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Bioaccumulation of heavy metals and ecophysiological responses to heavy metal stress in selected populations of *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L

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Abstract The aim of this study was to determine the concentrations of heavy metals (Cd, Pb, Zn, Fe, and Mn) in soil, and their bioavailability and bioaccumulation in *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. organs. Analysis also concerned the physiological responses of these plants from three polluted sites (immediate vicinity of a zinc smelter in Miasteczko Śląskie, ArcelorMittal Poland S.A. iron smelter in Dąbrowa Górnicza-Łosień, and Jaworzno III power plant in Jaworzno) and one pseudo-control site (Pazurek nature reserve in Jaroszowiec Olkuski). All of the sites are situated in the southern parts of Poland in the Śląskie or Małopolskie provinces. The contents of proline, non-protein thiols, glutathione, ascorbic acid, and the activity of superoxide dismutase and guaiacol peroxidase in the leaves of *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. were measured. In soil, the highest levels of Cd, Pb, and Zn (HNO_3 extracted and $CaCl_2$ extracted) were detected at the Miasteczko Śląskie site. At all sites a several times lower concentration of the examined metals was determined in the fraction of soil extracted with $CaCl_2$. Much higher Cd, Pb, Zn and Fe concentrations were found in *V. myrtillus* and *V. vitis-idaea* grown at the most polluted site (located near the zinc smelter) in comparison with cleaner areas; definitely higher bioaccumulation of these metals was found in lingonberry organs. Additionally, we observed a large capability of bilberry to accumulate Mn. Antioxidant response to heavy metal stress also differed between *V. myrtillus* and *V. vitis-idaea*. In *V. myrtillus* we found a positive correlation between the level of non-protein thiols and Cd and Zn concentrations, and also between proline and these metals. In *V. vitis-idaea* leaves an upward trend in ascorbic acid content and superoxide dismutase activity accompanied an increase in Cd, Pb, and Zn concentrations. At the same time, the increased levels of all tested metals in the leaves of *V. vitis-idaea* were accompanied by a decreased activity of guaiacol peroxidase. In both species increased Mn accumulation caused a decrease in antioxidant response.

Abbreviations

SPI	Single pollution index
NPI	Nemerow pollution index
NPTs	Non-protein thiols
GSHt	Glutathione total
AA	Ascorbic acid
SOD	Superoxide dismutase
GPX	Guaiacol peroxidase
TF	Translocation factor
MR	Mobility ratio
BCF	Bioconcentration factor

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Introduction

Vaccinium myrtillus L. (bilberry) and *Vaccinium vitis-idaea* L. (lingonberry) are the most frequent and abundant dwarf shrubs species in the understory of conifer forests in Europe

and Northern Asia, both belonging to the family Ericaceae and the genus *Vaccinium* (Liu et al. 2014; Rodriguez and Kouki 2015). Bilberry and lingonberry prefer silicate, nutrient-poor and humid soils. Although these species often co-occur, *V. myrtillus* generally requires moister conditions and shady sites, while *V. vitis-idaea* can also be found on more arid and exposed sites (Sebald et al. 1993; Polatschek 1999; Ganthaler and Mayr 2015). Dwarf shrubs provide provisioning (food), regulating (pollination), species habitat and they constitute one of the major drivers in the ecosystem dynamics of the boreal forest, affecting seedlings regeneration and the soil nutrient and carbon cycles (Nilsson and Wardle 2005; Kolari et al. 2006; Rodriguez and Kouki 2015). The major reason for the interest in *V. myrtillus* L. and *V. vitis-idaea* L. is that they can serve as model species of the forest floor of boreal forests, differing in their life strategies (Taulavuori et al. 2013). Bilberry is a deciduous species as it sheds its leaves during autumnal preparation for winter, whereas lingonberry, an evergreen species, retains its leaves for many years (Taulavuori et al. 2013; Parzych 2014). Because of these differences it is interesting to study whether or not these species vary in responses to any stress, including heavy metals stress.

Heavy metal pollution is one of the most important environmental problem these days. It is growing with inevitable pace, endangering humans, animals, and plants (Anjum et al. 2015a, b, 2016). The biological effects of individual metals are more or less known, but even though combinations of heavy metals are common in nature, their combined effects still need to be thoroughly investigated (Wilde et al. 2006; Cyjetko et al. 2010; Tkalec et al. 2014). All non-essential heavy metals, as well as essential ones, when present in higher concentrations than optimal, affect different cellular components, thereby interfering with the normal metabolic functions of plant cells (Tkalec et al. 2014). For example, elevated levels of heavy metals are associated with the increased generation of reactive oxygen species (ROS) (Emamverdian et al. 2015). These ROS interfere with various macromolecules and disrupt normal cellular functions and metabolism (e.g. resulting in lipid peroxidation, inactivation or damage of proteins and chlorophyll, DNA injury) (Anjum et al. 2016). Once formed, ROS must be detoxified as efficiently as possible to minimize damage. Antioxidant systems in plants are complex and involve an array of non-enzymatic and enzymatic mechanisms capable of preventing the cascades of uncontrolled oxidation (Gratão et al. 2005, 2008; Kandziora-Ciupa et al. 2013). The non-enzymatic antioxidants are generally small molecules that include tripeptide glutathione, proline, cysteine, ascorbate, non-protein compound rich in −SH groups, etc. The enzymatic antioxidant components contain the enzymes capable of removing, neutralizing, or scavenging ROS and the intermediates such

as superoxide dismutase (SOD), catalase (CAT), guaicaol peroxidase (GPX), and glutathione reductase (Das et al. 2015). Antioxidant systems in plants may be used as early indicators of environmental stress on target organisms preceding morphological or ultrastructural damage, and as warning indicators for the ecosystem (Białońska et al. 2007; Kandziora-Ciupa et al. 2016). Estimation of plant ecophysiological responses in a field study may be useful in pollution biomonitoring as well as in verifying the effect of metal contamination on plant physiology (Hu et al. 2014; Nadgórska-Socha et al. 2016).

Therefore, the objective of the current study was to determine metal accumulation efficiency in polluted and non-polluted areas and ascertain the influence of selected heavy metals (Cd, Pb, Zn, and Mn) on levels of antioxidants (proline, non-protein thiols, glutathione, ascorbic acid) and antioxidant enzymes activity (guaiacol peroxidase, superoxide dismutase) in *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. leaves. We hypothesized that (1) bioaccumulation of chosen heavy metals in the leaves, stems, and roots of *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. from the polluted sites would vary from those at a non-polluted site; (2) *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. would differ in responses to heavy metal stress; (3) the chosen and examined ecophysiological parameters are good indicators of oxidative stress caused by heavy metals in plants living under field conditions.

Materials and methods

Study area

The study was performed in four areas under different levels of anthropogenic stress [immediate vicinity (about 1 km from) of a zinc smelter "Miasteczko Śląskie" (M), iron smelter "ArcelorMittal Poland S.A." in Dąbrowa Górnicza-Łosień (L) (about 3.5 km from), power plant "Jaworzno III" in Jaworzno (J) (about 1 km from) and nature reserve "Pazurek" in Jaroszowiec Olkuski (P)]. All the sites are situated in southern Poland, in either the Śląskie or Małopolskie provinces. Accurate characterization and maps of the sites are given in a previous works by Kandziora-Ciupa et al. (2013) and Nadgórska-Socha (2016).

Because nature reserve "Pazurek" is situated in close proximity (but not directly) to a number of smelters, in additions to the east (in Poland westerly winds prevail) (Bolesław and Olkusz very strong Zn-Pb mines/smelters – 7 km, many local factories – 3.5 km, and large mine/smelter complex—Upper Silesian Industrial Region – 20 km) we couldn't considered this study area as completely good control-site, so we decided, in whole manuscript, that this reference site would be referred as "pseudo-control".

The study was conducted in middle-aged Scots pine forest, growing on sandy acidic soils, mixed with birch (*Betula pendula* L.), European beech (*Fagus sylvatica* L.), and Pedunculate Oak (*Quercus robur* L.), without or with small-scale forest management.

Sample collection

Vaccinium myrtillus L. and *Vaccinium vitis-idaea* L. leaves, stems, and roots and soil samples were collected in mid-June 2012. Each sampling site consisted of 25×25 m square in three replicates within which three subsamples bilberry and lingonberry organs and soil samples were randomly collected. The fully matured and undamaged leaves, and the stems and roots of each species were collected from at least 20 different specimens (*V. vitis-idaea* L. has not occurred at the site L so organs from this species at this site were not collected). After collection, the samples were covered with plastic bags, deposited in ice, immediately transported to the laboratory, and then frozen until analysis.

The soil samples, after removing surface litter, were taken in the neighborhood of the samples shrubs from a depth of 0–10 cm. At each site soil sub-samples were combined in a composite sample.

Analysis of metal concentration in the soil and samples of plants

The concentrations of Cd, Pb, Zn, Fe, and Mn were analyzed in individual soil fractions and in the leaves, stems, and roots of *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. The metal content in soil was estimated according to the method of Bouwman et al. 2001 and Ostrowska et al. (2001) in the air-dried soil samples, which were sieved through a 1 mm sieve. Metals were extracted from soil with 0.01 M $CaCl_2$ (potentially bioavailable elements) or with 2 M HNO_3 (acid extracted elements). For the $CaCl_2$ extraction, 5 g of soil with 50 ml of 0.01 M $CaCl_2$ solution was mechanically shaken for 2 h. The HNO_3-extractable fraction was obtained by shaking a 10 g soil sample with 100 ml of 2 M HNO_3 for 1 h. The content of metals was measured in the filtered extracts by inductively coupled plasma-atomic emission spectroscopy (Spectro Analytical Instruments).

For acid extracted elements, single pollution index and Nemerow pollution index were calculated.

Pollution with a given heavy metal (i) was evaluated with the single pollution index (Pi) calculated as the ratio between the metal concentration (Ci) in a soil sample and permitted standard of the same metal (Si):

$$Pi = C_i / S_i$$

(Lei et al. 2010; Hu et al. 2013).

Permitted standard for this study was recommended by the Regulation of the Ministry of Environment (2002) about the standards of soil and ground quality (Zn—300 mg kg^{-1}; Pb—100 mg kg^{-1}; Cd—4 mg kg^{-1}).

The overall pollution status of the surface soils by the heavy metals was assessed by the Nemerow pollution index (Pn) (Cheng et al. 2007; Hu et al. 2013):

$$Pn = \sqrt{\left(P_{ave}{}^2 + P_{max}{}^2\right)/2}$$

where P_{ave} is the average single pollution index of all metals and P_{max} is the maximum value of the single pollution index of all metals. Pollution of the surface soils by the heavy metals was classified into five grades based on the Nemerow pollution index (Pn < 0.7—non-pollution; Pn 0.7–1.0—pollution warning line; Pn 1.0–2.0—low level of pollution; Pn 2.0–3.0—moderate level of pollution; Pn > 3.0—high level of pollution) (Jiang et al. 2014).

In order to determine the heavy metal concentrations in the organs of bilberry and lingonberry, the plant material was washed in a tap with distilled water and dried at 105 °C. A 0.25 g portion of dried plant material was treated with 5 ml of concentrated nitric acid and left for 24 h. Next, the samples were digested at 110 °C until complete digestion was achieved. After digestion, the samples were diluted with deionized water to a volume of 25 ml. Concentration of Cd, Pb, Zn, Fe and Mn were measured using flame absorption spectrometry (Thermo Scientific iCE3500). The quality of the analytical procedure was checked using a reference material (Certified reference Material CTA-OTL-1 Oriental Tobacco Leaves) with the same quantities of samples.

Metal accumulation efficiency

To evaluate the metal accumulation in plants, translocation factor (TF), mobility ratio (MR), and bioconcentration factor (BCF) were calculated. TF is the ratio of metal concentration in shoots (leaves + stems) and roots. TF > 1 indicates that plants translocates metals effectively from root to shoot (Serbula et al. 2012). MR is the ratio of metal concentration in the shoot (leaves + stems) to its concentration in soil. MR > 1 indicates that the plant is enriched with metals (accumulator), MR1 indicates a rather indifferent behavior of the plant towards metals (indicator), and a MR < 1 shows that the plant excludes metals from uptake (excluder) (Mingorance et al., 2007; Serbula et al. 2012). The bioconcentration factor (BCF) was calculated to measure the ability of each organ (leaves, stems, and roots) to accumulate metals from soil (Hladun et al. 2015). BCF > 1 indicates that particular element is accumulated by leaves, stems, or roots from soil (Yoon et al. 2006; Serbula et al. 2013).

Analysis of the biochemical parameters of the plants

All ecophysiological parameters were measured in the leaves of *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L.

Proline content was determined by the method of Bates et al. ([1973](#)). The plant material (0.5 g of) was homogenized in 10 ml of sulfosalicylic acid (3 g per 100 ml) and the homogenate was filtered through Whatman No. 2 filter paper. The reaction mixture containing 2 ml of homogenate, 2 ml acid ninhydrin and 2 ml of glacial acetic acid was incubated at 100 °C for 1 h. The reaction mixture was placed on ice and extracted with 4 ml of toluene. The absorbance was read at 520 nm using toluene as the blank. The proline content was expressed in µmol proline g^{-1} fresh weight.

The content of non-protein thiols was estimated as described by Mass et al. ([1987](#)). The plant material was homogenized in a 5 vol/g mixture containing 5-sulphosalicylic acid (2 g per 100 ml) and 1 mM EDTA and sodium ascorbate (0.15 g per 100 ml). The samples were centrifuged at 20,000 g for 10 min at 4 °C. Then a 0.5 ml liquid supernatant, 0.5 ml of a 1 M sodium phosphate buffer (pH 8.0) and 100 µl of 10 mM 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) were put into test tubes. The absorbance at 415 nm was read 1 min after the addition of DTNB. The number of non-protein SH groups was established based on a curve prepared using L-cysteine and expressed as nmol –SH g^{-1} fresh weight.

To measure the total glutathione concentration (GSHt), plant parts (0.5 g) were homogenized in TCA (trichloroacetic acid, 5 g per 100 ml) and 0.125 mM phosphate buffer (pH 6.3) with 6.3 mM EDTA and were centrifuged at 10,000 g for 10 min at 4 °C. Supernatants were used for GSH determination using the DTNB - GSSG reductase recycling procedure according to Anderson ([1985](#)). The reaction mixture contained 0.2 ml of supernatant, 0.6 ml of 0.3 mM NADPH, 0.1 mL of 6 mM DTNB and 0.1 ml (0.5 IU ml^{-1}) of glutathione reductase. The linear changes in the absorbance of the reaction mixtures were measured at 412 nm and the GSHt was expressed as µmol GSH g^{-1} fresh weight.

Ascorbic acid content was calculated by the formula given by Keller and Schwanger [1977](#):

$$\text{Ascorbic acid} \left(mg \times g^{-1}\ f.w. \right) = \frac{(E_O - E_S - E_t)}{W \times 100} \times 100$$

where V is the volume of the extract, W is the weight of the leaf sample (g), and E_o, E_s and E_t are optical densities of a blank sample, a plant sample, and a sample with ascorbic acid, respectively.

The analysis of superoxide dismutase (SOD) was performed in a buffer with 3 mM $MgSO_4$, 1 mM dithiotreitol (DTT), 3 mM EDTA (1:5 ratio) and centrifuged at 12,000 g for 20 min. The entire procedure was carried out at 4 °C. The reaction was measured spectrophotometrically at 560 nm according to Beauchamp and Fridovich ([1971](#)). One unit of SOD was defined as the amount of enzyme activity that was able to inhibit the photoreduction of nitroblue tetrazolium (NBT) to blue formazan by 50%.

For the analysis of guaiacol peroxidase fresh plant material was homogenized in a 100 mM phosphate buffer (pH 6.8). The GPX activity was measured at 470 nm according to Fang and Kao ([2000](#)) and Liu et al. ([2004](#)) with guaiacol as the substrate. The GPX activity was measured in a reaction mixture (3 ml) containing a 50 mM phosphate buffer (pH 5.8), 1.6 µl H_2O_2, 1.5 µl guaiacol and 0.2 ml enzyme extract. The activity was calculated using the extinction coefficient (26 $mM^{-1}cm^{-1}$) for tetraguaiacol and was expressed in µmol tetra-guaiacol g^{-1} fresh weight min^{-1}.

Statistical analysis

The metal content in organs of the studied plants and in the soil and biochemical parameters was analyzed, checked for normality and equality of variance. One-way ANOVA was carried out to compare the differences in soil and plant variables from various sampling sites and significant statistical differences were established using the Tukey's test, $p < 0.06$ (ANOVA; Statistica 10 package, StatSoft, Inc.). Pearson's correlation coefficient was calculated for assessing the relationship between estimated metal concentrations and biochemical parameters in the bilberry and lingonberry leaves. CANOCO 4.5 was used to carry out Principal Component Analysis (PCA). Principal Component Analysis assessed the relationships between heavy metal concentrations and biochemical parameters in the leaves of *V. myrtillus* and *V. vitis-idaea*.

Results

Heavy metal content and their availability in soil

There were significant differences in the content of the metals studied (HNO_3 extracted and $CaCl_2$ extracted) between the polluted and pseudo-control site (Table [1](#)). Additionally, there was a clear difference in the concentrations of metals between the fraction of soil extracted with HNO_3 and the fraction extracted with $CaCl_2$. Among the metals examined, the highest concentrations of Fe, Pb and Zn were measured in the acid-extracted soil fraction. A several times lower concentration of the metals examined was determined in the fraction of soil extracted with $CaCl_2$. The highest level of Cd, Pb and Zn in both fractions was

Table 1 The concentration of selected metals (mg kg^{-1}) in fractions of the soils extracted with HNO$_3$ and CaCl$_2$ (mean values ± SE, $n = 9$). The different letters denote significant differences between the particular metal concentrations ($p < 0.05$)

Site	Cd		Pb		Zn		Fe		Mn		pH
	HNO$_3$	CaCl$_2$	HNO$_3$	CaCl$_2$	HNO$_3$	CaCl$_2$	HNO$_3$	CaCl$_2$	HNO$_3$	CaCl$_2$	
M	40.20 ± 5.54b	8.95 ± 1.30b	448.94 ± 26.48b	18.68 ± 5.11b	1362.17 ± 54.84b	249.79 ± 43.82b	1160.72 ± 100.99b	2.13 ± 0.37a	87.61 ± 20.42a	10.19 ± 1.30a	4.5
L	5.86 ± 1.45a	0.46 ± 0.11a	190.88 ± 64.50a	1.01 ± 0.05a	207.98 ± 60.58a	26.78 ± 7.42a	681.39 ± 47.20a	1.11 ± 0.32a	367.48 ± 35.97b	11.14 ± 1.02a	5.1
J	2.87 ± 0.87a	0.51 ± 0.23a	110.17 ± 44.54a	1.37 ± 0.31a	165.32 ± 71.45a	44.19 ± 1.10a	1018.15 ± 33.43b	5.47 ± 2.76a	69.87 ± 14.22a	13.07 ± 0.19a	5.1
P	2.65 ± 0.89a	0.66 ± 0.23a	182.08 ± 56.23a	2.62 ± 0.56a	117.22 ± 35.32a	33.04 ± 11.60a	1274.68 ± 93.35b	14.22 ± 5.30b	70.06 ± 31.35a	8.51 ± 1.39a	4.1

Table 2 Single pollution index (SPI) and Nemerow pollution index (NPI) (for acid extracted elements) in soil of investigated sites

Site	SPI-Cd	SPI-Pb	SPI-Zn	NPI
M	10.05	4.49	4.54	8.41
L	1.46	1.91	0.69	1.66
J	0.72	1.10	0.55	0.96
P	0.66	1.82	0.39	1.45

observed at site M (respectively: 40.20, 448.94, 1362.17 mg kg^{-1} after HNO$_3$ extraction; 8.95 mg kg^{-1}, 18.68, 249.79 after CaCl$_2$ extraction) (Table 1). The highest levels of Fe (HNO$_3$: 1274.68 mg kg^{-1}, CaCl$_2$: 14.22 mg kg^{-1}) were observed at site P and highest level of Mn we found at sites L and J (L – HNO$_3$: 367.48 mg kg^{-1}, J – CaCl$_2$: 13.07 mg kg^{-1}).

The following descending order of bioavailability was found among the heavy metals: Zn > Cd > Mn > Pb > Fe.

The highest single pollution of all investigated metals were found at site M. Nemerow pollution index ranged from 0.96 at J site to 8.41 in the soil of the most polluted site M (Table 2).

Metal bioaccumulation in leaves, stems, and roots

Metal bioaccumulation was analyzed in leaves, stems, and roots of *V. myrtillus* and *V. vitis-idaea* (Tables 3 and 4). The highest Cd, Pb, and Zn content in leaves, stems, and roots were found in the plants collected at the M site for both species; additionally at the same site we observed the highest Fe concentrations in all *V. vitis-idaea* organs. The highest bioaccumulation of Mn was noticed in *V. myrtillus* organs.

Metal accumulation efficiency

Mean values of translocation factor, mobility ratio, and bioconcentration factors in bilberry and lingonberry at all sampling sites are given in Tables 5 and 6. Effective translocation (TF > 1) in *V. myrtillus* was observed for Pb at J and P sites, for Zn at L site and for Mn at L, J and P sites; in *V. vitis-idaea* was observed for Pb, Zn, and Mn at P site, and additionally for Mn at J site. The values of mobility ratio for Cd, Pb, Zn, and Fe in shoots of both species from contaminated areas showed that their absorption from the soil was not considerable (except Pb at M site for lingonberry). However, we observed very high MR for Mn at all investigated sites for *V. myrtillus* and at J and P site for *V. vitis-idaea*. Also for Mn BCF was greater than one for all organs at all sites for *V. myrtillus* and at sites J and P for *V. vitis-idaea*. BCF also greater than one was observed for Pb in all lingonberry organs at most contaminated site (M) and

Table 3 The concentrations of heavy metals (mg kg^{-1} d.w.) in the various plant tissue of *V. myrtillus* (mean values ± SE, $n = 9$). The different letters denote significant differences between the particular metal concentrations in the same organ ($p < 0.05$)

Site	Plant organs	Cd	Pb	Zn	Fe	Mn
M	Leaves	7.68 ± 2.31b	222.02 ± 61.58b	308.70 ± 63.44b	121.41 ± 11.44a	133.15 ± 12.28a
	Stems	15.34 ± 2.83b	217.02 ± 42.38b	640.08 ± 20.05b	92.27 ± 17.74a	132.69 ± 28.19a
	Roots	22.91 ± 1.58b	837.47 ± 33.24b	886.75 ± 2.39c	148.47 ± 12.29ab	154.03 ± 2.93a
L	leaves	0.75 ± 0.39a	34.07 ± 13.23a	65.23 ± 15.55a	177.06 ± 4.15a	661.56 ± 10.79c
	Stems	0.97 ± 0.06a	39.99 ± 12.76a	299.80 ± 13.43a	135.89 ± 35.29a	418.28 ± 15.93b
	Roots	1.95 ± 0.07a	45.78 ± 18.06a	149.42 ± 7.53a	195.84 ± 32.05bc	415.35 ± 17.87bc
J	Leaves	0.22 ± 0.09a	20.93 ± 8.34a	57.93 ± 17.81a	164.62 ± 47.23a	873.11 ± 15.55d
	Stems	1.02 ± 0.11a	51.36 ± 37.25a	177.05 ± 14.28a	100.29 ± 15.73a	605.16 ± 76.19b
	Roots	1.10 ± 0.07a	33.86 ± 4.42a	139.42 ± 7.55a	214.27 ± 12.52c	411.16 ± 14.29b
P	Leaves	0.32 ± 0.15a	201.02 ± 98.97b	70.27 ± 1.43a	120.74 ± 32.17a	555.92 ± 56.91b
	Stems	0.24 ± 0.05a	27.23 ± 4.00a	191.40 ± 12.28a	116.69 ± 15.37a	583.90 ± 70.83b
	Roots	1.71 ± 0.04a	98.99 ± 38.21a	208.63 ± 3.40b	139.34 ± 5.84a	450.81 ± 15.95c

for this same species for Zn in roots from M and P sites. For *V. myrtillus* BCF > 1 for Pb and Zn in roots was found at M and P sites, respectively, and for Zn in stems from L, J, and P sites.

The biochemical status of the plants

There were no significant differences in the proline content between the contaminated and pseudo-control sites and between two investigated species (Fig. 1). We found significant correlations between the proline content and the concentrations of the Cd, Zn, and Mn in the leaves of *V. myrtillus* (Table 7, Fig. 2) and between proline content and the concentration of the Fe in the leaves of *V. vitis-idaea* (Table 8, Fig. 3).

The greatest concentration of non-protein −SH groups was found in the leaves of *V. myrtillus* at the most contaminated M site (47.78 nmol −SH g^{-1} fresh weight) (Fig. 4). This dependence was confirmed by a positive correlation between non-protein −SH groups and the content of Cd and Zn in the leaves of bilberry (Table 7). At the other sites and in *V. vitis-idaea* leaves the content of NPTs was definitely lower (Fig. 4).

In the leaves of both investigated species the highest content of glutathione (GSHt) (for *V. myrtillus*: 473.02 μmol GSH g^{-1} f.w.; for *V. vitis-idaea* 280.46 μmol GSH g^{-1}) and ascorbic acid (AA) (for *V. myrtillus* 0.71 mg g^{-1} f.w; for *V. vitis-idaea* 0.68 mg g^{-1} f.w) was detected in the most contaminated area (M) (Fig. 5, Fig. 6). A positive correlation was found between GSHt and Zn in leaves of *V. myrtillus* and between AA and Cd, Pb, Zn in leaves of *V. myrtillus* (Tables 7 and 8; Figs. 2 and 3). Additionally we found a strong negative correlation between Mn and GSHt and AA in *V. vitis-idaea* leaves, and also between Mn and AA in *V. myrtillus* leaves (Tables 7 and 8; Figs. 2 and 3).

An elevated SOD activity in the leaves of *V. myrtillus* and *V. vitis-idaea* in our study was recorded at M and L sites—the highest activity was observed in *V. myrtillus* at L site (260.21 U) (Fig. 7). We found positive correlations between Cd, Pb, Zn, Fe and SOD activity in *V. vitis-idaea* leaves (Table 8, Fig. 3).

Guaiacol peroxidase activity was by far the highest in the leaves of *V. myrtillus* from the L site (24.34 μmol tetra-guaiacol g^{-1} fresh weight min^{-1}) (Fig. 8). The lowest GPX activity was observed at M in leaves of both investigated species (for *V. myrtillus*: 6.39 34 μmol tetra-guaiacol g^{-1} fresh weight min^{-1}; for *V. vitis-idaea* 3.57 34 μmol tetra-guaiacol g^{-1} fresh weight min^{-1}) (Fig. 8). Guaiacol peroxidase activity in the leaves of lingonberry was negatively correlated with Cd, Pb, and Zn content (Table 8 and Fig. 3).

In *V. myrtillus*, the first two axes of PCA explained 98.3% physiological characteristic variability (85.6% by axis 1; 12,7% by axis 2) (Fig. 2) Similarly, in *V. vitis-idaea* the first two axis of PCA explained 100% of physiological characteristic variability (91% by axis 1 and 9% by axis 2) (Fig. 3).

Discussion

Heavy metal content and their availability in soil

Forest ecosystems are known for their high capacity to filter contaminants, including heavy metals (Jamnická et al. 2013). In this study we aimed at evaluating heavy metal accumulation and ecophysiological parameters in *V. myrtillus* and *V. vitis-idaea* growing naturally at contaminated and pseudo-control sites.

Many studies emphasize that the total concentration of heavy metals in soils are useful indicators of the extent of soil contamination but provides a poor indication of plant

Table 4 The concentrations of heavy metals (mg kg^{-1} d.w.) in the various plant tissue of *V. vitis-idaea* (mean values ± SE, $n = 9$). The different letters denote significant differences between the particular metal concentrations in the same organ ($p < 0.05$)

Site	Plant organs	Cd	Pb	Zn	Fe	Mn
M	Leaves	14.02 ± 2.96b	461.79 ± 49.04b	427.25 ± 36.32b	178.79 ± 2.31b	79.94 ± 2.10a
	Stems	54.47 ± 0.78b	1381.43 ± 133.38b	1067.95 ± 97.44b	412.02 ± 67.84b	56.87 ± 2.36a
	Roots	55.69 ± 9.28b	1041.58 ± 141.05b	2068.56 ± 253.78b	406.54 ± 70.25c	151.64 ± 14.33a
J	Leaves	0.19 ± 0.03a	27.44 ± 12.94a	62.91 ± 1.61a	112.23 ± 7.73a	126.40 ± 2.11b
	Stems	0.41 ± 0.01a	111.63 ± 2.96a	96.46 ± 4.19a	283.10 ± 2.11a	92.37 ± 3.76b
	Roots	1.23 ± 0.05a	90.84 ± 31.23a	116.18 ± 1.55a	270.14 ± 13.19b	106.29 ± 3.24a
P	Leaves	0.23 ± 0.08a	91.94 ± 36.15a	107.20 ± 5.14a	159.84 ± 5.60c	507.31 ± 0.08c
	Stems	0.27 ± 0.05a	100.93 ± 1.49a	140.56 ± 28.43a	214.08 ± 3.84a	386.12 ± 16.94c
	Roots	2.38 ± 0.78a	87.79 ± 3.46a	178.29 ± 43.39a	140.21 ± 40.16a	338.05 ± 74.68b

Table 5 Translocation factor (TF), Mobility ratio (MR) and Bioconcentration factors (BCF) (in various plant organs) calculated for *V. myrtillus* ($n = 9$)

			M	L	J	P
Cd	TF		0.51	0.44	0.57	0.17
	MR		0.29	0.15	0.22	0.11
		Leaves	0.19	0.13	0.08	0.12
	BCF	Stems	0.38	0.17	0.36	0.09
		Roots	0.57	0.33	0.09	0.65
Pb	TF		0.27	0.98	1.03	1.13
	MR		0.49	0.19	0.33	0.63
		Leaves	0.49	0.18	0.19	1.10
	BCF	Stems	0.48	0.21	0.47	0.15
		Roots	1.87	0.24	0.31	0.54
Zn	TF		0.53	1.21	0.85	0.63
	MR		0.35	0.88	0.71	1.12
		Leaves	0.23	0.31	0.35	0.60
	BCF	Stems	0.47	1.44	1.07	1.63
		Roots	0.65	0.72	0.84	1.78
Fe	TF		0.72	0.80	0.62	0.85
	MR		0.09	0.23	0.13	0.09
		Leaves	0.10	0.26	0.16	0.09
	BCF	Stems	0.08	0.20	0.10	0.09
		Roots	0.13	0.29	0.21	0.11
Mn	TF		0.86	1.30	1.80	1.27
	MR		1.52	1.47	10.58	8.13
		Leaves	1.52	1.80	12.50	7.93
	BCF	Stems	1.51	1.14	8.66	8.33
		Roots	1.76	1.13	5.88	6.43

Table 6 Translocation factor (TF), Mobility ratio (MR) and Bioconcentration factors (BCF) (in various plant organs) calculated for *V. vitis-idaea* ($n = 9$)

			M	J	P
Cd	TF		0.63	0.24	0.11
	MR		0.85	0.10	0.09
		Leaves	0.35	0.07	0.09
	BCF	Stems	1.36	0.14	0.10
		Roots	1.39	0.43	0.90
Pb	TF		0.89	0.83	1.09
	MR		2.05	0.63	0.53
		Leaves	1.03	0.25	0.50
	BCF	Stems	3.08	1.01	0.55
		Roots	2.32	0.82	0.48
Zn	TF		0.37	0.69	0.72
	MR		0.55	0.48	1.05
		Leaves	0.31	0.38	0.91
	BCF	Stems	0.78	0.58	1.19
		Roots	1.52	0.70	1.52
Fe	TF		0.73	0.73	1.41
	MR		0.25	0.19	0.15
		Leaves	0.15	0.11	0.13
	BCF	Stems	0.35	0.28	0.17
		Roots	0.35	0.27	0.11
Mn	TF		0.45	1.03	1.36
	MR		0.78	1.57	6.38
		Leaves	0.91	1.81	7.24
	BCF	Stems	0.65	1.32	5.51
		Roots	1.73	1.52	4.83

phytoavailability (Tokalioglu et al. 2000; Wang et al. 2004; Feng et al. 2005; Ortiz and Alcañiz 2006; Xiao et al. 2011; Dao et al. 2012; Boussen et al. 2013). A large part of bio-monitoring research consists in the comparison of bioavailable fractions of metals (extracted with CaCl$_2$) with their total concentration or pseudo-total (extracted, e.g. 2 M HNO$_3$) in soil (Ullrich et al. 1999; Peijnenburg and Jager 2003; Pueyo et al. 2004; Keller and Hammer 2004; Kandziora-Ciupa et al. 2013). Neutral salt extraction (e.g. with CaCl$_2$) provides the most useful indication of metal phytoavailability and is more effective for estimating plant availability (Lebourg et al. 1996; Boussen et al. 2013). In our study, higher concentrations of (acid- and CaCl$_2$-extracted) Cd, Pb, and Zn were found in the Miasteczko
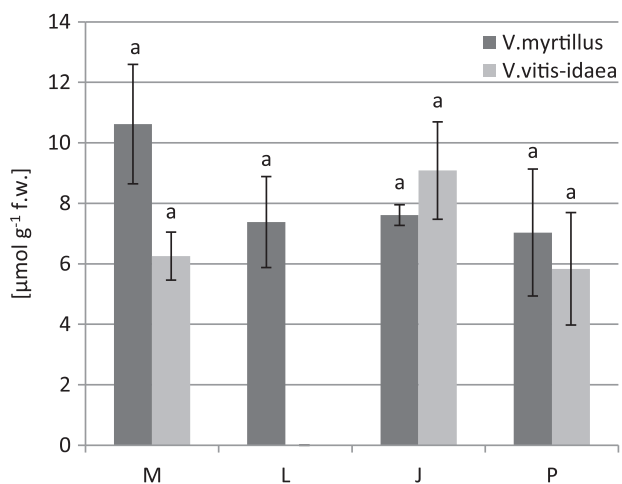
Fig. 1 Proline contents (μmol g^{-1} fresh weight) in *V. myrtillus* and *V. vitis-idaea* leaves (mean values ± SE, $n = 9$). *Different letters* above the columns indicate significant differences for the species ($p < 0.05$)

Table 7 The correlation coefficients between metal concentration and antioxidant measurements in the leaves of *V. myrtillus* ($p < 0.05$); NS-not significant

	Cd	Pb	Zn	Fe	Mn
PRO	0.79	NS	0.77	NS	−0.63
GSHt	NS	NS	0.58	NS	NS
−SH	0.91	NS	0.91	NS	NS
AA	NS	NS	NS	NS	−0.63
SOD	NS	NS	NS	NS	NS
GPX	NS	NS	NS	NS	NS
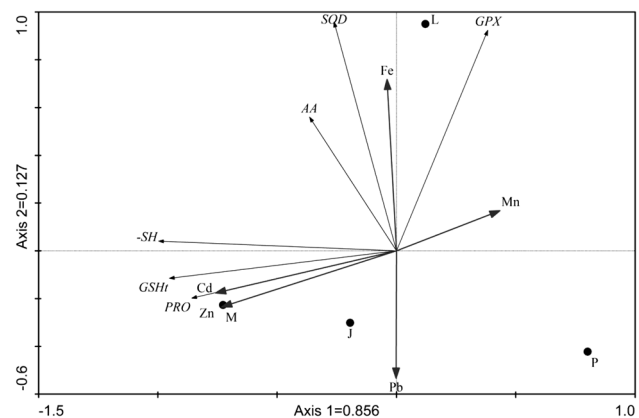
Fig. 2 Principal Component Analysis (PCA) biplot of sampling sites and heavy metal concentrations and biochemical parameters in the leaves of *V. myrtillus*

Śląskie area (M) in comparison to other investigated sites. These concentrations (acid extracted) were above permissible levels (Pb—100 mg kg^{-1}; Cd—4 mg kg^{-1} Zn—300 mg kg^{-1}) according to the Regulations by the Minister of the Environment (2002). Exceeded concentrations were also

Table 8 The correlation coefficients between metal concentration and antioxidant measurements in the leaves of *V. vitis-idaea* ($p < 0.05$); NS-not significant

	Cd	Pb	Zn	Fe	Mn
PRO	NS	NS	NS	−0.76	NS
GSHt	NS	NS	NS	NS	−0.95
−SH	NS	NS	NS	NS	NS
AA	0.80	0.70	0.75	NS	−0.78
SOD	0.77	0.85	0.84	0.91	NS
GPX	−0.71	−0.67	−0.70	NS	NS
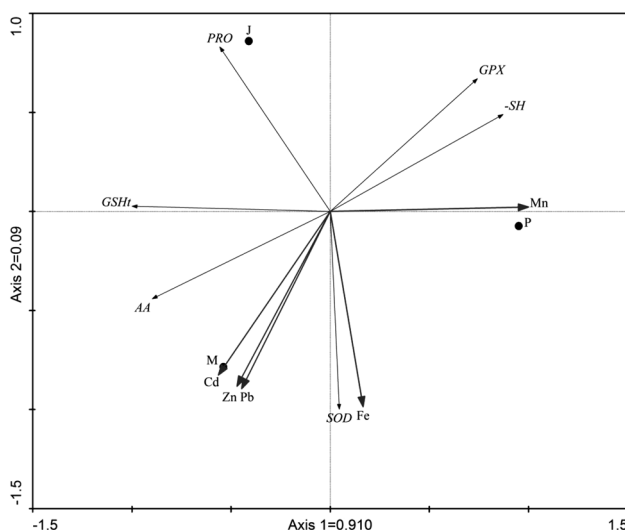
Fig. 3 Principal Component Analysis (PCA) biplot of sampling sites and heavy metal concentrations and biochemical parameters in the leaves of *V. vitis-idaea*
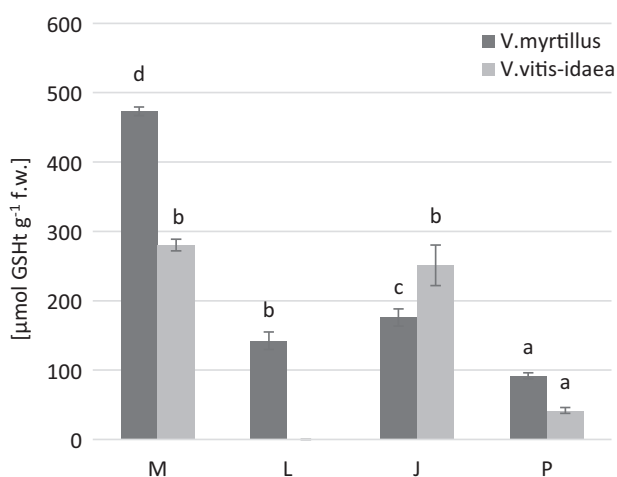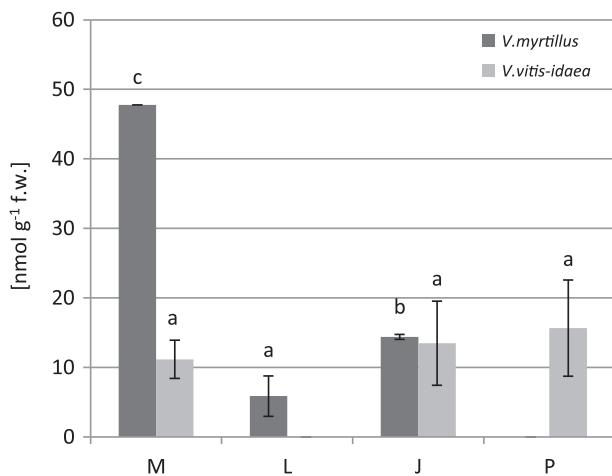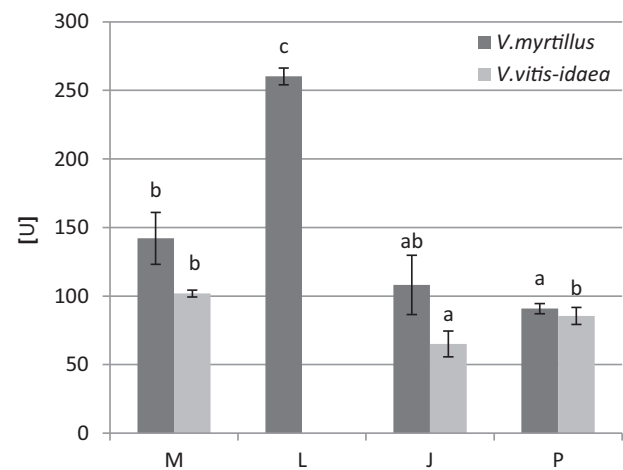

Fig. 4 Total glutathione contents (μmol GSHt g^{-1} fresh weight) in *V. myrtillus* and *V. vitis-idaea* leaves (mean values ± SE, $n = 9$). *Different letters* above the columns indicate significant differences for the species ($p < 0.05$)
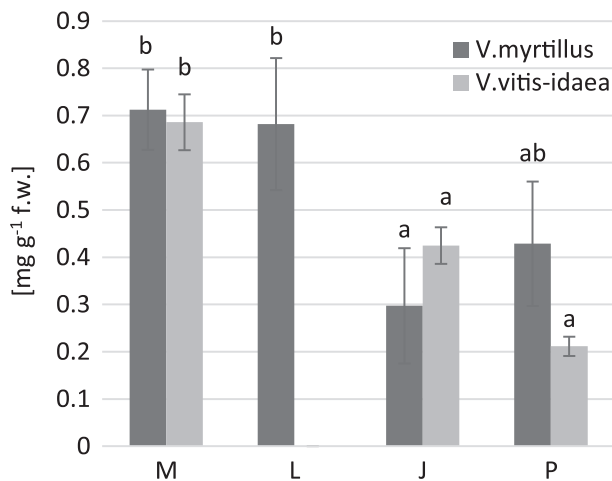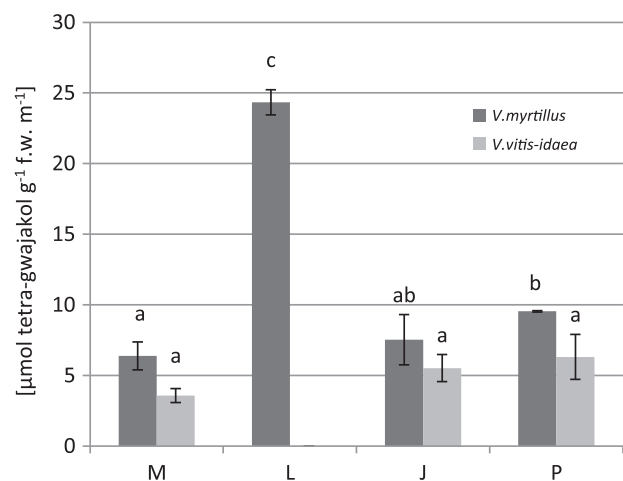
Fig. 5 Non-protein −SH groups contents (nmol −SH g^{-1} fresh weight) in *V. myrtillus* and *V. vitis-idaea* leaves (mean values ± SE, $n = 9$). *Different letters* above the columns indicate significant differences for the species ($p < 0.05$)

Fig. 7 Superoxide dismutase activity (mg g^{-1} fresh weight) in *V. myrtillus* and *V. vitis-idaea* leaves (mean values ± SE, $n = 9$). *Different letters* above the columns indicate significant differences for the species ($p < 0.05$)

Fig. 6 Ascorbic acid contents (mg g^{-1} fresh weight) in *V. myrtillus* and *V. vitis-idaea* leaves (mean values ± SE, $n = 9$). *Different letters* above the columns indicate significant differences for the species ($p < 0.05$)

Fig. 8 Guaiacol peroxidase activity (μmol tetra-guaiacol g^{-1} fresh weight min^{-1}) in *V. myrtillus* and *V. vitis-idaea* leaves (mean values ± SE, $n = 9$). *Different letters* above the columns indicate significant differences for the species ($p < 0.05$)

found for Cd at L site and for Pb at all investigated sites. These results are similar to our previously studies carried out at the same investigated areas (Kandziora-Ciupa et al. 2013; Nadgórska-Socha et al. 2016). Also the single pollution index and Nemerow pollution indices showed serious pollution with heavy metals at M site. The levels of heavy metals at the most polluted site were lower than those found in heap soils left by the historical Zn-Pb ore mining (Stefanowicz et al. 2016).

Metal bioaccumulation in leaves, stems, and roots

The vascular plants of the forest floor may be good bioindicators to assess the degree of natural environmental pollution, and research on their response to pollution could contribute to a better understanding of the mechanism and factors affecting element bioavailability and plant uptake (Wittig 1992; Jamnická et al. 2013). In this study we found significantly higher levels of Cd, Pb, and Zn in all organs of investigated species collected at the site located near the zinc smelter (M). The levels of these three metals were much higher than values considered as normal, and also exceeded levels considered toxic for plants (according to Kabata-Pendias and Pendias 2001). Higher bioaccumulation of these three metals was found in the lingonberry (especially in leaves) which is related to the fact that it is an evergreen species, retaining its leaves for many years. The obtained concentrations of Cd, Pb, and Zn were largely

similar to those reported in field studies by Nadgórska-Socha et al. (2013a) and Stefanowicz et al. (2016) on other species. Definitely lower Cd, Pb, and Zn contents than in the present study were found in the leaves of bilberries by Białońska et al. (2007).

Average Fe concentration in both plants at all investigated sites oscillated around the reference value according by Markert (1992)—150 mg kg^{-1}. These results were similar to those given by Parzych (2014) for bilberry shoots from the Słowiński National Park and by Mróz and Demczuk (2010) in *V. myrtillus* shoots from a copper smelter area. Also Nadgórska-Socha et al. (2016) reported similar Fe contents in *Robinia pseudoacacia* and *Melandrium album* leaves from the same investigated areas.

The concentration of Mn in the shoots and roots of *V. myrtillus* and *V. vitis-idaea* at sites classified as less polluted (J and P) in most cases exceeded values considered as toxic to plants (400–1000 mg kg^{-1} according to Kabata-Pendias and Pendias 2001). The results obtained in this study indicate excessive accumulation of Mn in both species' shoots, with higher concentrations found in *V. myrtillus*. Mróz and Demczuk (2010) suggested that *V. myrtillus* is an accumulator of Mn and its high concentrations suggest a possibility that bilberry uses Mn for some beneficial purposes. Many other authors also mentioned the large capability of bilberry to accumulate Mn (Reimann et al. 2001; Kozanecka et al. 2002; Salemaa et al. 2004; Boyd 2007; Mróz and Demczuk 2010; Kandziora-Ciupa et al. 2013; Parzych 2014). Reeves and Baker (2000) included *V. myrtillus* as manganese hyperaccumulator.

Metal accumulation efficiency

In our study, metal accumulation efficiency in plant tissues differed considerably between the elements, plant species, and organs. Cd, Pb, Zn, and Fe were accumulated mainly in the roots of *V. myrtillus* and *V. vitis-idaea*. Both species behave as excluders of Cd, Pb, Zn, and Fe—plants that restrict heavy metal transport from roots to shoots (Stefanowicz et al. 2016). TF, MR, and BCF for Mn in most cases were higher than 1, especially in bilberry, which confirmed its tendency to elevated manganese accumulation.

The biochemical status of the plants

In our study we also compared the antioxidant response in *V. myrtillus* and *V. vitis-idaea* leaves to verify the influence of heavy metals on the physiology of both species in various contaminated environments. Biomaterials such as leaves of higher plants—the site of major physiological processes—have been used to detect the deposition, accumulation, distribustion of metal pollution and physiological responses (Serbula et al. 2012; Kandziora-Ciupa et al. 2013; Deepalakshmi et al. 2014; Nadgórska-Socha et al. 2014).

Proline plays multifarious roles in plant tissues exposed to abiotic stress such as nutritional reserve for growth, stabilization of protein and membranes, osmoprotection, and free radical scavenging (Zouari et al. 2016). This low molecular compounds accumulating in high concentrations in response to a variety of abiotic stress including heavy metals (Kishor and Sreenivasulu 2014). Induction of proline in plants' response to heavy metals is to a great extent concentration-dependent, and organ and metal specific (Emamverdian et al. 2015). Therefore, the accumulation of proline in plants under heavy metal pollution has been reviewed by many authors (Tantrey and Agnihotri 2010 in *Cicer arietinum* L.; Kumar et al. 2010 in *Rosa hybrida* L.; Kandziora-Ciupa et al. 2016 in *Pinus sylevstris* L.—field study). Our tests found a positively significant effect of Cd and Zn on the accumulation of proline only in the leaves of *V. myrtillus*. In the leaves of this species the highest proline content was observed the vicinity of the zinc smelter (site M) (Fig. 1). An increase in proline level during environmental contamination was also found in *Philadelphus coronarius* leaves (Kafel et al. 2010—field study) and in the needles of *Pinus sylvestris* (Kandziora-Ciupa et al. 2016—field study). Singh et al. (2014) reported an increase in proline accumulation in *Vigna unguiculata* seedlings in response to increasing Zn concentration. On the other hand, our present study found a decreased proline concentration in relation to increased Fe concentration in *V. vitis-idaea* leaves and increased Mn concentration in *V. myrtillus* leaves. Capability of a specific heavy metal to induce proline accumulation may depend on the concentration and specificity of heavy metals, their toxicity threshold, and plant species employed in the trials (Emamverdian et al. 2015). According to Riscitti et al. (2011), an increase in heavy metals concentration raised the proline content to a specific level, and inhibition of proline accumulation occurs beyond a certain threshold of the metal.

Glutathione (GSH), a nonenzymatic antioxidant, is a low molecular weight thiol implicated in a wide range of metabolic process and constitutes an important plant defense system against environmental stresses, including heavy metals (Hossain et al. 2012). Glutathione protects potentially susceptible cysteine-rich proteins from binding free metal ions and respectively affecting their function. After forming nontoxic complexes with metals, GSH simplifies their sequestration away from sensitive sites in cells (Foyer and Noctor 2005; Herbette et al. 2006; Verbruggen et al. 2009; Józefczak et al. 2012). The changes in GSHt levels are dependent on the metal and the part of plant (Arya et al. 2008; Nadgórska-Socha et al. 2013a; Kandziora-Ciupa et al. 2013, 2016).

Similar to previous research on pine needles (Kandziora-Ciupa et al. 2016—field study) and on bilberry leaves (Kandziora-Ciupa et al. 2013—field study), we found a decline in GSHt due to an increased concentration of Mn in lingonberry leaves (Table 8 and Fig. 3). Also Srivastava and Dubey (2011) reported a decline in the level of GSH in Mn-treated rice seedlings. Additionally, many authors reported that exposure to other heavy metals initially resulted in a severe depletion of glutathione (Cd in pine—Schützendübel et al. 2001; Pb in *Raphanus sativus*—El-Beltagi, Mohamed (2010); Pb in *Vicia faba* and *Phaseolus vulgaris*—Piechalak et al. 2002). The decline in the glutathione content of plants may result from the inhibition of enzymes involved in glutathione synthesis by toxic metal ions. In addition, the depletion of the glutathione pool may also be considered to play some role in the synthesis of phytochelatins and other low molecular weight, thiol-rich compounds, or might be attributed to an increased regeneration of ascorbate from dehydroascorbate (Madhava Rao, Sresty 2000; Yadav 2009; Hossain et al. 2012). Pietrini et al. (2003) suggested that higher heavy metals concentration could overload the defense capacity of glutathione. In our study, we also found a positive correlation between GSHt and Fe content in *V. myrtillus* leaves. The same dependence (induction of GSH by exposure to metals) was demonstrated by many authors (Pietrini et al. 2003 in *Phragmites australis* L.; Nadgórska-Socha et al. 2013a in *C.arenosa* and *P.lanceolata*- field study; Anjum et al. 2016 in maize; Nadgórska-Socha et al. 2016 in *R.pseudoaccacia* and *M.album*- field study).

In addition to glutathione, chelation of heavy metals in plants is achieved by cysteine containing metal-binding ligands, including phytochelatins and metallothioneins (Di Baccio et al. 2005). Non-protein thiols contain a high percentage of cysteine sulfhydryl residues and play an important role in metal detoxification process in plants (Sun et al. 2013). Non-protein compounds rich in −SH groups are involved in metal detoxification and/or metal allocation between different organs of the plant, because their main task is to bind metal ions and form non-toxic complexes with metals which are transported from the cytoplasm into the vacuole (Andrade et al. 2010; Yadav 2009; Kandziora-Ciupa et al. 2016—field study). In our study, the highest NPTs content was noticed in *V. myrtillus* leaves at the most polluted site (M); in addition, the content of non-protein thiols was positively related with concentrations of Cd and Zn in bilberry leaves. These results are similar to our previous studies where we observed an increase in NPTs in the leaves of bilberry (Kandziora-Ciupa et al. 2013—field study) and in the needles of pine (Kandziora-Ciupa et al. 2016—field study). Also Nadórska-Socha et al. (2011, 2016—field studies) found a positive correlation between NPTs and selected heavy metals in the leaves of *Silene vulgaris* and *Robinia pseudoacacia*. Our results were also similar to Sun et al. (2013) who found a significantly increased concentration of NPTs in cabbage treated with Cd. According to Mishra et al. (2006), an increase in NPTs content indicates an ability to tolerate the cellular metal load.

Ascorbic acid is the most abundant, powerful, and water soluble antioxidant preventing or minimizing the damage caused by ROS in plants (Gill and Tuteja 2010). On one hand, in our study we observed its highest levels (in the leaves of both investigated species) at the most contaminated M site and we found a positive correlation between AA content and Cd, Pb, and Zn levels in lingonberry leaves. On the other hand, we found a decrease in AA levels under Mn excess in *V. myrtillus* and *V. vitis-idaea* leaves. In a study of Rai et al. (2013), the ascorbic acid content was higher in the leaves of plants at an industrial site than at a non-industrial site. The same relationship was found by Meerabai et al. (2012) in *Cajanus cajan* L. leaves. Also Demirevska-Kepova et al. (2004) found a significantly higher AA content at elevated Cu concentration in barley plants, compared to control. Increased levels of ascorbic acid have a positive effect on pollution tolerance, which may be due to the defense mechanism of respective plants (Rai and Panda 2014; Nadórska-Socha et al. 2016—field study). However, Nadórska-Socha et al. (2016—field study) observed a negative correlation between ascorbic acid content and Pb and Cd concentrations in the leaves of *Robinia pseudoaccacia*, and between AA and Mn in *Melandrium album* leaves. Similar to our results, Demirevska-Kepova et al. (2004) reported a reduction in ascorbic acid under Mn excess in *Hordeum vulgare*. According to Smirnoff (2000), ascorbic acid is the main source of oxalic acid in some species. Immobilization of excess Mn in oxalate crystals has been suggested as a possible detoxification mechanism (El-Jaoual and Cox 1998; González and Lynch 1999), so an AA decrease under Mn redundancy could be explained by an enhanced synthesis of oxalate at the ascorbic acid (Demirevska-Kepova et al. 2004).

The balance between ROS and the antioxidant system is crucial for survival and adaptation of plants growing in soils with relatively constant levels of heavy metals (Słomka et al. 2008). Research on activity of the antioxidant enzymes is important for a better understanding of antioxidant protection (Nadórska-Socha et al. 2013a). In this study we also examined an antioxidant defense system formed by SOD and GPX.

SOD is known to catalyze to conversion of O_2^- to less toxic H_2O_2 and O_2, and is considered to be the first line of defense against elevated levels of ROS in plants (Zouari et al. 2016). The response of SOD to heavy metal stress varies considerably, depending on plant species, tissue, stage of development, utilized metal, and exposure time (Gratão et al. 2005). In our study, an increase in SOD

activity under influence of Cd, Pb, Zn, Fe was observed only in the leaves of *V. vitis-idaea*. Nadgórska-Socha et al. (2013a) also found, in field study, an increase in SOD activity under exposure to Fe in the leaves of *Cardaminopsis arenosa* and *Plantago lanceolata*. Zouari et al. (2016) reported that SOD activity increased significantly in the leaves and roots of Cd-treated young olive trees. Also the results of Verma and Dubey (2003) showed an increase in SOD activity in rice plants under the toxic levels of Pb. According to Sharma et al. (2012), an increased activity of SOD often correlated with increased tolerance of plants against environmental stresses.

Peroxidases are antioxidant enzymes which are significant for plants' growth and development. Activities of these enzymes are changed under both biotic and abiotic stress conditions, and so are used as a potential indicator of metal toxicity (Radotić et al. 2000; Macfarlane and Burchett 2001; Baycu et al. 2006; Doğanlar and Atmaca 2011; Kandziora-Ciupa et al. 2013). Guaiacol peroxidase is associated with many important biosynthetic processes and defense against abiotic and biotic stresses and is widely accepted as a "stress enzyme" (Erofeeva 2015). Despite the fact that many authors report increased GPX activity in response to elevated heavy metal concentrations (Doğanlar and Atmaca 2011; Kafel et al. 2010; Nadgórska-Socha et al. 2013a; Nadgórska-Socha et al. 2013b—all field studies) in our study, we observed a significant decrease correlated with increased Cd, Pb, and Zn contents, although again only in lingonberry leaves. We found a similar dependence in our previous studies where we reported a decrease in GPX activity under the exposure to Cd, Pb, and Zn in bilberry leaves (Kandziora-Ciupa et al. 2013) and exposure to Zn in spruce needles (Kandziora-Ciupa et al. 2016—field study). Also Nadgórska-Socha et al. (2013a—field study) found a negative correlation between GPX activity and Cd content in *Cardaminopsis arenosa*.

Stress intensity may be linked to an increase or decrease in oxidative metabolism. It is generally accepted that enzymatic activity decreases in higher heavy metal concentrations (Słomka et al. 2008).

Conclusions

In connection to our hypothesis, we may conclude that much higher Cd, Pb, Zn, and Fe concentrations were found in *V. myrtillus* and *V. vitis-idaea* grown at the most polluted site (located near the zinc smelter) in comparison with cleaner areas, and definitely higher bioaccumulation of these metals was found in lingonberry organs. Increased Mn accumulation was found at less polluted sites (including the outskirts of the Pazurek Nature Reserve) and we noticed the large capability of bilberry to accumulate Mn.

Anti-oxidant response to heavy metal stress differed between *V. myrtillus* and *V. vitis-idaea*. However, we did not find a single general marker of heavy metal contamination in the two investigated species. In bilberry, elevated heavy metal concentrations caused an increase in proline, GSHt, and non-protein −SH groups content. In lingonberry the increased heavy metal accumulation induced an increase in ascorbic acid content, SOD activity, and a decrease in GPX activity. In both species increased Mn accumulation caused a decrease in anti-oxidative response.

The selected ecophysiological parameters may be good biochemical markers of stress caused by heavy metal and we do realize that in natural conditions these parameters are influenced by various factors, which we weren't studied. Therefore, it is important to continue this type of research on plants growing in natural conditions.

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