SOD and POD activities, and implicitly, the protein content in the investigated plant species. The values of these activities for all three species of plants grown in areas A and B can be seen in Table 10. Moreover, it could be observed that when the pollution was stronger (area A), the protein content significantly decreased in all three plant parts ($p<0.05$). This protein content consequently led to an increase in enzymatic activities, and so the SOD activity was more intense in the roots, followed by inflorescence and then the aboveground part of plants. The same variation pattern was noticed for POD activities, too. A similar increase in SOD activities also was found by Guala et al. [53] in their research (up to 60% compared to the control area) when mixtures of Ni+Cd, Ni+Zn, and Ni+Mn were added. Mehes-Smith et al. [52] stated that metal toxicity decreased the level of SOD and ascorbate peroxidase (APX), but increased the activity of catalase (CAT) and glutathione reductase (GR), while Dazy et al. [8] reported an increase in SOD and CAT activities for all investigated species, but working only on leaves on strong gradients of soil metal pollution. For the biochemical variables, statistically significant differences were registered between the pollution areas A and B in most parts of the three species of plants. The content of proteins, SOD, and POD in roots and inflorescence was statistically different between areas. Also, in the aboveground part the content of proteins, SOD, POD, carotenoids, chl a, and chl b were statistically different between areas. We also remarked that there were differences between species, as indicated by p-values ($p<0.05$) and 95% Confidence Intervals (when they do not include zero). These differences were similar to the ones found when comparing the areas, except for the content of SOD in the aboveground part, which was not statistically different between species. A statistical analysis on LP could not be performed as this was below the detection limit (<0.02 µM MDA) for the plantain species.

The statistical increase of P in plants is correlated with a higher content of protein and a lower enzymatic (SOD and POD) activity. Neagoe et al. [54] found similar positive correlations between protein and P nutrition in the case of four plant species grown on a metal-contaminated soil. Also, in a recent study, [55, 56] it was shown that P nutrition strongly influenced the development of *Agrostis capillaris* on a mine tailing substrate, alleviating the oxidative stress and increasing the protein content. We found the same variation pattern in the case of the enzymatic activities, but not in the case of lipid peroxides in plantain, whose concentration was below the detection limit of the used method (Table 10). Another measured biochemical variable was the content of photosynthetic pigments assessed in the aboveground part of plants. Surprisingly, both chlorophyll *a* and *b* and the carotenoids content registered slightly higher values in area A than in the less polluted area B. Sánchez-Viveros et al. [57] proved that when a concentration of metals was present, both the chlorophyll and the carotenoids contents were affected.

These findings could be partially explained through the negative impact of the metals on the perturbation of photosystem II. Moreover, Maleva et al. [14] observed a slight decrease of the chlorophyll contents after applying a combined treatment of Mn and Zn on *Elodea* (Egeria) *densa*, whereas when Cd was added, the chlorophyll contents slowly increased (by up to 9% compared to the control area). The same authors demonstrated the decrease (up to 1.5%) of the carotenoids content after adding Cu separately or combined with Mn and the increase (up to 24%) of their content when Cd was added. The stimulation of the carotenoids as a result of heavy metal pollution was also noticed by Mascher et al. [58], who stated that this effect could be attributed to a defense mechanism against the oxidative stress induced by the presence of high heavy metal concentrations. In addition, Kanoun-Boulé and Vicente [59] stated that the inhibitive or stimulating effects of metals on photosynthetic pigments content could be the result of sensitivity or tolerance particular to each species of plant. In our case, the polymetallic pollution had a clear effect of stimulating the photosynthetic pigments when there was a higher heavy metal concentration in area A.

It can be concluded that the physiology of plants has a great influence on their response to the stress induced by the presence of toxic elements. Even if the plants were sampled during the same vegetation period but in areas with different degrees of pollution (A and B), they had different growth conditions, which were influenced by temperature, different levels of humidity, shadowing, different nutrients content, etc. Neagoe et al. [54] reported that the P content in plants is a decisive factor in diminishing the oxidative stress caused by the presence of metals. There were statistically significant differences between the P concentration in area A and the P concentration in area B, as indicated by the statistical analysis carried out (Tables 8 and 9). The plants in area A showed, on average, lower P concentrations in the entire plant (all three species and parts of plant) as compared to area B (Table 7). It must be mentioned that P registered a value below the recommended limit in area A, whereas the concentrations in area B were within the sufficiency interval of 2000-5000 $\mu g \cdot g^{-1}$ P, according to Marschner [60].

Following the results obtained in terms of biochemical variables of three species and three plant parts grown in two areas with different levels of pollution, we support the idea that after more extensive investigation, these variables could be included in the monitoring program. By using these biochemical variables as bioindicators, the early detection of changes in the structure and function of biological systems could be possible as we believe that a timely reaction of the governments would prevent the irreversible damaging of the ecosystems. The advantage of using such bioindicators instead of instrument monitoring lies in the fact that they have the capacity to offer a response to the combined effect of various pollutants as is the case with our research area, where polymetallic pollution exists.

Conclusions

The three species of plants responded to the changes in soil pollution with toxic elements in a similar way, recording an increase of protein concentrations in all species and plant parts coupled with a decrease of SOD and POD activity. Moreover, the polymetallic pollution had a clear effect of stimulating the photosynthetic pigments when there was a higher concentration of both pseudo-total and bioavailable forms of elements with toxic potential. However, these results were inferred by changes in phosphorus availability in soil. The three plant species which have been investigated have been shown to be good bio-indicators of oxidative stress, but to support the idea that they may be used as a tool for early warning detection of alterations in metabolism, it is necessary to continue research on a greater number of plants and on the most varied areas of pollution gradients. It is desired that a permanent monitoring be established in the future, with a focus on the integrated effects of the multiple stressors, including the oxidative stress variables. These stressors could help to make a timely identification of potential alterations in plant metabolism without destroying the crops or the pastures. Even more so, it is necessary that the climate changes be taken into consideration both seasonally and all year round, since seasonal sampling is not sufficient to assess the level of pollution. Before making recommendations on the use of plant biochemical variables in monitoring, it is essential to clarify how the available major nutrients (N, P) modulate the bioaccumulation of toxic elements and the effects they might have on plant biochemical variables, in particular on oxidative stress.

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