DISTRIBUTION OF ZINC AND CADMIUM IN TISSUES OF GIANT REED (*Arundo donax* L.): SEQUENTIAL EXTRACTION - RADIOMETRIC STUDY

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Abstract: Heavy metals are taken up by the vascular plant root system from water solutions in cationic forms. Subsequently, during both short and long distance transport to other plant tissues, cation forms are incorporated to many bioorganic compounds differing in stability, ionic character and physico-chemical properties such as solubility in lipid structures and mobility across cell membrane systems. Many sequential and single step extraction methods have been elaborated for characterization of the role of individual components of plant cells components in transport and detoxication of heavy metals. In our study, dry biomass of giant reed (*Arundo donax* L.) grown in hydroponic media spiked with $^{65}ZnCl_2$ and $^{109}CdCl_2$ was treated with dithizone solutions as complexing ligand in order to convert free Zn^{2+} and Cd^{2+} ions to corresponding dithizonates. Treatment with dithizone showed that up to 67 % of the total plant Cd and 56 % of the total plant Zn were transformed to dithizonate complexes extracted with chloroform. Extraction of biomass with Folch reagent showed that up to 48 % of the total root cadmium and up to 18 % of the total shoot cadmium is bound in lipid fraction. Zinc was not found in lipid fraction of root and shoot. Derivatization of the dried root and shoot lipid fraction by dithizone showed that two third of Cd in root and practically all Cd in shoot lipid fraction could be transformed to Cd-dithizonate. Methods of biomass treating with complexing ligands and a method of sequential extraction procedures with non-polar organic solvents and radiotracer methodology seem to be useful methods for the study of metal speciation and distribution in vascular plants.

Key words: ^{65}Zn, ^{109}Cd, *Arundo donax* L., sequential extraction, dithizonate, speciation

1. Introduction

Contamination of environment by heavy metals such as Cu, Cd, Pb, Hg and Zn is a global problem. Heavy metals and radionuclides spreading from the source of air pollution are dissolved by rainfall water and sorbed by leaf surface of higher plants or directly sorbed onto soil components. Reversibility of the metal sorption at the end of vegetation period determine the next transport of contaminants to the soil and consequently into the food chain in the following vegetation period. Plants can bind contaminants from soil and according to the way of binding, these metals are released to the environment in different speed.

Giant reed (*Arundo donax*) can be considered both as an energy plant and as a tool for toxic metal removal from contaminated land. The plant can survive in wetlands

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when the concentration of Cu^{2+}, Pb^{2+}, $\mathbf{Cd^{2+}}$**,** $\mathbf{Zn^{2+}}$, Ni^{2+} and Hg^{2+} is 100 mg/kg and Cr^{6+} 50 mg/kg (HAN and HU, 2005). The concentration of heavy metals in soil declines with the growth of the plant due to the translocation of heavy metals from peripheral soil to rhizosphere and the phytoextraction and phytovolatilization. The characters of large biomass, exuberant root and good adaptability of *A. donax* suggested its great potential in remediation of polluted soils.

Giant reed can be considered also as a sensitive biomonitor of the presence of heavy metals in water and sediments. BONANNO (2012) showed that concentrations of metals in plant tissues were significantly dependent on the kind of organ and element. Trace element concentrations decreased according to the pattern of root > leaf > stem, implying that roots acted as the main centres of bioaccumulation, and stems as transit organs as a consequence of the general high translocation from roots to leaves. *A. donax* showed a significant capacity of bioaccumulation in agreement with ecologically similar macrophytes. Positive correlations were found between trace concentrations in plant organs and sediment (Al, Cr, Mn, Ni, **Zn)**, and water (Cu, Ni, **Zn**). The results of this study suggested that *A. donax* acts as an ecological indicator of environmental conditions, thus, its application may prove a useful tool during monitoring campaigns of wetlands.

In the quantification of the efficiency of energy production processes based on biomass, energy returned on invested energy is an important parameter to be considered. In this context, *A. donax* showed the best results, thanks to the great yields reached in low input cultivation conditions, with respects to other herbaceous perennial species (PILU *et al*., 2012; TAKAHASHI *et al*., 2010). The annual stem production can reach from 12 to 23 t DM/ha (MAVROGIANOPOULOS *et al.,* 2002). Giant reed is therefore considered an important representative of energy plants.

For extraction of heavy metals from soils and sediments, a variety of single and sequential extraction techniques can be used (TESSIER *et al*., 1979). These techniques were recently reviewed by BABEL and DACERA (2006). However, owing to the wide range of extractants used in these extraction techniques, the results obtained were not comparable. In 1987, the Community Bureau of Reference (BCR) started a programme to harmonize the methodology used in sequential extraction schemes for determination of metals in soils and sediments (URE *et al*., 1993). The methodology was also successfully applied to different types of biomass. The type of applied sequential extraction scheme can lead to the anomalies in the results, as sequential extraction schemes are greatly influenced by the type of extractants used, the operating conditions (pH, contact time and temperature) and the sequence in which the extraction steps are applied. VAN HULLEBUSCH *et al*. (2005) compared the sequential extraction procedure by TESSIER *et al*. (1979), the procedure according to STOVER *et al*. (1976), the BCR protocol and discussed the results of the fractionation of anaerobic metabolism of important metals (Co, Ni, Cu, **Zn**, Mn and Fe).

The phytofiltration of Cd and Zn by giant reed (*A.* donax L.) was described and published in previous papers (HORNÍK, *et al*. 2011; DÜREŠOVÁ *et al*., 2013; DÜREŠOVÁ *et al*., 2013). Sequential extraction has been successfully used for speciation study of elements in soils and other inorganic matrices (TESSIER, 1979; MEHLICH, 1953; MEHLICH, 1984; LINDSAY, 1978). However practical applicability for behaviour of toxic metals in plant tissues is rather limited. The use of method of sequential extraction of heavy metals in sewage sludge was reported in our previous paper FRIŠTÁK *et al*. (2012). The aim of this paper was to characterize mode of heavy metals binding in the process of root uptake and translocation to shoots in vascular plants. Giant reed (*A. donax* L.) grown in nutrient media spiked with ^{65}Zn and ^{109}Cd was used as a model plant. Sequential extraction of dried biomass was used in the presence of ligands able to bind heavy metal cations to complexes differing in stability constants *log K* and estimated in extracts by gamma-spectrometry.

2. Material and methods

2.1 Chemicals

Deionized water (conductivity 0.054 μS/cm) was prepared with Simplicity 185 (Millipore, F). Commercially available chemicals of analytical grade, without pretreatment were used: dithizone ($C_{13}H_{12}N_4S$; CAS: 60-10-6), chloroform, methanol.

2.2 Cultivation media

Hoagland medium (mg/dm^3): NH_4NO_3 – 160.1; H_3BO_3 – 8.5; $Na_2MoO_4.2H_2O$ – 0.06; $MnSO_4.5H_2O$ – 5.0; $ZnSO_4.7H_2O$ – 0.66; $CuSO_4.5H_2O$ – 0.8; $MgSO_4.7H_2O$ – 369.7; KNO_3 – 404.4; $CaCl_2$ – 443.9; $NaH_2PO_4.2H_2O$ – 291.7; $Na_2HPO_4.12H_2O$ – 46.5; $FeSO_4.7H_2O$ – 17.9; $NaNO_3$ – 339.9; NH_4Cl – 213.9, pH 6.0. Basal medium was diluted with deionized water in the ratio 1:3.

Mineral medium (MM) (mg/dm^3): $CoCl_2.6H_2O$ – 0.3; $FeSO_4.7H_2O$ – 5.8; KH_2PO_4 – 23.4; $MgSO_4.7H_2O$ – 41.0; $CO(NH_2)_2$– 91.7; NH_4Cl – 12.8; CH_3COONH_4 – 79.4. Microelements (μg/dm^3): $CuSO_4.5H_2O$ – 80; H_3BO_3 – 850; $MnSO_4.5H_2O$ – 500; $Na_2MoO_4.2H_2O$ – 6.0; $ZnSO_4.2H_2O$ – 66, pH 5.5.

2.3 Plants

Arundo donax L. (var. *versicolor*) was obtained from The Centre for Research of Plant Production (Piešťany, Slovak Republic). Plants originated from tissue cultures grown in nutrition media (MURASHIGE *et* SKOOG, 1962) supplemented with synthetic cytokinine 6-benzylaminopurine and α-naphthylacetic acid for micropropagation of plants. In the next step, plants were cultivated for 2-4 weeks in 25 % Hoagland nutrition media (HOAGLAND, 1920) in cultivation box Binder (Germany), type KBWF 720. Photoperiod light/dark 16h/8h at light intensity max 11 450 lx (stepwise 0, 40, 60 and 100 %) and at 28/15 °C, relative humidity 60-80 %.

2.4 Zinc and cadmium root uptake

Pre-cultivated 20 plants (each approx. 20 cm, 40 mg d.w., shoot to root ratio 4:1 d.w.) were transferred to test tubes and cultivated in the first step for 24 h in mineral medium (MM), in the second step for 24 h in the same mineral medium supplemented with $^{65}ZnCl_2$ and $^{109}CdCl_2$ at 20 °C at day/night light cycle. Roots were washed in surplus of deionized water, roots and shoots were air dried, weighted and radioactivity was measured by gamma-spectrometry.

2.5 Extraction procedures

Dried biomass (approx. 200 - 250 mg) divided to roots and shoots was separately treated according to scheme in Fig. 2 and Fig. 3. Presented data are averages of three independent experiments.

2.5.1 Sequential extraction of lipid fraction

Shoot or root biomass (approx. 110 mg) were subsequently extracted with 2 mL Folch solution ($CHCl_3$: MeOH = 2 : 1) for 1 h at 25 °C according to the scheme in Fig. 3. Aliquots of extract solution removed from biomass and in both extract and biomass were ^{65}Zn and ^{109}Cd radioactivity by gamma-spectrometry measured. After this, all samples of biomass and FOLCH extracts were dried for 1 day at 25 °C. Subsequently, the dried samples were extracted with 2 cm^3 1 mmol/dm^3 chloroform solution of dithizone for 1 h at 25 °C. Extracts were removed from biomass and samples of wet biomass were weighted and retained volume of extractants was calculated by subtraction of dry biomass used in experiments. Concentration ratio of Zn and Cd species was calculated from the radioactivity of extracts and remaining biomass by gamma-spectrometry.

2.5.2 Sequential extraction combined with derivatization to dithizonates

Biomass of *A. donax* (approx. 200-250 mg, d.w.) was separated to roots and shoots, air dried and extracted according to the scheme in Fig. 2. Shortly, in the first step, biomass was extracted with 2 cm^3 0.1 mmol/dm^3 dithizone for 1 h at 25 °C, in the next step biomass was extracted with chloroform solution of 1 mM dithizone, and in the last step with 0.1 mol/dm^3 HCl. After each extraction the extract was removed, samples of wet biomass were weighted and retained volume of extractants was calculated by subtraction of dry biomass used in experiments. Concentration ratio of Zn and Cd species was calculated from the radioactivity of extracts and remaining biomass by gamma-spectrometry.

2.6 Radiometric analysis

For determination of ^{65}Zn and ^{109}Cd gamma-spectrometric scintillation detectors 54BP54/2-x and 76BP76/3 with well type crystal Na(Tl) (Scionix, NL) and data processing software ScintiVission-32 (Ortec, USA) were used. A library of radionuclides was built by selecting characteristic γ-ray peaks (88.04 keV for ^{109}Cd and 1115.52 keV for ^{65}Zn) for energy and efficiency calibration. Standardized solution of $^{65}ZnCl_2$ (4.90 MBq/cm^3; 0.05 g/dm^3 $ZnCl_2$ in 3 g/dm^3 HCl) and $^{109}CdCl_2$ (3.94 MBq/cm^3; 0.05 g/dm^3 $CdCl_2$ in 3 g/dm^3 HCl) was provided from the Czech Metrological Institute (Prague, CR).

3. Results and discussion

Giant reed (*A. donax)* was grown in liquid nutrient media spiked with ^{65}Zn and ^{109}Cd at subtoxic concentrations 11.0 μmol/dm^3 $ZnCl_2$ and 11.0 μmol/dm^3 $CdCl_2$. It was found that zinc and cadmium were distributed between roots and shoots in the ratio 6.6:1 for Cd and 5.4:1 for Zn. The ratio of specific radioactivity (Bq/g, d.w.) of root biomass to specific radioactivity of shoots biomass calculated from 20 plants of *A. donax* was 33.7:1 for Cd and 11.9:1 for Zn. This phenomenon can be explained by higher mobility of zinc as microelement comparing with mobility of phytotoxic cadmium, which is retained in root biomass with higher efficiency.

Speciation of zinc and cadmium in plants

Speciation of heavy metals entering the plant tissues plays decisive role in the both long distance and short distance transport of these metals in plant and is responsible for phytotoxic behaviour of heavy metals and participation in metabolic processes. For solving these problems in our experiments we combined sequential extraction procedures and derivatization of plant zinc and cadmium by complexing ligands (Fig. 1).

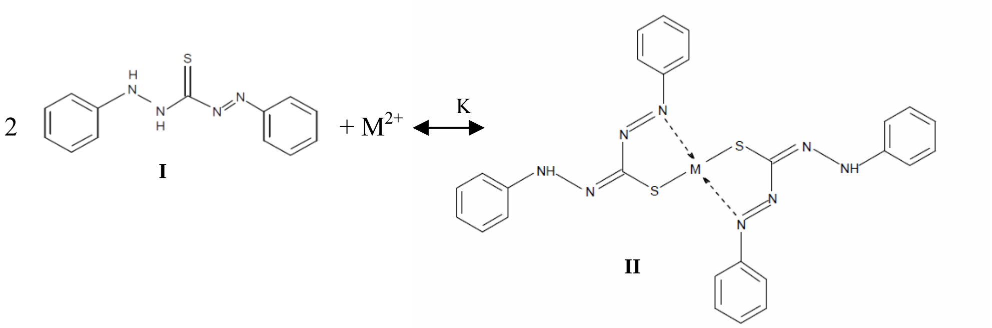

Fig.1. Reaction of dithizone (I) with bivalent metals M^{2+}resulting in metal dithizonates formation (II).

Dithizone is able to react with free Zn^{2+} and Cd^{2+} ions and also with zinc and cadmium bound in complexes with many organic and inorganic compounds bearing anionic groups such as carboxylic acids, amino acids, inorganic and organic phosphates. Concentration equilibrium of this substitution reaction depends on *log K* values of stability constants of corresponding metal ligands. Stability constants of synthetic complexing ligands such as EDTA, NTA and dithizone are higher within orders than stability constants for components of plant tissues (Table 1). Synthetic complexing ligand could therefore serve as an efficient tool for speciation analysis of microelements and toxic metals entering plant tissues. The main advantage of dithizone comparing with EDTA is high solubility of Zn and Cd dithizonate in organic solvents e.g. in chloroform and it can be quantitatively separated from reaction mixture and at utilization of radiotracer methods determined by common radio-analytical equipments.

Table 1. Stability constants *log* K of naturally occurring and synthetic metal-ligand complexes.

Ligand	***log* K**	
	Zn^{2+}	**Cd^{2+}**
Naturally occurring		
Acetic acid	1.59 [c]	1.7 [c]
Glutamic acid	5.45 [a]	4.78 [b]
Histidine	6.6 [c]	5.65 [b]
Cysteine	9.8 [c]	10.30 [a]
ADP	4.28 [c]	-
ZnH_3Phytate	7.81 [d]	-
Triphosphates	9.7 [c]	-
Synthetic		
NTA	10.45 [c]	9.8 [b]
EDTA	16.5 [b]	16.5 [b]
Other organic ligands	0.8-21.9 [e]	0.9-23.3 [e]

[a] BOTTARI and FESTA (1997); [b] CHRISTENSEN and IZATT (1983); [c] MARTELL (1964); [d] CREA *et al.* (2008); [e] SOLOVJEV *et al.* (2012)

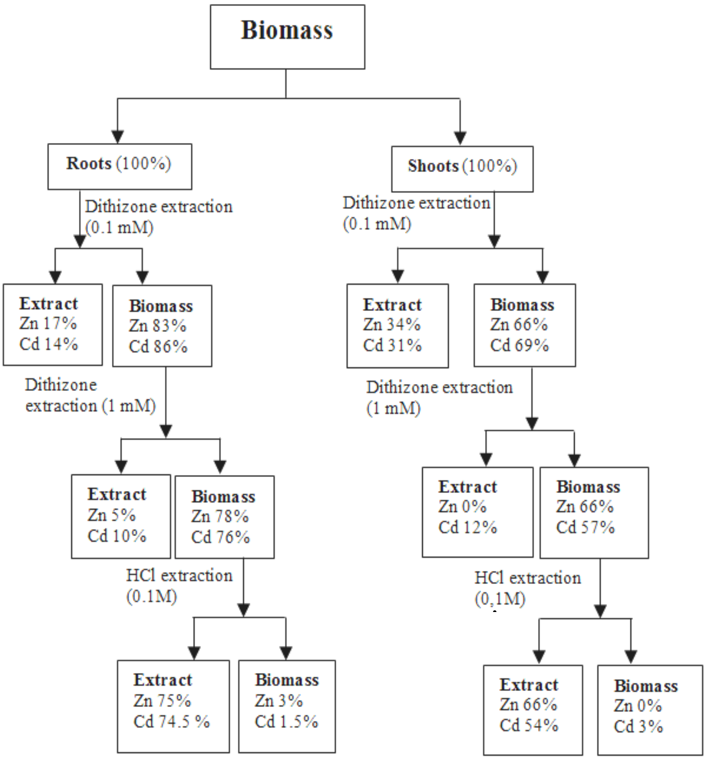

Fig. 2. Sequential extraction of dried *A. donax* biomass (~ 110 mg, d.w.) combined with Zn and Cd complexation with dithizone *in situ* without removal of lipid fraction. Plants grown in mineral medium (MM) at $C_0 = 11$ µmol/dm^3 $ZnCl_2$ and 11 µmol/dm^3 $CdCl_2$.

In our experiments (Fig. 2), dried plant biomass was treated with 0.1 and 1.0 mmol/dm^3 dithizone in chloroform at 20 °C. This procedure leads to conversion of Zn and Cd species to corresponding dithizonates and their extraction to chloroform phase. Gamma-spectrometry showed, that in root biomass after double extraction was 78 % Zn and 76 % Cd in non-ion-exchangeable form, which was soluble in 0.1 mol/dm^3 HCl. Similar ratios were obtained in shoot biomass, where after double extraction was in non-ion-exchangeable form 66 % Zn and 57 % Cd. Residual proportion in biomass after extraction with 0.1 mol/dm^3 HCl represented ≤3%, which defines metal binding to metalloproteins or other biomass compartments, which can be for example for steric reasons unavailable in reaction with such effective ligand as HCl. The data are presented in Table 2.

Table 2. Ion-exchangeable (dithizonate) and non-ion-exchangeable (non dithizonate) forms of Zn and Cd in *A. donax* estimated by *in situ* conversion of metals to corresponding metal-dithizone complexes with the following extraction into chloroform.

Fraction	Root (%)		Shoot (%)	
	Zn	**Cd**	**Zn**	**Cd**
Total biomass	100	100	100	100
Dithizonate [(a)]	22	24	34	43
Non dithizonate	78	76	66	57
HCl soluble	75	74.5	66	54
HCl insoluble	3	1.5	0	3

[a] Sum of the data of the subsequent reaction with 0.1 mmol/dm^3 and 1 mmol/dm^3 dithizone in chloroform.

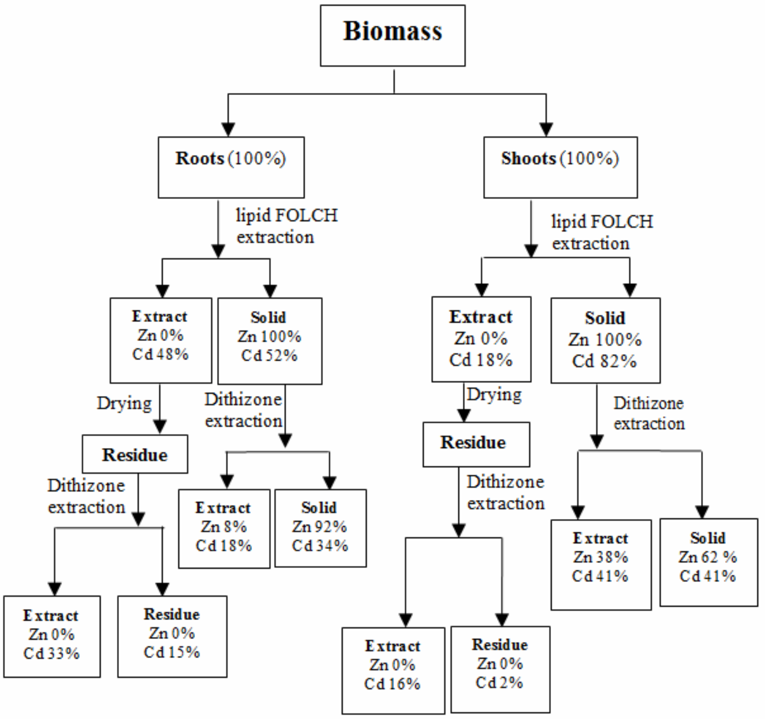

Fig. 3. Sequential extraction of dried *A. donax* biomass (~ 110 mg d.w.) combined with Zn and Cd complexation with dithizone *in situ* after removal of lipid fraction by Folch reagents.

After removal of lipid fraction from biomass (Fig. 3) we found, that Zn entering the plant *via* root of *A. donax* L. from very diluted solutions (11 µmol/dm^3 $ZnCl_2$ and 11 µmol/dm^3 $CdCl_2$) was not distributed in root and shoot lipid fraction, or only in small quantities. Zn was localized in non-lipid fraction, in forms which are not available for reaction with dithizone in non-polar organic solvent immiscible with water, or is bound to a chemical forms in ion non-exchangeable form (e.g. proteins, nucleic acid…).

Cadmium is bound in high quantities in root lipid fraction (48 %) and shoot lipid fraction (18 %). Derivatization of dried root and shoot lipid fraction by dithizone showed that two third of Cd in root and practically all Cd in shoot lipid fraction could be transformed to Cd-dithizonate. In shoot lipid fraction, substantial part of cadmium is in ion-exchangeable form (16 % total). In root, the proportion of metal in non-ion-exchangeable form is higher (15 %). In shoot non lipid fraction, Cd in ion-exchangeable form is practically in comparable amount as Zn (Cd 41 % and Zn 38 %), but in root non-lipid fraction is higher (Zn 8 % and Cd 18 %). Prevailing part of the zinc in root (up to 92 % of the total) and cadmium (up to 34 % of total) remaining in non-lipid fraction was not convertible to dithizonate. Similarly, high proportion of zinc and cadmium remained in non-lipid fraction of shoots (62 % of total zinc and 41 % of total cadmium). All data are presented in Table 3.

Table 3. Ion-exchangeable and non-ion-exchangeable forms of Zn and Cd in lipid and non-lipid fraction of root and shoot tissues of *A. donax*. See Table 2 for details.

Fraction	**Root (%)**		**Shoot (%)**	
	Zn	**Cd**	**Zn**	**Cd**
Total biomass	100	100	100	100
Lipid fraction	**0**	**48**	**0**	**18**
Dithizonate	0	33	0	16
Non dithizonate	0	15	0	2
Non lipid fraction				
Dithizonate	8	18	38	41
Non dithizonate	92	34	62	41

Many papers confirmed (see e.g. HIROSHI *et al.*, 1994) that both Zn and Cd dithizonate in chloroform extract can be completely converted to water soluble form by re-extraction with EDTA solution what gives the next opportunity of speciation analysis of microelements and toxic metals entering the plant tissues *via* root and foliar uptake as well as for speciation analysis of metal ions sorbed on sorbents with many different metal binding active centres.

4. Conclusions

Experiments with giant reed (*A. donax*) plants grown in the presence of non-phytotoxic concentration of $^{65}ZnCl_2$ and $^{109}CdCl_2$ in nutrient media showed that metals are transported from roots to shoots reaching root-to-shoot ratio 6.6:1 and 5.4: 1 for cadmium and zinc, respectively. Treatment of dried plant biomass with dithizone showed that up to 24 % of Cd and 22 % of Zn incorporated in root and up to 43% of Cd and 34% of Zn incorporated in shoot can be transformed to dithizonate complexes.

Sequential extraction of plants biomass, treatment of extracts and solids by complexing ligands provide metal-ligand complexes of various stabilities. Radiotracer methods can be a powerful tool for the study of long distance transport and speciation analysis of bivalent metals entering the plant tissues *via* root system.

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