

Salicylic acid accumulation as a result of Cu, Zn, Cd and Pb interactions in common reed (*Phragmites australis*) growing in natural ecosystems

Kinga Drzewiecka¹ · Mirosław Mleczek¹

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Abstract Common reed (*Phragmites australis* (Cav.) Trin. ex Steud.) plants were harvested from four natural water ecosystems of the Bogdanka river catchment (Poznań, Poland) four times throughout the 2014 vegetative season. Over the year, average metal contents followed different decreasing trends according to the analyzed tissue: Zn > Cu \approx Pb > Cd (rhizomes) and Zn > Pb > Cu > Cd (leaves), and mean translocation ratios (leaves/rhizomes) were found as follows: 0.93, 0.70, 0.65, 0.40 for Zn, Pb, Cd and Cu, respectively. Metal content increased gradually during the growing season, and in the case of Cu, Cd and Pb exceeded the upper limit of average concentration detected in plants from natural ecosystems. However, the content of salicylic acid did not follow the increase of metal accumulation. In rhizomes, the highest production of the metabolite was observed in May and reached 324 ng g⁻¹ fresh weight (FW) (mean value). Afterwards, a significant drop to 50 ng g⁻¹ FW was observed. Simultaneously, the highest values of total salicylic acid in *P. australis* leaves were observed in July and accompanied the intensive development of the aboveground biomass of the plant (11.3 μ g g⁻¹ FW—mean value). Subsequently, its content in leaves showed a significant decrease down to 2.1 μ g g⁻¹ FW in November. Multivariate regression analysis revealed significant interactions between analyzed metals influencing the plant response to metal-derived stress. Cu and Zn showed antagonistic properties

considering their uptake and the induction of salicylic acid biosynthesis, whereas non-essential metals (Pb and Cd) acted similarly and stimulated the formation of salicylic acid glucoside.

Keywords Heavy metals · Oxidative stress · *Phragmites australis* · Salicylic acid

Introduction

Among the various phenolic compounds, the relatively small molecule of salicylic acid (SA) is a widely distributed secondary metabolite exhibiting a hormone-like function. The compound has a complex role in plants' growth and development (regulating seed germination, leaf and root elongation, flowering and thermogenesis, etc.), and in response mechanisms against numerous environmental stress factors of biotic and abiotic nature (Raskin 1992; Khan et al. 2015; Wani et al. 2016). The regulatory function of SA combines signaling, transcriptional and post-transcriptional control of numerous genes, as well as interactions with other phytohormones (Bari and Jones 2009; Pieterse et al. 2012; Xia et al. 2015). The best recognized function of SA is induction of the hypersensitive response (HR) to biotrophic pathogens and some insects (e.g., pea aphid), followed by a suicidal programmed cell death (PCD) able to restrict the infection site. Furthermore, it serves as an intra- and interplant signal to develop systemic acquired resistance (SAR) with the induction of pathogenesis-related proteins (PRs) in uninfected organs and neighboring plants (Popova et al. 2008). Upon oxidative stress, SA shares/induces the transduction pathways of reactive oxygen species (ROS) involving NPR1 (NON-EXPRESSOR OF PR GENES 1) and a network of the

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✉ Kinga Drzewiecka
kinga.drzewiecka@gmail.com

¹ Chemistry Department, Poznań University of Life Sciences, Wojska Polskiego, 75 60-625 Poznań, Poland

NPR1-independent MAPK cascade (Samuel et al. 2005; Backer et al. 2015). In this manner, it influences the activity of antioxidative enzymes, glutathione level and expression of mitochondrial genes modulating the production of ROS, and finally the oxidative state of the cell (Berkowitz et al. 2016; Singh et al. 2016a).

The functioning of salicylic acid in plant immune strategies has gained scientific interest for the last two decades. However, some aspects of mechanisms involving the metabolite are still unclear and remain the subject of ongoing debate. Genetic engineering, obtaining gain/loss-of-function mutations combined with genomic tools and advanced analytical methods, allows the further understanding of the complex role of the compound in regulation and control processes within plants (An and Mou 2011). So far, numerous studies have been conducted under controlled conditions and concerned salicylic acid accumulation upon single stressor treatment, the effect of plants' pretreatment with the metabolite, and lately its function in cross-tolerance mechanisms (Arasimowicz-Jelonek et al. 2014; Janda et al. 2014). Still, investigations performed in natural ecosystems or under ambient conditions are generally rare. Therefore, evaluation of the cumulative effect or interactions of co-existing stressors on salicylic acid biosynthesis and the possibility of using this metabolite as a biomarker of overall plant stress are of prime concern. Since salicylic acid is an unspecific metabolite induced by various adverse environmental factors, its level may reflect the real impact of the combined stressors on plant condition.

In the present study, we investigated the simultaneous effect of major metallic pollutants (Cd, Cu, Pb and Zn) on salicylic acid accumulation in rhizomes and leaves of common reed (*P. australis*). Recent studies showed moderate to very high levels of contamination factor (CF) evaluated for these metals in water and sediments of both left and right tributaries of the Warta river (up to 95 in the case of Zn concentration in water) (Borowiak et al. 2016; Kanclerz et al. 2016). Common reed is one of the most distributed macrophytes in aquatic ecosystems, showing bioaccumulation abilities towards numerous metals and generally exposed to their mixtures (Vymazal et al. 2009; Bonanno 2011). Therefore, naturally occurring *P. australis* can be employed in biomonitoring studies as an accumulative indicator of low level water contamination with toxic elements (Maddison et al. 2009; Bonnano and Giudice 2010; Fawzy et al. 2012). In our study, *P. australis* plants were collected over the year from four water reservoirs of various size differentially affected by human pressure to obtain a variety of metal accumulation and plant biochemical response.

Only a few studies have focused on interactions between metals due to their simultaneous presence in the

environment and their impact on *P. australis* physiology (Alfadul and Al-Fredan 2013; Ait Ali et al. 2004). Furthermore, salicylic acid content in the macrophyte growing in natural conditions under multi-metal stress still remains unstudied. Thus, the aims of the paper were:

1. to quantify the contents of free and glucoside-bound salicylic acid in *P. australis* from natural water ecosystems;
2. to assess differences in salicylic acid content according to season/time and plant organ (rhizomes and leaves);
3. to indicate metals responsible for the increased salicylic acid accumulation in *P. australis*;
4. to evaluate the interactions between metals in inducing the elevation of salicylic acid biosynthesis.

Materials and methods

Investigation sites

Investigations were carried out in Poznań (Poland) (52°24'N 16°56'E) in the Bogdanka river catchment during the growing season of 2014. The Bogdanka river is one of the most heavily polluted rivers in Poland (class III watercourse) and collects the majority of left bank rainwaters of the city and surrounding areas. It is a tributary of the Warta river—the third longest river in Poland—and runs through three water reservoirs (Strzeszyńskie Lake—S2, Rusalka Lake—S3, and Sołacz Pond—S4). Kierskie Lake (S1) was also chosen for the study, being an alternative source of the watercourse and the biggest water reservoir located in the upper left bank of the Warta river. The location of sampling sites within the area of the city of Poznań was based on previously documented biomonitoring studies of water pollution with heavy metals using macrophytes (*Typha angustifolia* and *P. australis*) performed over the years 2006–2013. Thus, detail characteristics of the Bogdanka river catchment, water reservoirs and investigation sites were previously described by Borowiak et al. (2016) and Drzewiecka et al. (2011).

Plant material

Common reed (*Phragmites australis* (Cav.) Trin. ex Steud.) plants were harvested four times during the vegetative season (20th May, 16th July, 15th September and 10th November 2014). The largest homogeneous stand of the species was selected from the littoral zone of each water reservoir and five plants in three randomized replicates were harvested. Leaves and newly developed rhizomes together with hairy roots without visible signs of injury or disease were collected for the analysis.

Metal accumulation

Collected plants were separated into rhizomes and leaves, washed with distilled water and dried using an electric oven SLW 53 STD (Pol-Eko, Wodzisław Śląski, Poland) within 90 h at 105 ± 4 °C. Dried samples (~ 0.5 g) were ground using a laboratory Cutting Mill SM 200 (Retsch GmbH, Haan, Germany) for 5 min. Ground material was digested using the microwave mineralization system CEM Mars 5 Xpress (CEM, Matthews, NC) with 7 mL of 65% HNO_3 (Sigma-Aldrich) in 55 mL vessels. Digestion was composed of three stages: (1) 7 min at 100 °C; (2) 8 min at 140 °C; (3) 10 min at 200 °C. Obtained solutions were filtered through 45-mm filters and supernatants were diluted to a final volume of 50 mL using ultrapure water (Milli-Q, Millipore, St. Louis, USA).

Content of Cd, Cu, Pb and Zn in plant material was analyzed with flame atomic absorption spectrometry (FAAS) using an AA Duo—AA280FS/AA280Z spectrometer (Agilent Technologies, Mulgrave, Victoria, Australia). To minimize the error of the complex matrix, deuterium background correction was applied. Calibration curves were prepared daily for five replicates per each element concentration out of stock solution of 1000 mg L^{-1} (Romil), and the precision was evaluated at the level of 4%. All analyses used hollow-cathode lamps (HCL, Varian) and single-element lamps were exclusively used. The key experimental conditions of FAAS were as follows: wavelength (nm)—228.8, 324.8, 217.0 and 213.9, slit width (nm)—0.5, 0.5, 1.0 and 1.0 and lamp current (mA)—4, 3, 8 and 5, for Cd, Cu, Pb and Zn, respectively. The quality of obtained results was verified with the certified reference material NCS DC 73348 (bush branches and leaves) analyzed in every tenth assay system. Additionally, validation of results was performed for randomly selected samples using the inductively coupled plasma optical emission spectrometer Agilent 5100 ICP-OES (Agilent, USA).

Salicylic acid content

For salicylic acid analysis, plant rhizomes and leaves were washed, dried and in situ frozen in liquid nitrogen, transported and stored at -80 °C until analysis. Samples (0.5 g) were ground in liquid nitrogen with a chilled mortar and pestle to obtain a homogeneous powder and salicylic acid was extracted twice with methanol (90% followed by straight solvent) according to the procedure of Yalpani et al. (1991). Methanolic extracts were combined, mixed and then divided into two equal volumes to evaluate the contents of free salicylic acid (SA) and its glucoside (SAG) calculated as the difference between total and free salicylic acid (TSA—SA). The solvent was evaporated under a stream of nitrogen,

and the residue was dissolved in 5% TCA (w/v). After centrifugation, SA was partitioned three times against the organic phase composed of ethyl acetate:cyclopentane:isopropanol (100:99:1, v/v/v). For TSA determination, enzymatic degradation of SAG was performed with 40 units of β -glucosidase per sample in NaAc/HAc buffer (0.1 M, pH 5.2). The enzyme solution was added to the second part of the dry residue and incubated for 90 min at 37 °C. The reaction was terminated by the addition of 5% TCA and TSA was extracted as described above.

After solvent evaporation under a stream of N_2 , the dry residue was dissolved in the mobile phase (0.2 M KAc/HAc buffer, 0.5 mM EDTA, pH 5.0), centrifuged and analyzed with the HPLC method using a Waters Alliance 2695 Chromatograph coupled with a Waters 2475 Multi- λ fluorescence detector. The separations were performed in a Spherisorb ODS2 column (10×4.6 mm, $3 \mu\text{m}$) at a flow rate of 1.5 mL min^{-1} , and detection parameters were as follows: $\lambda_{\text{Ex}} = 295 \text{ nm}$ and $\lambda_{\text{Em}} = 405 \text{ nm}$.

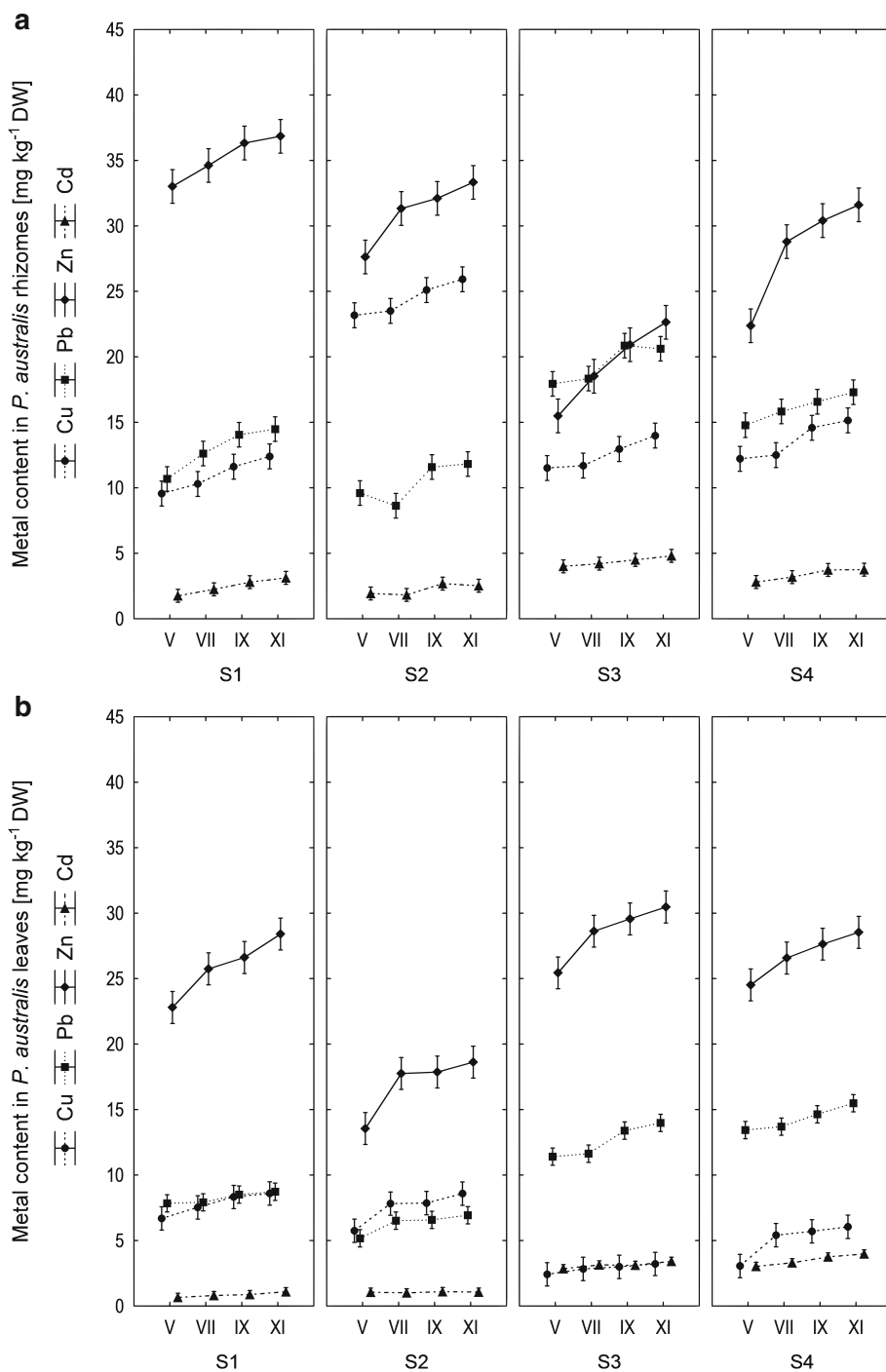
Statistical analysis

Standard statistical methods were used for data evaluation including multi-factor analysis of variance (ANOVA) for collection time, site and plant organ single and fixed effects. Metal accumulation and salicylic acid content were presented in the form of charts for time \times site fixed effects (leaves and rhizomes separately) and single effects of term or plant organ. A heat map was used for visualization of correlations between variables. Furthermore, multivariate regression analysis was performed for salicylic acid accumulation with metal contents in plant organs as predictors. Selection of the final model was achieved with the backward selection method according to Akaike Information Criterion. Only significant regressions (with R higher than for simple correlation) are presented. All statistical calculations were performed with Statistica 12 and R software.

Results and discussion

The results of metal concentration in rhizomes and leaves of *P. australis* are presented in Fig. 1. Obtained values differed significantly ($p \leq 0.001$) between investigation sites, collection times (seasons) and plant organs for all studied metals; however, only for Zn and Pb the interaction effect of the three mentioned experimental factors was found significant ($p \leq 0.05$) (Table 1). Over the year, average metal contents followed different decreasing trends according to the analyzed tissue: $\text{Zn} > \text{Cu} \approx \text{Pb} > \text{Cd}$ (rhizomes) and $\text{Zn} > \text{Pb} > \text{Cu} > \text{Cd}$ (leaves), and mean translocation ratios (leaves/rhizomes) at the end of the observation were as follows: 0.93 ± 0.34 ,

Fig. 1 Mean values of metal (Cu, Zn, Pb, Cd) contents in *Phragmites australis* organs (**a** rhizomes, **b** leaves) regarding to sites along the Bogdanka river catchment (S1 Kierskie Lake, S2 Strzeszyńskie Lake, S3 Rusałka Lake, S4 Sołacz Pond) and times of sample collection during the growing season. Vertical bars represent 95% confidence intervals for “site × time” fixed effect according to two-way ANOVA



0.70 ± 0.13 , 0.65 ± 0.29 , 0.40 ± 0.19 for Zn, Pb, Cd and Cu, respectively. This observation proved stronger sequestration of all analyzed metals in favor of the belowground organs (rhizomes and roots) previously documented by Bonnano and Giudice (2010) for *P. australis* sampled from natural aquatic habitats. Similarly, Alfadul and Al-Fredan (2013) observed higher concentrations of all four metals in roots than in shoots of *P. australis* treated with single metals and their combinations in a greenhouse

experiment. Our results are partly in agreement with Vymazal et al. (2009), who reported the highest concentration ratio between roots and leaves for Pb, moderate for Cd and Cu, and the lowest for Zn for *P. australis* cultivated in constructed wetlands for wastewater treatment. However, the plants were not exposed to intense metal deposition from the air as in our studies.

The content of all metals tested showed a slow gradual increase during the vegetation season, reaching peak values

Table 1 Testing of general hypothesis in three-way analysis of variance (three-way ANOVA) for Cu, Zn, Pb, and Cd accumulation in *Phragmites australis* according to the collection site (S1 Kierskie Lake, S2 Strzeszyńskie Lake, S3 Rusałka Lake, S4 Sołacz Pond), time

(20th May, 16th July, 15th September and 10th November 2014) and analyzed organ (rhizomes and leaves) at $\alpha = 0.05$ (df degrees of freedom, p empirical significance level, SA free salicylic acid, SAG salicylic acid glucoside, TSA total salicylic acid)

Source of variation	df	Metal accumulation				Salicylic acid content		
		Cu	Zn	Pb	Cd	SA	SAG	TSA
Site (S)	3	***	***	***	***	***	***	***
Time (T)	3	***	***	***	***	***	***	***
Plant organ (O)	1	***	***	***	***	***	***	***
S \times T	9	*	*	*	ns	***	***	***
S \times O	3	***	***	***	***	***	***	***
T \times O	3	**	**	*	*	***	***	***
S \times T \times O	9	ns	*	*	ns	***	***	***
Residuals	64							

ns not significant

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

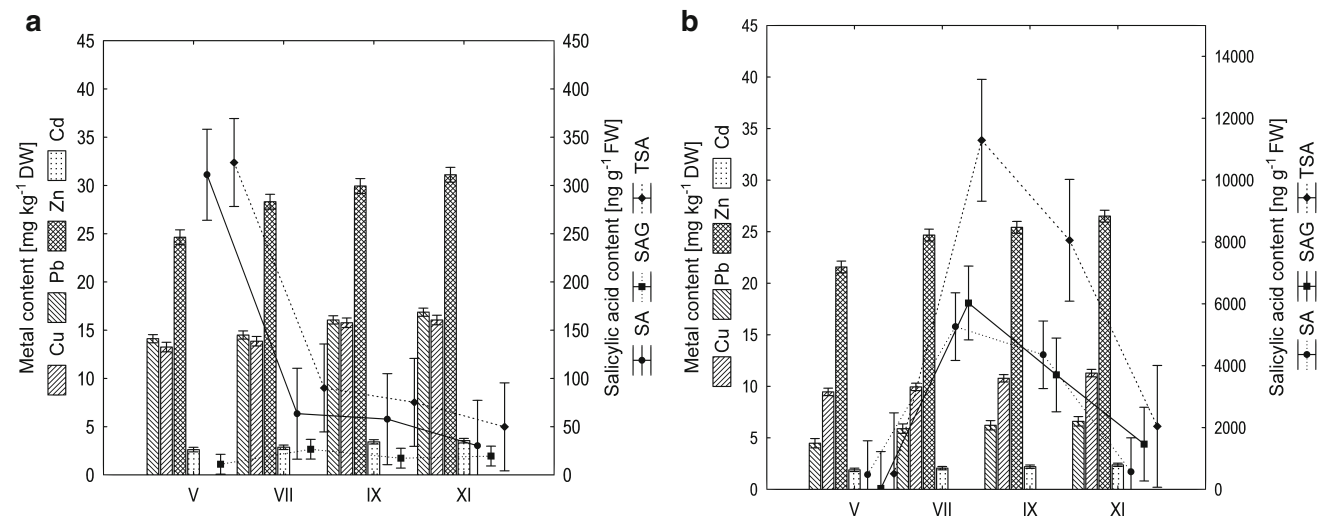


Fig. 2 Mean values of metal (Cu, Zn, Pb, Cd) and salicylic acid contents in *Phragmites australis* organs (**a** rhizomes, **b** leaves) according to collection time during the growing season. Vertical bars

represent 95% confidence intervals for “time” effect according to one-way ANOVA (SA free salicylic acid, SAG salicylic acid glucoside, TSA total salicylic acid) and mobility within plants are relatively low in most species (Kabata-Pendias 2011). The observed accumulation of Pb in aerial organs of common reed was previously documented by Fawzy et al. (2012), and was probably a result of airborne Pb deposition emitted from nearby intense car traffic. Similarly, in our studies, the highest Pb concentrations in *P. australis* leaves were observed in S3 and S4 located close to the city center (16.17–19.44 and 12.61–14.32 mg kg⁻¹ DW in rhizomes and leaves, respectively) (Fig. 1).

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The second non-essential and highly toxic metal in the study was cadmium. Similarly as for Pb, the highest Cd contents were observed in plants harvested from water reservoirs located close to the Bogdanka river sink, and its

maximum values were 4.38 (S3) and 3.51 (S4) mg kg^{-1} DW in rhizomes and leaves, respectively. According to Outridge and Noller (1991), Cd content in most plants does not exceed the value of 1.9 mg kg^{-1} DW, but many authors have documented higher values in plants collected from metal-contaminated environments as a result of intensive industrialization and amendment of agricultural soils with Cd-contaminated fertilizers and bio-solids (Westfall et al. 2005; Fawzy et al. 2012). Cd shows the greatest mobility among investigated metals. Therefore, its enhanced accumulation downstream of the Bogdanka catchment is highly probable (Fig. 1).

Zn is a transition metal and a co-factor of numerous enzymes, and—as a structural component—is predominant in the transcriptional and translational machinery in plants (Finney and O'Halloran 2003). Despite its crucial function, elevated concentrations of Zn (above the level of $100\text{--}300 \mu\text{g g}^{-1}$ DW) usually cause severe toxic effects (Rout and Das 2009). Although the mobility rate of Zn in the environment is rather low, higher concentrations of the element can be noted in areas close to incineration facilities, smelters, mines and electroplating industry as a consequence of dry and wet deposition of airborne Zn-rich particles. Intense urbanization, domestic waste water discharge, building industry and vehicles are also significant sources of Zn (Rout and Das 2009). In our study, Zn concentration was the highest among the four metals analyzed (37.5 and 31.5 mg kg^{-1} DW, in rhizomes and leaves, respectively), but the peak values did not reach the phytotoxicity threshold (Fig. 1). The values correspond with concentrations documented by Fawzy et al. (2012) and Maksimovic et al. (2014) in *P. australis* sampled from natural environments, but were two times lower than in reed plants collected from the Słupia River (Pomerania region, Poland) (Parzych et al. 2015). The highest Zn concentrations in belowground organs of *P. australis* were observed in sites S1 and S2, located relatively far from the city center and exposed to landfill leachate, sanitary domestic effluents and waste water discharge. Simultaneously, a relatively high Zn load in leaves was found at sites S3 and S4, probably as a result of fuel and domestic waste incineration and emission of airborne Zn (Fig. 1).

The second investigated mineral micronutrient was Cu. Compared to Zn, the number of Cu-requiring enzymes (mainly oxidases) is relatively small. However, the metal is essential for redox reactions of regular metabolic processes, as well as those of the stress response machinery. Cu is a transition metal possessing a dual oxidation state and may occur in the form of mono- or divalent Cu cations. The concentration of Cu required for plants ranges from 1 to 5 mg kg^{-1} DW, and its toxicity threshold was found at the level of $20\text{--}30 \text{ mg kg}^{-1}$ DW (Marschner 1995). In our study, the maximum Cu concentration was noted at the

level of 25.62 and 9.65 in rhizomes and leaves, respectively (Fig. 1). In addition, Cu showed the lowest translocation factor among analyzed elements. The values are comparable to the results of Vymazal et al. (2009) for constructed wetland, but are higher than levels observed in natural habitats in Poland (Parzych et al. 2015). High Cu release in suburban areas probably originates from domestic waste water, wood production and preservation, phosphate fertilization of agricultural soil and also domestic combustion of waste and biomass (Georgopoulos et al. 2001). The highest Cu concentrations in leaves were observed in S1 and S2 (city surrounding areas), and were accompanied by the lowest Zn contents, proving their antagonistic interactions (Arredondo et al. 2006). However, the highest Cu level in rhizomes was noted in S2 together with relatively high Zn concentration (Fig. 1).

Taking into consideration results collected throughout the growing season, Cu uptake by *P. australis* rhizomes was negatively correlated with Zn content in leaves ($r = -0.76$). Simultaneously, for Cu in leaves and Zn in rhizomes a strong direct proportional relation ($r = 0.93$) was observed, proving their antagonistic properties (Fig. 3). An inverse relationship was also observed for Cu and Pb or Cd, while Cd and Pb were strongly correlated ($r = -0.58, -0.66, 0.94$ for leaves, respectively). Furthermore, the non-essential metals were negatively related to Zn content in rhizomes and positively with Zn load in leaves ($r = 0.79$ and 0.63 for Pb and Cd, respectively) indicating a significant contribution of airborne particles to metal accumulation in aboveground tissues (Fig. 3).

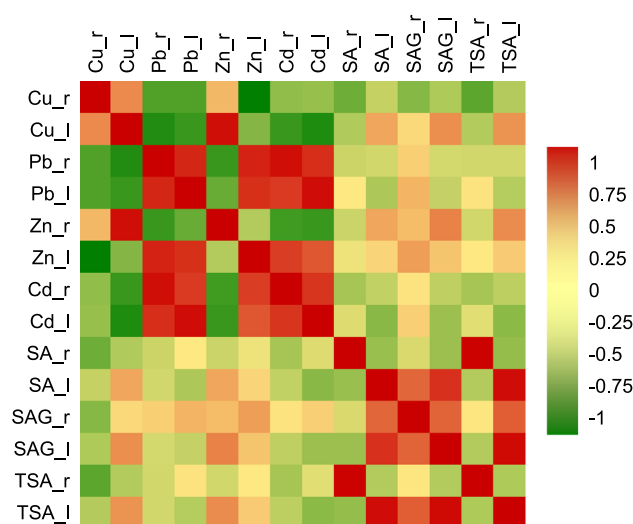
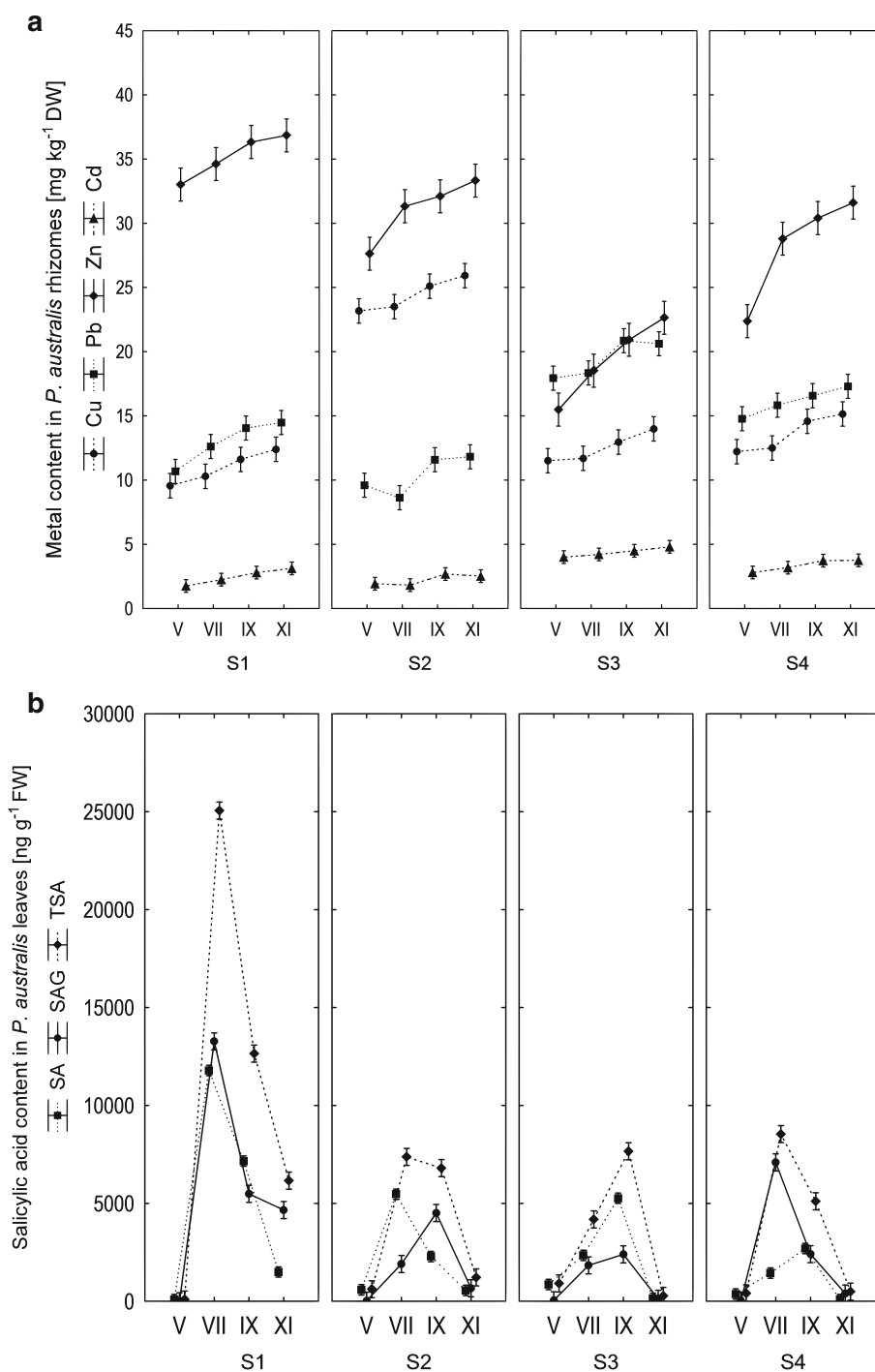


Fig. 3 Heat map of Pearson's simple correlation coefficient (r) between metal (Cu, Zn, Pb, Cd) and salicylic acid contents in *Phragmites australis* collected from natural water ecosystems along the Bogdanka river catchment throughout the growing season (SA free salicylic acid, SAG salicylic acid glucoside, TSA total salicylic acid, *r* rhizomes, *l* leaves)

Simultaneously with metal concentration, we investigated the accumulation of salicylic acid in rhizomes and leaves of common reed. According to the ANOVA analysis, all experimental factors, as well as their fixed effects, showed significant variance ($p \leq 0.001$) in the contents of free and glucoside-bound salicylic acid (Table 1), proving the influence of pollution level, collection time and organ on the defense reaction of the macrophyte. In previous studies, enhanced biosynthesis of salicylic acid (up to

~1000-fold higher compared to control plants) was observed in plants challenged with both biotic or abiotic stressors, i.e., fungi (*Fusarium proliferatum* and *F. oxysporum*), oomycetes (*Phytophthora infestans*), insects (pea aphid), tropospheric ozone and metal ions (Arasimowicz-Jelonek et al. 2014; Mai et al. 2014; Drzewiecka et al. 2012a, b; Gąsecka et al. 2012; Dobosz et al. 2011). Among these treatments, the highest induction of the metabolite was noted in leaves of *Salix viminalis* L. treated

Fig. 4 Mean values of salicylic acid content in *Phragmites australis* organs (**a** rhizomes, **b** leaves) according to sites along the Bogdanka river catchment (S1 Kierskie Lake, S2 Strzeszyńskie Lake, S3 Rusałka Lake, S4 Sołacz Pond) and times of sample collection during the growing season. Vertical bars represent 95% confidence intervals for “site \times time” fixed effect according to two-way ANOVA (SA free salicylic acid, SAG salicylic acid glucoside, TSA total salicylic acid)



hydroponically with Ni^{2+} at 3 mM and reached $\sim 60 \mu\text{g g}^{-1}$ FW. In the present study, the highest contents of salicylic acid were found at ~ 25 and $0.6 \mu\text{g g}^{-1}$ FW for leaves and rhizomes, respectively (Fig. 1). However, its content did not follow the gradual increase of metal level in plant tissue (Fig. 2). In rhizomes, the highest production of the metabolite (TSA) was observed at the beginning of the *P. australis* growing season and reached 324 ng g^{-1} FW (mean value for all investigated sites). Afterwards, a significant drop of its content down to 50 ng g^{-1} FW was noted, probably as a result of active transport of free SA up to the photosynthetic organs (Fig. 4a). Simultaneously, the highest values of TSA content in reed leaves were noted in July and accompanied the intensive development of the aboveground biomass of the plant ($11.3 \mu\text{g g}^{-1}$ FW—mean value for all investigated sites). Subsequently, its content in leaves showed a significant drop down to $2.1 \mu\text{g g}^{-1}$ FW in November, probably as a consequence of catabolic reactions of the metabolite, its release as a volatile methyl ester or polymerization to lignin (Fig. 4b). Among investigated sites, the highest biosynthesis of the metabolite was noted in S1 and was accompanied with the highest content Zn in *P. australis* rhizomes (Fig. 2a), and the lowest in S2 where the highest Cu levels were observed (Fig. 2b). This may suggest an antagonistic effect of Cu and Zn in the induction of salicylic acid and proves the competitive relation between the two metals in the environment (Arredondo et al. 2006).

Salicylic acid function in plant tolerance of toxic metals was recently reviewed by Singh et al. (2016b). The authors concluded that the metabolite evokes an acclimatization effect and enhances the resistance towards heavy metal stress due to the adjustment of metabolic processes mainly by induction of the antioxidant capacity and production of non-protein thiols. Accordingly, Freeman et al. (2005) observed the elevated accumulation of endogenous salicylic acid and its up- and downstream metabolites in numerous species of *Thlaspi* showing Ni/Zn hyperaccumulation abilities. The authors surmised that the elevated tolerance of some plants to potentially toxic levels of some metals is mediated by glutathione (GSH) and signaled by constitutively elevated SA. GSH plays a key role not only in metal detoxification (as a precursor of phytochelatins—PCs), but also as an antioxidant protecting plant cells from excessive ROS accumulation. Consequently, the increased GSH pool allows plants to survive the metal-induced oxidative stress (Freeman et al. 2004). Additionally, the study of Pál et al. (2002) revealed that SA blocks the activity of phytochelatin synthase (PCS) to maintain the effective GSH level in the cell cytosol to act efficiently as an antioxidant. In contrast, according to Simek et al. (2016) cucumber treatment with Cd salts lowered the accumulation of endogenous SA. However, the authors did not

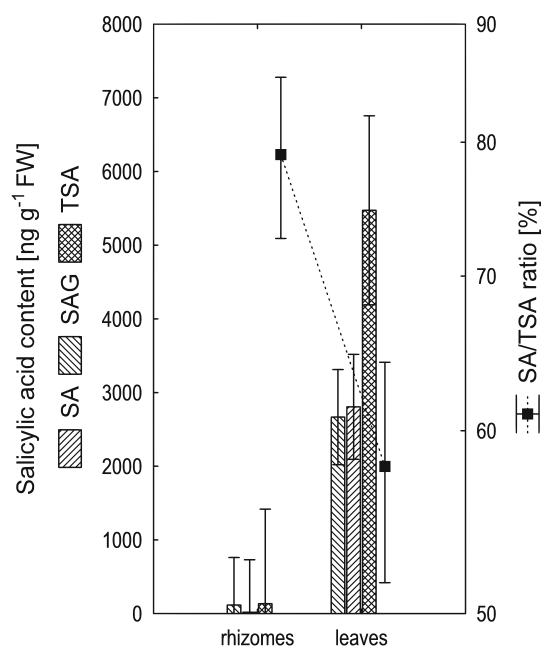


Fig. 5 Mean values of salicylic acid content in *Phragmites australis* according to the analyzed organ. Vertical bars represent 95% confidence intervals for “organ” effect according to one-way ANOVA (SA free salicylic acid, SAG salicylic acid glucoside, TSA total salicylic acid)

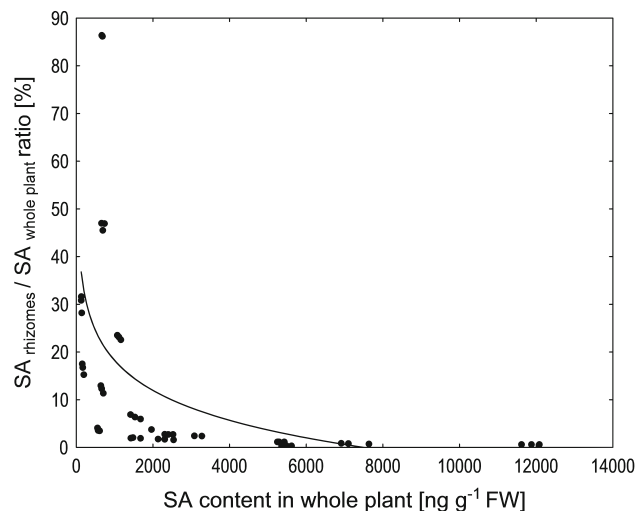


Fig. 6 The relation between free salicylic acid (SA) content in rhizomes and its average content in whole *Phragmites australis* plants

analyze the content of the glucoside-derived salicylic acid, which may achieve up to 95% of TSA, and the SA/SAG ratio depends mainly on the level of stress factor (Raskin 1992; Drzewiecka et al. 2012a). In the present study, in the case of *P. australis* rhizomes the content of SA reached $\sim 80\%$ on average of TSA content, but in leaves a lower SA/TSA ratio was noted ($\sim 58\%$) (Fig. 5). This indicates the existence of rapid SA transport up to the aboveground

Table 2 Analysis of significance for multivariate linear regressions displaying relationships between salicylic acid accumulation and metal (Cu, Zn, Pb, Cd) contents in *Phragmites australis* organs taking into consideration all collection sites ($\alpha = 0.05$; a the “Y intercept”, i.e., the value of Y when X is zero, b estimate of regression coefficient,

R^{2*} adjusted coefficient of determination, P empirical level of significance for the variable, p empirical level of significance for the model, SA free salicylic acid, SAG salicylic acid glucoside, TSA total salicylic acid)

Dependent variables (Y)	Independent variables (X)	Multivariate linear regression coefficients and analysis of their significance				
		a	b	R^{2*}	P	p
Rhizomes (May)						
SA	Cu_rhizomes	63.91	−0.86	0.9944	***	***
	Zn_rhizomes		0.78		***	
	Cd_rhizomes		0.242		**	
TSA	Cu_rhizomes	177.67	−0.89	0.9951	***	***
	Zn_rhizomes		0.69		***	
	Cd_rhizomes		0.18		*	
Leaves (July)						
SA	Cu_rhizomes	1242.55	−0.31	0.9876	*	***
	Pb_leaves		−1.0		***	
	Zn_rhizomes		0.487		***	
	Zn_leaves		0.53		**	
SAG	Cu_rhizomes	1269.73	−0.68	0.9969	**	***
	Cu_leaves		0.37		**	
	Pb_rhizomes		−0.96		*	
	Pb_leaves		0.74		**	
	Zn_leaves		0.70		*	
	Cd_leaves		−0.67		**	
	TSA		Cu_rhizomes		−2636.53	−0.57
	Pb_rhizomes		−0.59		***	
	Zn_rhizomes		0.71		***	
	Zn_leaves		0.34		***	

Only significant regressions ($p \leq 0.05$) are presented in the table. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$

organs followed by its transformation into glucoside (SAG) predominantly in plant leaves. Furthermore, the increase of average SA content in the whole plant caused the logarithmic decrease of rhizomes/whole plant ratio (Fig. 6). This corresponds with the results of Pál et al. (2005). The authors observed elevated biosynthesis of SA upon Cd stress in leaves of maize, but the metabolite content in roots remained at a constant and significantly lower level. The observation confirms a key role of the metabolite in protecting the photosynthetic tissue against adverse environmental conditions.

We assume that in conditions of multi-metal stress, SA has a complex function, i.e., serving as a signaling molecule and regulator of the oxidative burst, and probably as a metal chelator in detoxification mechanisms, thus influencing simultaneously metal uptake and plant resistance. As previously documented, the exogenous application of salicylic acid to rice (Choudhury and Panda 2004; Panda and Patra 2007) or mustard (Ahmad et al. 2011) seeds significantly lowered Cd accumulation and reduced metal

toxicity. Similar results were reported by Belkadhi et al. (2012) for flax seedlings. Seed priming with SA alleviated Cd toxicity decreased production of non-protein thiols and reduced Cd bioaccumulation. Further, SA + Cd pre- and co-treatment of maize seedlings lowered metal accumulation in roots and ameliorated Cd content in leaves (Gondor et al. 2016). Additionally, a significant elevation of antioxidant enzyme activity and thiol (GSH and PCs) production by SA-Cd application was observed and depended on the plant organ and the form of salicylic acid applied (free acid or sodium salt). However, in the study of Kováčik et al. (2009), simultaneous treatment of chamomile plants with SA and selected heavy metals increased or decreased their accumulation in photosynthetic tissue depending on the metal (in the case of Ni and Cd, respectively). This indicates distinct, metal-specific modes of SA-metal interactions. Additionally, exogenous SA influenced the activity of phenolic metabolism-related enzymes; i.e., shikimate dehydrogenase (SKDH) and cinnamyl alcohol dehydrogenase (COD) were strongly

induced by SA in the case of Cd, but in Ni-treated plants, SKDH activity was significantly lower than in plants cultivated without SA. Consequently, significant changes in phenolics profile were observed between treatments (i.e., in benzoic, cinnamic and endogenous SA contents), reflecting differences between Cd and Ni uptake and toxicity.

The simple correlation analysis performed over the growing season to evaluate the impact of metal pollution on plant response revealed relatively weak positive and inverse relations between salicylic acid and metal contents in *P. australis* organs ($-0.48 \leq r \leq 0.44$) (Fig. 3). Therefore, we performed a multivariate regression analysis for selected times of plant collection (May for rhizomes, July for leaves) reflecting the highest contents of salicylic acid to analyze the simultaneous effect of all analyzed metals on its biosynthesis and translocation within investigated plants (Table 2). The obtained correlation coefficients were each time higher for simultaneous than for single metal considerations (excluding SAG content in rhizomes). The analysis proved a strong positive influence of Zn accumulation in rhizomes on SA and TSA contents in leaves, while leaf Zn also influenced SA conjugation with glucose. This observation confirms the existence of SA active transport from roots up to aboveground organs in conditions of metal stress (Rocher et al. 2009). Furthermore, Cu decreased Zn-induced accumulation of salicylic acid in rhizomes and leaves during multi-metal stress, proving distinct mechanisms of Cu and Zn toxicity. This observation is in agreement with our previous results. In *Salix* sp., Cu treatment led to elevated accumulation of total phenolic compounds (TPC) and sucrose; however, the level of salicylic acid remained at a relatively low level compared to plants treated with Ni (Drzewiecka et al. manuscript submitted). This indicates a strong influence of Cu on primary metabolism and sucrose signaling rather than SA-dependent responses. Cd enhanced the accumulation of SA and TSA in reed rhizomes, while Pb depleted salicylic acid content in leaves and induced its binding with glucose in photosynthetic tissue (Table 2).

Conclusions

The pollution of natural ecosystems with metallic elements induces the response machinery of plants along with the elevation of salicylic acid biosynthesis among numerous phenolic compounds with antioxidant and chelation properties. The resulting level of the metabolite, the rate of its translocation from roots/rhizomes up to aboveground organs as well as binding with glucose to form an inactive SA depot are the effect of antagonistic and synergistic interactions between metals. Among investigated metals, Zn exhibited the strongest abilities to induce SA

biosynthesis both in rhizomes and leaves of *P. australis*, whereas Cu depleted the Zn-caused effect. Simultaneously, Cd acted mainly in plant roots and increased salicylic acid accumulation, while Pb diminished the level of the metabolite in photosynthetic tissue and stimulated its binding with glucose.

Author contribution statement KD planned the experiment, collected the samples of *P. australis* from natural water ecosystems, performed the analyses of free and glucoside-bound salicylic acid in collected material, performed the statistical analysis of the data, prepared the manuscript. MM performed the analysis of metal (Cu, Zn, Pb and Cd) content in plant material.

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