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Impact of organic pollutants on metal and As uptake by helophyte species and consequences for constructed wetlands design and management

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ABSTRACT

Various industrial processes and anthropogenic activities in urban areas induce a release of metals, metalloids and organic pollutants. Phytoremediation of co-contaminated waters in constructed wetlands is a promising solution for reducing the impact on natural environments. In order to improve the design and management of constructed wetlands, more knowledge is needed concerning the effect of organic pollutants on plant metal and metalloid uptake. In this study, the effects of a mixture of organic pollutants commonly found in industrial effluents (hydrocarbons, polycyclic aromatic hydrocarbons, anionic detergent) on the uptake of ten metals and metalloids (MM), i.e. Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn, by five helophytes having a wide European distribution were studied. Main effects of plant species and pollutant conditions on metal uptake and interactions between factors were determined by a statistical treatment of a microcosm experiment. Overall, the order of element uptake in plants was Fe > Al > Mn > Cr, Ni, Zn, > Cu > As, Cd, Pb, which was consistent with relative concentrations in the rhizosphere environment of microcosms. Larger amounts of metals were retained in belowground biomass of plants than in aboveground parts. Statistical analysis showed that organic pollutants enhanced the accumulation of Mn in whole plants and the retention of Fe in belowground parts, while they reduced the accumulation of Cd, Ni, and Zn in whole plants and the retention of Cu in belowground parts. For the other MM (Al, As, Cr, Pb), effects were variable, depending on the plant species. Among the five plants tested, *Carex cuprina* generally removed the highest

quantities of MM, which was the result of both a high metal accumulation capacity and high biomass production. Nevertheless, no significant proportion of the MM total loading could be removed in plants' aboveground parts.

1. Introduction

Various industrial processes and anthropogenic activities in urban areas induce the release of metals and metalloids (MM) in natural environments (Yadav et al., 2012). MM are currently considered as the main toxic and genotoxic compounds present in the hydrosoluble fraction (Maceda-Veiga et al., 2013) and cannot be degraded through biological processes. Depending on their forms (complexed, adsorbed onto particles or dissolved), they may enter the trophic web or sorb to the sediments where they remain until the physical and chemical conditions change (Devallois et al., 2008). MM and organic pollutants are often found together in contaminated waters because they are discharged from the same sources, e.g. vehicle emissions, industrial processes or waste incineration plants (Sun et al., 2011; Zhang et al., 2012). The effects of organic-inorganic pollutants on fauna and flora are difficult to predict (Zhang et al., 2011) but it is accepted that co-occurrence of these pollutants may induce higher toxicity (Chen et al., 2004) and requires urgent remediation of the contaminated water.

Among remediation techniques, constructed wetlands (CWs), that were designed to optimize the natural processes involving wetland vegetation, soils and associated microbial assemblages for treating wastewaters (Vymazal, 2005), are increasingly used worldwide to successfully remove MM from many types of effluents (Marchand et al., 2010; Stottmeister et al., 2003). Phytoremediation of MM is based on the soil's ability to trap elements as well as on plant element uptake and microorganism contribution in immobilizing elements or facilitating plant absorption (Pilon-Smits, 2005). While it is recognized that soil acts as the main sink for MM (Ye et al., 2001), plants may significantly contribute to MM removal through uptake in biomass (Guittonny-Philippe et al., 2014). Nevertheless, little information is available regarding the qualitative and quantitative effects of organic pollutants on plant MM uptake (Kabata-Pendias, 2011). Organic pollutants may change metal speciation (directly by formation of soluble or insoluble metal-organic complexes, or indirectly by induction of changes in biological and physico-chemical conditions in the rhizosphere) or they may alter plant growth and metabolism (Chen et al., 2004; Lin et al., 2008). Depending on the pollutant characteristics, on plants species, and on experimental conditions, it has been shown that metal uptake could either be inhibited (Lin et al., 2008), or favored (Almeida et al., 2008; Zhang et al., 2011) by the presence of organic pollutants. These changes of metal uptake rates may modify the metal quantities exportable from the CW through plant harvesting. Thus, phytoremediation of MM and organic pollutants may require adaptation of the design (i.e. plant species selection and arrangement) and the management practices of the CW (e.g. plant harvesting and biomass value-added reuse) (Guittonny-Philippe et al., 2014; Liu et al., 2007).

The aim of this study was therefore to assess the qualitative and quantitative effects of organic pollutants on MM uptake in belowground (BG) and aboveground (AG) parts of five common helophyte species, with a view to gaining knowledge relevant to the treatment of industrial effluents in CW.

2. Material and methods

2.1. Planted microcosm set-up

The experiment was designed in order to distinguish the effects of helophyte species as well as the pollutant conditions on metal and metalloid (MM) uptake and the possible interaction effects between the two factors, using a full factorial design (Fig. 1A), as recommended by Lewis et al. (1999). This type of experimental design can be used to quantify the relationship between the output variables (responses) and the input variables (factors) and interaction effects between the factors, meaning that the influence of some factors could be different according to the value of the other factors (Kaifas et al., 2014).

Plantlets from five common native helophytes species, i.e. *Alisma lanceolatum* With., *C. cuprina* (Sandor ex Heuff.) Nendtv. ex A. Kern., *Epilobium hirsutum* L., *Iris pseudacorus* L. and *Juncus inflexus* L., were collected from the wetland Les Paluns, south of the Berre lagoon, south-east France (43°35′90″09 N; 06°42′65″19 E). High concentrations of MM (Al, As, Cd, Cr, Cu, Fe, Ni, Pb and Zn) and organic pollutants (hydrocarbons, polycyclic aromatic hydrocarbons) were previously reported in the wetland sediments (Guittonny-Philippe et al., 2015). *J. inflexus* has already been used in constructed wetlands (CWs) for treating mining effluents (White et al., 2011) and seems to be a good accumulator species for As (Ruiz-Chancho et al., 2008). *E. hirsutum* has been shown to hyperaccumulate Cu (Ghaderian and Ghotbi Ravandi, 2012), but to be sensitive to Fe toxicity (Wheeler et al., 1985). *I. pseudacorus* is currently used in CWs treating metallic effluents (Soda et al., 2012). No practical feedback has been reported regarding *A. lanceolatum* and *C. cuprina* MM tolerance and their use in CWs.

Following a four-month period of vegetative reproduction of the plants in a greenhouse, twenty-four plant individuals of each species were planted in four rectangular plastic-microcosms (413 × 345 × 294 mm; 6 individual per microcosm) filled with 22 kg of pozzolan ($\emptyset = 7-12$ mm) (Fig. 1B), as previously described (Guittonny-Philippe et al., 2015). These microcosms constituted low scale CWs and were situated in a

Fig. 1 – (A) Experimental design of the microcosm trial; (B) Photos of planted microcosms during the first test-phase. A = A. lanceolatum; C = C. cuprina; E = E. hirsutum; I = I. pseudacorus; J = J. inflexus; MPM = MM pollutants mixture; OPM = organic pollutants mixture; OMPM = organic and MM pollutants mixture.

greenhouse. Pozzolan, a siliceous material of volcanic origin (the material used in this study comes from Puy-de-Dôme, France), was selected because its mechanical characteristics (low density, high porosity, large specific surface area and low ability to retain water i.e. 19%) enhance water filtration processes (Dumont et al., 2008). Following an acclimation period of 42 days, three microcosms per plant species were exposed separately to three types of pollutant mixtures: a MM pollutant mixture (named "MPM"), an organic pollutant mixture (named "OPM"), or an organic and MM pollutant mixture (named "OMPM"). A fourth microcosm per species, left uncontaminated, was used as control, yielding a total of 20 planted microcosms for this study. After the first input of pollutant mixtures, the microcosms were monitored during 113 days. All plants survived, grew, and exhibited contrasting effects of pollutants on their health (Guittonny-Philippe et al., 2015).

2.2. Chemicals and pollutant exposure

The selection of metals and metalloids (MM) and organic pollutants, and their relative aqueous concentrations in the mixture solutions (Table 1), were based on the former European environmental quality standards for water discharge in aquatic bodies (Directive 76/464/EEC).

The MM pollutant mixture (MPM) consisted of an aqueous mixture of 11 MM salts: $AlCl_3.2H_2O$; AsO_3; $CdSO_4.8H_2O$; $K_2Cr_2O_7$; $CuSO_4$; $Fe_2O_{12}S_3$; $MnSO_4.4H_2O$; $NiSO_4.7H_2O$; $Pb(NO_3)_2$; $SnCl_2$; $ZnCl_2$ (Table 1). The organic pollutant mixture (OPM) consisted of total hydrocarbons (THC) (i.e. Blend Arabian Light petroleum topped at 250 °C (BAL 250)), as well as phenanthrene (PHE) and pyrene (PYR) obtained in reagent quality from Merck (Germany) and an anionic detergent Linear Alkylbenzene Sulfonate (LAS) named CARPHEM®. The organic and MM pollutant mixture (OMPM) was prepared by mixing together both solutions. In all the microcosms, 25 mL of an organo-mineral fertilizer (NutriActiv®, NF U 42-001 produced by FLORENDI JARDIN SAS) was added at the beginning of the experiment, containing 3% of total nitrogen, 3% of total P_2O_5 and 3% of water-soluble K_2O.

The exposure of plants to pollutants was conducted in the microcosms for 113 days as previously described (Guittonny-Philippe et al., 2015) and the highest levels of pollutant concentrations were ten times higher than the European quality standards.

2.3. Sampling and chemical analysis

In every microcosm, one PVC pipe was placed upright about 150 mm from the centre of the tank for collection of water samples and immersion of measuring probes. Electrical conductivity (EC), dissolved oxygen (DO), and temperature (T) were monitored with WTW® equipment and pH was monitored in the water column with a portable pH meter (Hanna Instruments®).

Before enriching microcosms, three water aliquots were taken from the pollutant mixtures in order to determine the true initial pollutant concentrations added in the microcosms.

Table 1 — Maximum target and measured (mean ± SD, $n = 6$) metal and As, and organic pollutant concentrations (mg/L) in the pollutant mixtures.

Chemicals	Chemical forms/commercial name	Maximum target concentrations in pollutant mixtures (mg/L)	Maximum measured concentrations in pollutant mixtures (mg/L)
Al	$AlCl_3 . 2H_2O$	25	17 ± 2
As	AsO_3	0.5	0.33 ± 0.06
Cd	$3CdSO_4 . 8H_2O$	2	2.1 ± 0.3
Cr	$K_2Cr_2O_7$	5	4.5 ± 0.4
Cu	$CuSO_4$	5	4.4 ± 0.2
Fe	$Fe_2O_{12}S_3$	25	17 ± 1
Mn	$SO_4Mn . 4H_2O$	10	11.7 ± 0.7
Ni	$NiSO_4 . 7H_2O$	5	4.9 ± 0.2
Pb	$Pb(NO_3)_2$	5	3.6 ± 0.6
Sn	$SnCl_2$	20	n.m.
Zn	$ZnCl_2$	20	16 ± 2
THC	BAL 250	100	38.6 ± 3.7
PYR	PYR	0.5	0.34 ± 0.03
PHE	PHE	0.5	0.32 ± 0.04
Anionic detergent	CARPHEM®	10	4.7 ± 1.6

n.m.: not measured.

At the end of the experiment, water samples were collected in the 15 contaminated microcosms for determination of the residual pollutant concentrations (Sn was not quantified because of analytical constraints linked to the presence of spectroscopic interferences with other elements and to the low sensitivity obtained with the available apparatus).

Water samples as well as pozzolan samples in the rhizospheric zone were taken at the end of the experiment in each of the 20 microcosms in order to determine MM and organic pollutant concentrations. At this time, five plant individuals per microcosm were harvested for MM analysis in plant biomass. Plant aboveground (AG) parts (including shoot, leaves and possibly inflorescence) and belowground (BG) parts (including roots and possibly rhizomes) were washed separately with tap water to remove particles. The BG parts were not desorbed to preserve the fraction of metals and metalloids (MM) adsorbed to cell walls, which would also be collected after harvesting in constructed wetland (CW) (Caldelas et al., 2012). Plant and pozzolan samples were dried at 80 °C during one week. Plant samples (AG and BG parts separately) were pooled by microcosm ($n = 5$) as well as rhizospheric pozzolan samples ($n = 6$) before being ground to 0.2 mm (RETSCH zm 1000 blender with tungsten blades and titanium sieve). Three replicates from each pooled sample were then mineralized with *aqua regia* (1/3 HNO_3 + 2/3 HCl) in a microwave oven (Milestone Start D) before Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES Jobin Yvon Horiba, Spectra 2000) analysis (Affholder et al., 2013). Quality assurance-quality controls and accuracy were checked using standard reference materials with accuracies within 100 ± 10% (CRM 049-050 from RTC-USA for the substratum, and DC 73349 from NCS-China for the plant).

The water samples were filtered with a 0.45 μm mesh and then analyzed for Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn by ICP-AES according to DIN EN ISO 11885 (2009). The concentration of anionic surfactants was monitored by MBAS analysis according to EPA 425.1 method (Clesceri et al., 1998).

Briefly, for the analysis of phenanthrene (PHE), pyrene (PYR) and total hydrocarbons (THC), water samples were extracted by liquid—liquid extraction with n-hexane. Pressurized liquid extraction with a mixture of n-hexane/dichloromethane (1/1) was used for solid samples (i.e. pozzolan or plants). Sample extracts were reduced to 1 mL and analyzed using a gas chromatograph (7890A GC System, Agilent Technologies, USA) coupled to a 7000 Triple Quad mass spectrometer, equipped with an HP-5MS silica fused capillary column (30 m × 0.25 mm inner diameter × 0.25 μm film thickness). The quantification of PHE and PYR was performed by using chrysene-D12 as surrogate and phenanthrene-D10 as internal standard. The amount of THC was determined as the sum of resolved and unresolved components eluted from the GC capillary column between the retention times of n-decane and n-tetracontane. The specific conditions used for extractions together with the chromatographic and mass spectrometry parameters have been previously detailed (Guittonny-Philippe et al., 2015).

2.4. Statistical analysis

Mean values of pollutant concentrations in water (pooled by condition of pollution, $n = 5$) were compared using two-tailed Mann—Whitney U-test ($p \leq 0.05$). Mean values of metal and metalloid (MM) concentrations in biomass (belowground (BG) or aboveground (AG) parts; $n = 3$) were compared between conditions for a same plant species using one-tailed Mann—Whitney U-test ($p \leq 0.1$).

Designs of experiments which are a group of mathematical and statistical techniques that can be used to best organize the experiments to quantify the relationship between the output variables (responses) and the input variables (factors) were achieved. Two experimental parameters, noted A and B in the mathematical model, respectively the nature of the pollutant mixture and the plant species, were considered and twenty responses, i.e. A_metal (MM concentration in AG parts) and B_metal (MM concentration in BG parts) for the ten MM
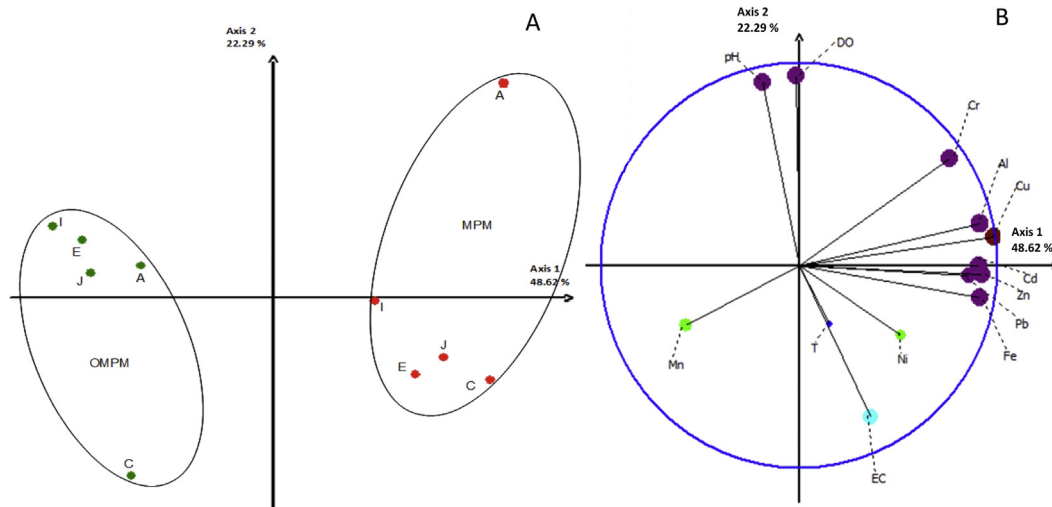
Fig. 2 – **(A) Two dimensional plots (axis1 - axis2) and (B) associated loadings investigated in principal component analysis. Species: A = A. lanceolatum; C = C. cuprina; E = E. hirsutum; I = I. pseudacorus; J = J. inflexus; EC = electrical conductivity; DO = dissolved oxygen; pH = potential of hydrogen; T = temperature. Metal: concentration in microcosm water at the end of the experiment; MPM = metallic pollutants mixture; OMPM = organic and metallic pollutants mixture.**

studied, were analyzed. Thus, for the quantitative study, according to the experimental design, only the mixtures of pollutants containing MM were considered. Two levels were attributed to factor A (i.e. pollutant mixture: the MM pollutant mixture (MPM) and the organic and MM pollutant mixture (OMPM) conditions) and five levels were assigned for factor B (i.e. plant species noted A, C, E, I, and J respectively for *A. lanceolatum*, *C. cuprina*, *E. hirsutum*, *I. pseudacorus* and *J. inflexus*). In order to evaluate the effects of these factors and interactions between them, a full factorial design was applied. Numerical treatment and analysis of the data were carried out with GraphPad Prism version 6.00 for Windows or NEMRODW (Mathieu et al., 2009).

2.5.   Geochemical modelling

The saturation indices of minerals and the speciation of MM in aqueous solution at the target concentration in presence of humic and fulvic acids were simulated for *in situ* conditions in aerobic and anoxic water using the code PHREECC (vs. 3.0.6, USGS, 2013). The modelling is described in more detail in the Supplementary Materials (Appendix A-B).

3.   Results

3.1.   Variations of physico-chemical conditions and metal and metalloid (MM) concentrations in microcosm's water

3.1.1.   Overview of physico-chemical conditions and MM concentrations in microcosm's water at the end of the experiment
Principal component analysis (PCA) of physico-chemical characteristics and dissolved metal and metalloid (MM) concentrations, underscored the major chemical trends of the

microcosms contaminated with the MM pollutant mixture (MPM) and the organic and MM pollutant mixture (OMPM) at the end of the experiment (Fig. 2). Dissolved oxygen (DO) and pH appeared to be correlated ($r = 0.779$) and pH and electrical conductivity (EC) inversely correlated ($r = - 0.728$), but these parameters were not correlated with MM concentrations in microcosms. Microcosm data were clustered depending on the condition of pollution (MPM and OMPM microcosms are represented in red and green, respectively in Fig. 2), even if in *A. lanceolatum* MPM microcosm and in *C. cuprina* OMPM microcosm, physico-chemical conditions were slightly different from the others. Average MM concentrations in water at the end of the experiment could thus be compared between microcosms of the same pollutant condition for the purpose of highlighting the effects of organic pollutants on MM removal.

3.1.2.   MM concentrations in water of control microcosms
In control microcosms in which water meeting drinking water criteria was added during the experiment, the presence of Al, Cr, Cu, Fe, Mn, Ni and Zn at low concentrations was detected in water at the end of the experiment (Table 2). Mean dissolved concentrations of Al, Ni and Mn in control microcosms were slightly higher than the European regulatory limits for drinkable water set at 0.2, 0.02 and 0.05 mg/L, respectively (Directive 98/83/EC), thus suggesting that pozzolan released these metals.

3.1.3.   MM concentrations in water of MPM microcosms compared to those of control microcosms
In MPM microcosms, all MM decreased significantly compared to their maximum initial values in water, but at the end of the experiment all MM, except As, remained above the detection limit (Table 2). It is worth noting that mean Mn concentration was within the same order of magnitude in MPM and control microcosms, resulting in a removal higher than 99% for this metal.

Table 2 – Contaminant concentrations in water (mg/L) of control and contaminated microcosms at the end of the experiment (means ± SD, $n = 5$). For a considered MM, means followed by a same letter are not significantly different between the MPM and OMPM microcosms at $p \leq 0.05$ (Mann–Whitney analysis).

Contaminant	Contaminant concentrations in microcosm water (mg/L)			
	Control	OPM	MPM	OMPM
Al	0.24 ± 0.20	n.m.	2 ± 0.71b	0.25 ± 0.27a
As	<0.03	n.m.	<0.03	<0.03
Cd	<0.01	n.m.	0.10 ± 0.03	<0.01
Cr	0.025 ± 0.01	n.m.	0.07 ± 0.05	<0.02
Cu	0.02 ± 0.01	n.m.	0.11 ± 0.03b	0.01 ± 0.01a
Fe	0.19 ± 0.29	n.m.	0.57 ± 0.12b	0.17 ± 0.07a
Mn	0.15 ± 0.29	n.m.	0.09 ± 0.07a	1.21 ± 1.11b
Ni	0.03 ± 0.03	n.m.	0.43 ± 0.16	0.28 ± 0.20
Pb	<0.04	n.m.	0.05 ± 0.01	<0.04
Sn	n.m.	n.m.	n.m.	n.m.
Zn	0.04 ± 0.03	n.m.	0.93 ± 0.25b	0.17 ± 0.11a
PHE	$0.27.10^{-3} \pm 0.03.10^{-3}$	$0.68.10^{-3} \pm 0.23.10^{-3}$	n.m.	$0.85.10^{-3} \pm 0.24.10^{-3}$
PYR	$0.10.10^{-3} \pm 0.003.10^{-3}$	$1.18.10^{-3} \pm 0.58.10^{-3}$	n.m.	$0.76.10^{-3} \pm 0.11.10^{-3}$
THC	0.23 ± 0.03	0.55 ± 0.29	n.m.	0.79 ± 0.30
Anionic detergent	n.m.	0.24 ± 0.05	n.m.	0.22 ± 0.06

n.m. = not measured. MPM = MM pollutants mixture; OPM = organic pollutants mixture; OMPM = organic and MM pollutants mixture.

For Al, Cd, Ni and Zn, average concentrations were high in MPM microcosms compared to those of control microcosms at the end of the experiment (from 8 times higher for Al to 23 times higher for Zn), and for Cr, Cu and Fe, average concentrations were from three to five times higher in MPM microcosms than in controls (Table 2). Nevertheless, for these MM, removals were high, as shown by the lower mean removal that was 88% for Al.

3.1.4. MM concentrations in water of OMPM microcosms compared to those of control and MPM microcosms

The mean dissolved fractions of Cd, Cu, Cr, Fe, Ni, Zn and especially Al were lower (differences are significant at $p \leq 0.05$ for Al, Cu, Fe and Zn; Mann–Whitney U test) in OMPM microcosms than in MPM microcosms; and dissolved fraction of Mn was significantly higher (Table 2). Al, As, Cd, Cr, Cu, Fe and Pb dissolved concentrations in OMPM microcosms were within the same range as in control microcosms. This highlights a high removal for these MM in presence of organic pollutants. Mn average concentration in OMPM microcosms was eight and thirteen times higher than in control and MPM microcosms, respectively.

3.2. Variation of metal and metalloid (MM) concentrations in plant biomasses

3.2.1. Overview of MM concentrations in plants and statistical approach

The experimental responses (ER) are defined as the metal and metalloid (MM) concentrations in aboveground (AG) parts (A_metal) and in belowground (BG) parts (B_metal) of plants in each microcosm and are presented in Table 3.

Principal Component Analysis (PCA) of ER underscored the major trends of MM uptake by the five helophytes in the MM pollutant mixture (MPM) and the organic and MM pollutant mixture (OMPM) conditions (Fig. 3). The microcosm data were clustered together, depending on the condition of pollution (i.e. MPM or OMPM) in the plot 1–2 (Fig. 3A–B) and depending on the plant species in plot 1–3 (Fig. 3C–D), indicating that plant MM uptake changed according to pollutant condition and plant species. In order to check the possible dependence between the effects of the factors, the pollutants (factor A) and the species (factor B), interaction effects were considered. To quantify these interaction effects between the "pollutant" (A) and the "species" (B) factors, datasets were statistically treated with a synergic model, and coefficients were estimated using a multilinear regression. In the case of one factor with two levels (factor A) and one factor with five levels (factor B), the mathematical model can be written as follows:

$$\eta = \beta_0 + \beta_{1A}X_{1A} + \beta_{2A}X_{2A} + \beta_{2B}X_{2B} + \beta_{2C}X_{2C} + \beta_{2D}X_{2D}$$
$$+ \beta_{1A2A}X_{1A}X_{2A} + \beta_{1A2B}X_{1A}X_{2B} + \beta_{1A2C}X_{1A}X_{2C} + \beta_{1A2D}X_{1A}X_{2D}$$

with $X_{1A} = +1$ if the factor A is present at level 1 and $X_{1A} = -1$ if the factor A is present at level 2. For the factor B:

- $X_{2A} = +1$ and $X_{2B} = X_{2C} = X_{2D} = 0$ if the factor B is present at level 1,
- $X_{2B} = +1$ and $X_{2A} = X_{2C} = X_{2D} = 0$ if the factor B is present at level 2,
- $X_{2C} = +1$ and $X_{2A} = X_{2B} = X_{2D} = 0$ if the factor B is present at level 3,
- $X_{2D} = +1$ and $X_{2A} = X_{2B} = X_{2C} = 0$ if the factor B is present at level 4,
- $X_{2A} = X_{2B} = X_{2C} = X_{2D} = -1$ if the factor B is present at level 5.

Moreover, for each ER, values were plotted as a function of other ER, in order to evaluate the correlation between ER. Among the possible combinations, several were highly correlated, indicating that these ER were controlled by the same factors and interactions. For this reason, in the following sub-sections, results are presented with groups of ER that exhibit similar patterns.

Table 3 – Experimental responses (ER): MM concentrations in plant biomass (mg/kg DW; means ± SD, $n = 3$) in MPM and OMPM conditions.

ER: Metal and As concentrations in plant belowground (B_metal) and aboveground (A_metal) biomasses (mg/kg DW)

Pollutant	ER	Species	Al	As	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn
MPM	B_metal	A	1283 ± 147	8 ± 1	**259 ± 32**	130 ± 20	**135 ± 17**	2574 ± 300	1762 ± 230	**431 ± 61**	47 ± 8	**915 ± 106**
		C	17,331 ± 459	13 ± 1	**131 ± 5**	491 ± 12	161 ± 6	21,659 ± 558	995 ± 17	443 ± 11	75 ± 2	703 ± 40
		E	**3801 ± 561**	21 ± 3	**187 ± 14**	160 ± 14	**199 ± 19**	6984 ± 863	1370 ± 121	**653 ± 35**	74 ± 7	**1317 ± 82**
		I	**1189 ± 80**	9 ± 1	**93 ± 2**	**85 ± 3**	176 ± 9	2553 ± 134	494 ± 11	**185 ± 4**	**40 ± 1**	**489 ± 13**
		J	9857 ± 1008	23 ± 3	**131 ± 12**	318 ± 35	160 ± 13	15,788 ± 1451	915 ± 83	384 ± 45	115 ± 13	**856 ± 77**
	A_metal	A	**158 ± 9**	<2.5	27 ± 1	26 ± 2	24 ± 1	**326 ± 26**	583 ± 5	**50 ± 2**	<1.2	225 ± 4
		C	930 ± 92	<2.5	**12 ± 1**	26 ± 2	19 ± 1	1455 ± 137	268 ± 18	**74 ± 4**	8 ± 1	157 ± 8
		E	140 ± 7	<2.5	**5.2 ± 0.1**	3.5 ± 0.5	12 ± 1	239 ± 5	253 ± 2	**26 ± 1**	2.0 ± 0.2	138 ± 3
		I	232 ± 1	<2.5	15 ± 0	10.4 ± 0.1	9.0 ± 0.1	524 ± 16	168 ± 5	43 ± 1	<1.2	80 ± 1
		J	885 ± 143	<2.5	11 ± 1	35 ± 4	16 ± 1	1538 ± 192	274 ± 8	**76 ± 4**	4.2 ± 0.3	156 ± 3
OMPM	B_metal	A	**2778 ± 62**	**29 ± 1**	45 ± 3	133 ± 9	63 ± 2	8603 ± 443	2101 ± 122	173 ± 10	**84 ± 5**	392 ± 14
		C	**24,195 ± 807**	**16 ± 1**	31 ± 1	**645 ± 7**	165 ± 5	**30,068 ± 841**	1005 ± 18	436 ± 4	77 ± 5	359 ± 7
		E	3107 ± 161	**77 ± 2**	85 ± 2	**194 ± 8**	122 ± 4	**11,867 ± 565**	2960 ± 68	348 ± 13	**144 ± 4**	643 ± 26
		I	877 ± 57	**11 ± 1**	20 ± 1	58 ± 2	36 ± 2	**2704 ± 77**	1322 ± 66	73 ± 3	19 ± 1	330 ± 15
		J	**15,972 ± 511**	23 ± 3	50 ± 1	**588 ± 10**	131 ± 1	**26,051 ± 292**	1191 ± 5	392 ± 3	121 ± 4	407 ± 8
	A_metal	A	89 ± 6	<2.5	4.1 ± 0.3	9.0 ± 0.6	21 ± 2	210 ± 17	**1869 ± 153**	21 ± 1	1.3 ± 0.1	107 ± 30
		C	857 ± 59	3.4 ± 0.4	4.6 ± 0.1	**42 ± 1**	**21 ± 1**	1587 ± 81	**571 ± 6**	58 ± 1	**13 ± 1**	98 ± 5
		E	**284 ± 7**	<2.5	2.1 ± 0.1	**19 ± 1**	**14 ± 1**	**532 ± 33**	452 ± 8	21 ± 1	**3.3 ± 0.1**	95 ± 15
		I	78 ± 24	<2.5	0.76 ± 0.03	5.5 ± 0.5	5.2 ± 0.8	181 ± 5	**447 ± 10**	7.8 ± 0.6	<1.2	36 ± 1
		J	1054 ± 66	<2.5	5.6 ± 0.2	**48 ± 1**	14 ± 1	**1950 ± 73**	640 ± 9	62 ± 2	**6.3 ± 0.1**	142 ± 3

A = *A. lanceolatum*; C = *C. cuprina*; E = *E. hirsutum*; I = *I. pseudacorus*; J = *J. inflexus*. DW = dry weight; MPM = MM pollutants mixture; OMPM = organic and MM pollutants mixture; A_metal = concentration in aboveground parts; B_metal = concentration in belowground parts. For a same plant species, ER in bold are significantly higher than the corresponding ER in the other pollutant condition (one-tailed Mann–Whitney U-test; $p \leq 0.1$).
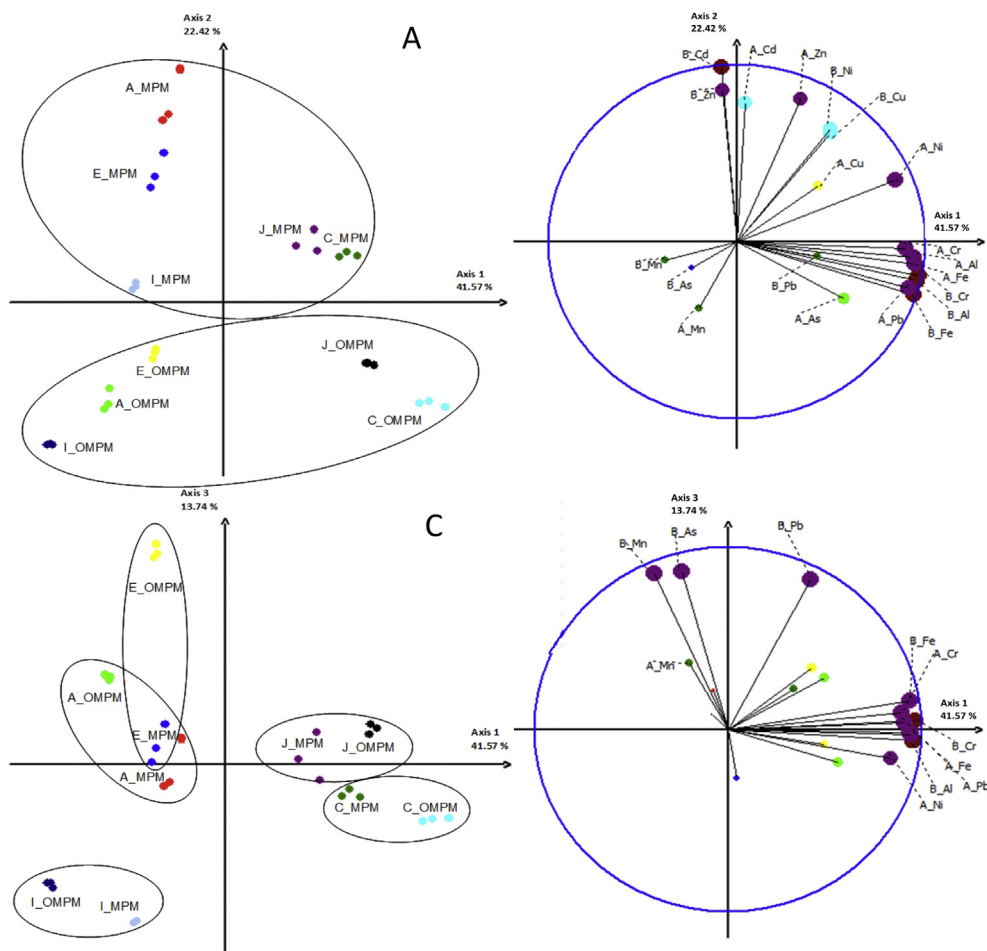
Fig. 3 − Two dimensional plots (A−B: axis 1 - axis 2; C−D: axis1 - axis 3) and associated loadings investigated in principal component analysis. Species_MPM = species in the MM pollutants mixture; Species_OMPM = species in the organic and MM pollutants mixture; Species: A = *A. lanceolatum*; C = *C. cuprina*; E = *E. hirsutum*; I = *I. pseudacorus*; J = *J. inflexus*; A_metal = MM concentration in aboveground parts; B = MM concentration in belowground parts; MPM = MM pollutants mixture; OMPM = organic and MM pollutants mixture.

3.2.2. Variation of Al, Cr, Fe (in AG and BG parts) and Pb (in AG parts) concentrations

The uptakes of Al, Cr, Fe in plants as well as Pb in AG parts were generally slightly influenced by the pollutant conditions and strongly influenced by the plant species, with low interaction effects between the two factors (Fig.1SM and Fig.2SM). Moreover, the ER of Al, Fe and Cr concentrations as well as Pb concentration in AG parts were highly correlated (see the *r* values, Table 1SM). For this group of metals, the concentrations were very high in *C. cuprina* and *J. inflexus* plants in comparison with the three other plant species (Fig.2SM). In the two former plant species, a significant effect of organic pollutants was observed, corresponding to a large increase of Al, Cr and Fe concentrations in plant BG biomass (Table 3). Moreover, in AG parts, Al, Cr, Fe and Pb concentrations were enhanced in presence of organic contaminants in *E. hirsutum* (Table 3).

3.2.3. Variation of Cd, Ni and Zn concentrations (in AG and BG parts)

The uptake of Cd, Ni and Zn was generally strongly influenced by the pollutant conditions and slightly influenced by the plant species, with some interaction effects between the two factors (Fig. 4).

For all plant species, the concentrations of Cd and Zn in AG as well as in BG biomasses were significantly reduced in presence of organic pollutants (Table 3). This effect was particularly strong for *A. lanceolatum* as well as for *I. pseudacorus* (AG) and *E. hirsutum* (BG) for Cd and Zn, respectively (Fig. 4A−D). Moreover, Cd and Zn ER were highly correlated (see the *r* values, Table 2SM), indicating that the effects of plant species, pollutants and, interactions between the two factors were generally comparable for both metals (Fig. 4A−D). The uptake of Ni was also significantly reduced in presence of organic pollutants in all plant species AG biomasses (Table 3), as well as in *A. lanceolatum*, *E. hirsutum* and *I. pseudacorus* BG biomasses.

3.2.4. Variation of Mn (in AG and BG parts) and of As and Pb (in BG parts) concentrations

The concentration of Mn was significantly enhanced in presence of organic pollutants (Table 3) in all plant species AG and BG parts (except in *C. cuprina* BG parts). This effect
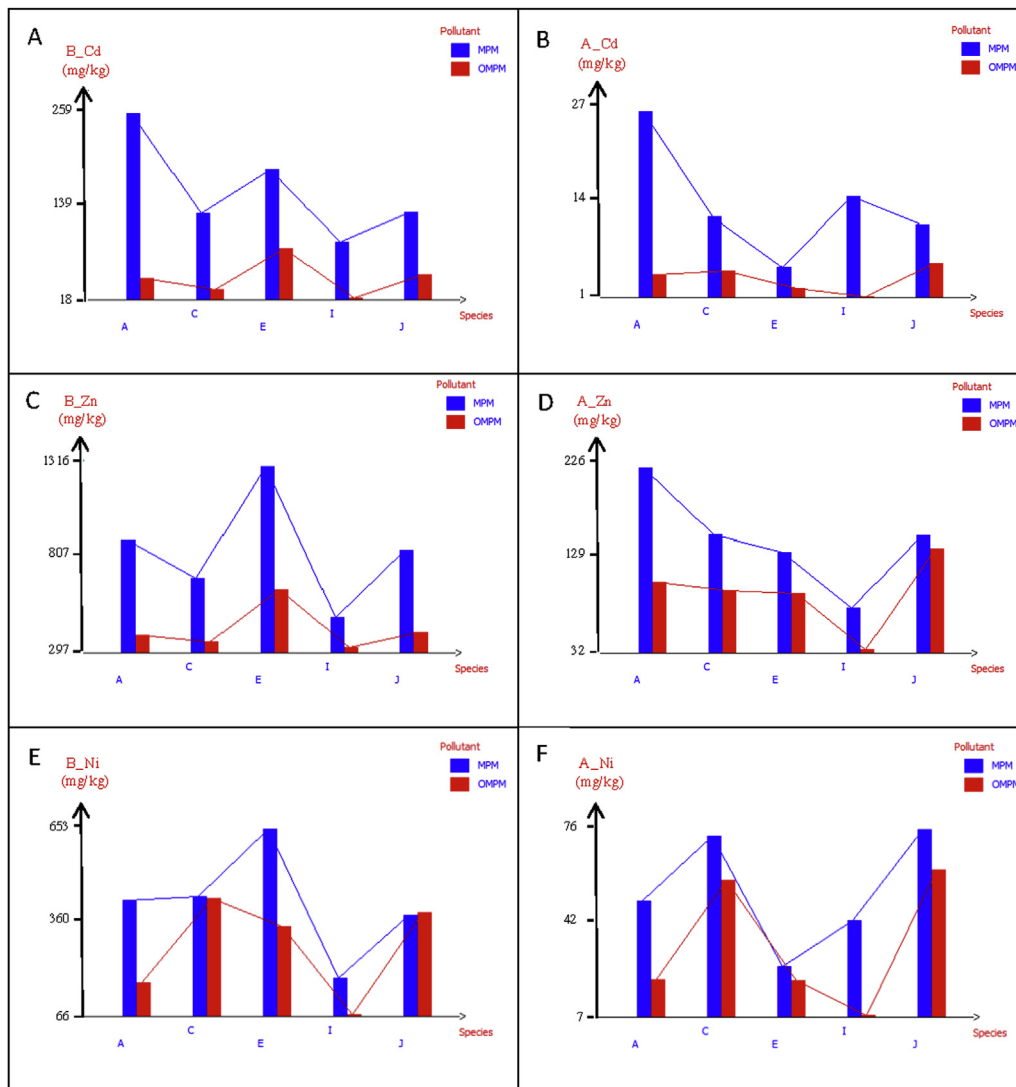
Fig. 4 − Representation of the interaction effects between MPM or OMPM conditions (Pollutant) and the five species for the responses (A) B_Cd, (B) A_Cd, (C) B_Zn, (D) A_Zn, (E) B_Ni, (F) A_Ni. The Y-axis represents the metal concentration (mg/kg): B_metal = metal concentration in belowground parts; A_metal = metal concentration in aboveground parts. Species: A = A. lanceolatum; C = C. cuprina; E = E. hirsutum; I = I. pseudacorus; J = J. inflexus; MPM = MM pollutants mixture; OMPM = organic and MM pollutants mixture.

was particularly high for *A. lanceolatum* (AG) and *E. hirsutum* (BG) (Fig. 5A−B). As shown by the PCA analysis (Fig. 3), the patterns of Mn uptake appeared to be very different from those of the other MM, except those of As in BG parts ($r = 0.805$ between B_As and B_Mn) and to a lesser extent, those of Pb in BG parts as well (Fig. 3D). The uptake of Mn, As and Pb in BG biomass was more or less strongly influenced by the pollutant condition, depending on the plant species (Fig. 5A−D), revealing interactions between factors. In particular, the uptake of these MM was strongly enhanced in the BG biomass of *E. hirsutum*, in presence of organic pollutants.

3.2.5. Variation of Cu concentrations (in AG and BG parts)
The concentrations of Cu were significantly reduced in the presence of organic pollutants in *A. lanceolatum, E. hirsutum, I.*

pseudacorus and *J. inflexus* BG biomass (Table 3, Fig. 3SM), while no such an effect was observed in AG biomass.

3.3. Variation of metal and metalloid (MM) uptake in plant biomass

3.3.1. Variation of biomass production and allocation
In addition to variation of metal and metalloid (MM) concentrations in plant biomasses, variation of biomass production and allocation of biomass between conditions may also contribute to changes in MM uptake abilities of plants. The total belowground (BG) dry weight of plants varied from 90 g for *J. inflexus* in the control microcosm to 704 g for *C. cuprina* in the organic and MM pollutants mixture (OMPM) microcosm after 133 days of growth (Table 4). The total aboveground (AG) dry weight ranged between 205 g for *A. lanceolatum* in the
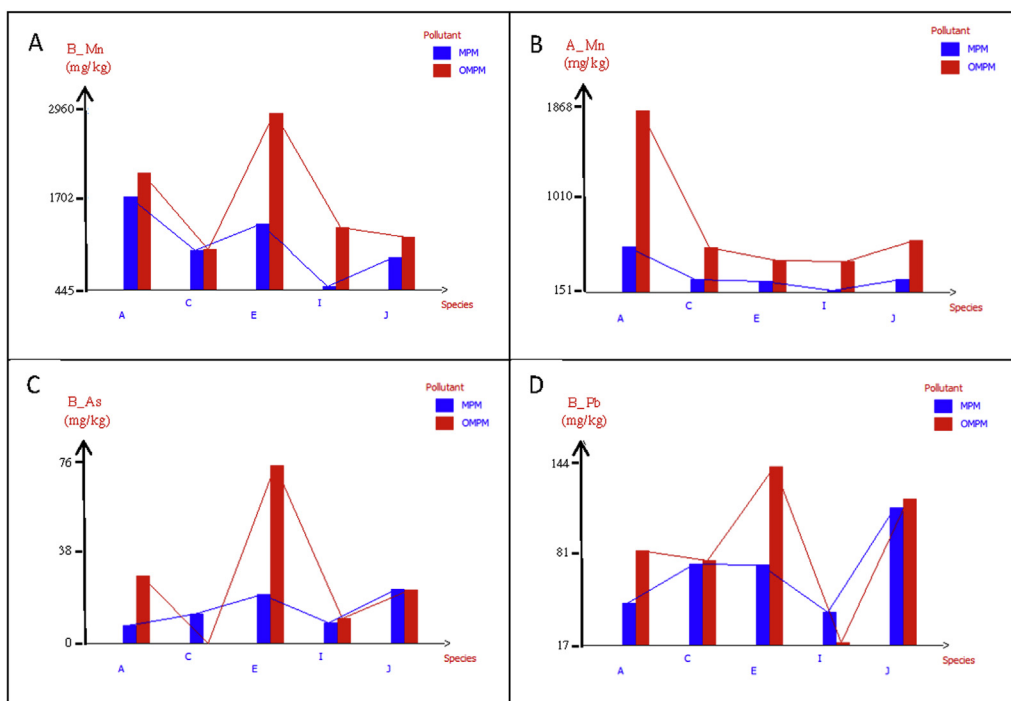
Fig. 5 – Representation of the interaction effects between MPM or OMPM conditions (Pollutant) and the five species for the responses (A) B_Mn, (B) A_Mn, (C) B_As, (D) B_Pb. The Y-axis represents the MM concentration (mg/kg): B_metal = MM concentration in belowground parts; A_metal = MM concentration in aboveground parts. Species: A = A. lanceolatum; C = C. cuprina; E = E. hirsutum; I = I. pseudacorus; J = J. inflexus; MPM = MM pollutants mixture; OMPM = organic and MM pollutants mixture.

OMPM microcosm and 585 g for C. cuprina in the control microcosm.

3.3.2. MM amounts removed in plants

The amounts of MM removed in the whole plant biomass of each microcosm as well as in AG and BG biomasses were calculated (Table 3SM and 4SM). Generally, in C. cuprina microcosms, the amounts of MM removed in plants were greater than in the other microcosms, whatever the condition or biomass type (Table 3SM and 4SM; Fig. 6). In particular, the total amount of Fe and Al removed in C. cuprina in the OMPM condition (i.e. 2200 ± 63 mg and 1748 ± 60 mg) was higher than the total amount of Fe and Al added in microcosm water during the experiment (i.e. 468 mg and 487 mg). These results highlight that a significant amount of MM in plants (at least Fe and Al) comes from pozzolan dissolution.

Thus, the net percentages of the total MM loadings removed in plant biomass were calculated after having subtracted the corresponding element concentration in plants from control microcosms (Table 5), assuming that it represents the quantity of the element brought by pozzolan dissolution. High net proportions of Al and Fe total loadings were removed in plant biomass (>50% in the OMPM condition), and a significant proportion (>5%) of Cd, Cr, Mn, Ni and Zn, depending on the pollutant conditions (Table 5). On average (in the five species whole plant biomass), the quantity of Al, Cr, Fe, Mn and Pb removed in plant biomass in the OMPM condition was higher than in the MPM condition, and the inverse trend was observed for Cd, Cu, Ni and Zn (Table 5).

4. Discussion

4.1. Metal and metalloid (MM) speciation changes induced by the presence of organic pollutants

Organic pollutants may induce changes of metal speciation by altering physico-chemical conditions in the rhizosphere through root exudation or microbial activity induced changes (Chen et al., 2004; Lin et al., 2008). In microcosm water, MM concentrations varied according to the condition of pollution and were slightly influenced by the helophyte species planted. The main observable effect concerns Mn that was much more highly concentrated in the water of the organic and MM pollutant mixture (OMPM) microcosms than in the water of the MM pollutant mixture (MPM) microcosms, whatever the species planted (Table 2). Under the oxidizing conditions of MPM microcosms (Table 5SM), Mn precipitated supposedly in the form of various Mn oxides, as revealed by a simulation performed with the PHREEQC software. The presence of degradable organic pollutants may have induced the formation of anoxic zones in OMPM microcosms even if this could not be revealed by the measurements performed in microcosm water, that do not allow for detection of small scale variations of physico-chemical conditions e.g. inside pozzolan micro-pores and around plant roots. In the absence of O_2, Mn oxides are not stable and can serve as electron acceptor in reactions of organic pollutants oxidation mediated by micro-organisms (Ponsin et al., in press), resulting in the reduction of

Table 4 — Total belowground (BG DW) and aboveground (AG DW) biomasses production in control, MPM (MM pollutant mixture) and OMPM (organic and MM pollutant mixture) conditions for the five species.

Condition	Species	BG DW (kg)	AG DW (kg)
CONTROL	A	0.0328	0.0219
	C	0.0371	0.0585
	E	0.0187	0.0491
	I	0.0459	0.0554
	J	0.0090	0.0380
MPM	A	0.0297	0.0237
	C	0.0336	0.0540
	E	0.0131	0.0551
	I	0.0512	0.0472
	J	0.0119	0.0484
OMPM	A	0.0292	0.0205
	C	0.0704	0.0530
	E	0.0126	0.0246
	I	0.0575	0.0431
	J	0.0201	0.0391

A = A. Lanceolatum; C = C. cuprina; E = E. hirsutum; I = I. pseudacorus; J = J. inflexus. DW = dry weight.

Mn oxides and release of Mn^{2+} in the microcosm water. This could explain the high variation of Mn concentrations observed between the MPM and OMPM conditions (Table 2). Another hypothesis is that dissolution of manganese oxides by humic acids, as well as by low molecular weight compounds such as phenolic acids (Baker, 1973; Stone and Morgan, 1984), occurred in OMPM microcosms. This hypothesis is supported by the fact that exudation of organic acids may be enhanced in presence of organic pollutants, as it has been reported by Phillips et al. (2012) with *Elymus angustus* under co-contamination of pyrene and phenanthrene.

In anoxic zones of OMPM microcosms, Cr may also have changed its speciation from Cr(VI) to Cr(III), favoring its co-precipitation with Fe or as Cr(III)-oxide, as revealed by the simulation performed with the PHREEQC software. These simulations yielded high supersaturation for most of MM at the concentration of target concentrations even under presence of complexing agents such as humic and fulvic acids: under aerobic conditions, only As, Cr and Ni should not precipitate, whereas all others could potentially form a mineral such as an oxide, a carbonate or a mixed phase. Under anoxic conditions, the PHREEQC simulations suggested a potential
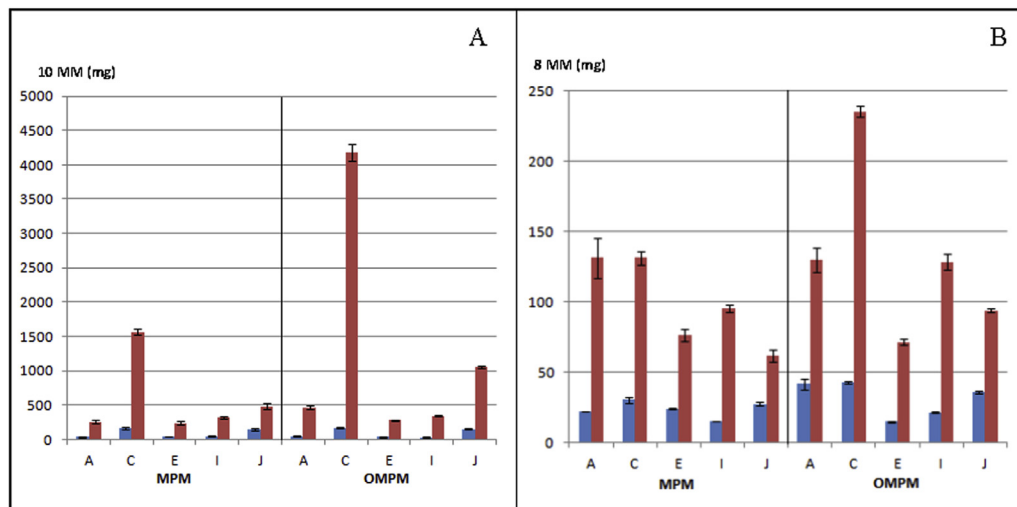

Fig. 6 — Sum of (A) the 10 MM (mg) or (B) the 8 MM (all MM except Al and Fe; mg) in aboveground biomass (blue sticks) and in whole plant biomass (red sticks). A = A. lanceolatum; C = C. cuprina; E = E. hirsutum; I = I. pseudacorus; J = J. inflexus; MPM = MM pollutants mixture; OMPM = organic and MM pollutants mixture.

Table 5 — Net percentages of the metal total loadings (%) removed in whole plant (WP) and in aboveground (AG) biomass.

Species	Biomass	Pollutant	Net percentage of the metal total loadings (%) removed in biomass								
			Al	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn
C. cuprina	WP	MPM	67.3	8.4	5.1	4.0	68.7	7.7	7.5	2.9	5.9
		OMPM	232	3.3	18.5	7.7	250.2	11.8	10.1	5.2	3.9
5 species (av.)	AG	MPM	1.6	0.96	0.15	0.25	2.6	2.1	1.2	0.1	1.0
		OMPM	1.4	0.03	0.08	0.001	0.7	2.4	0.004	0.02	0.1
5 species (av.)	WP	MPM	18.4	8.0	2.7	3.6	20.6	7.3	6.9	1.7	5.2
		OMPM	54.0	1.9	5.8	2.2	71.9	13.6	3.7	2.1	2.2

av. = average; MPM = metallic pollutants mixture; OMPM = organic and metallic pollutants mixture.

precipitation of Cr as Cr(III), a precipitation of other MM could occur as carbonate or sulfide, and dissolution of oxides. Humic and fulvid acids in the model complexed Cu primarily, but even this did not prevent it from being oversaturated under both oxic and anoxic conditions, especially with respect to a mixed Cu—Fe phase (Cupric Ferrite) (Table 6SM). Ion exchange, which was not included in the model, could furthermore have depleted the concentrations of MM, especially of those which are primarily in cationic form (Ni, Zn, Cd). For As, a potential co-precipitation with iron oxides is a tentative explanation for a strong concentration decrease. For all these MM, the removal variations observed between the MPM and OMPM conditions are most likely explained by a difference of oxidizing/reducing conditions created by the presence of organic pollutants (Muratova et al., 2009).

4.2. Metal and metalloid (MM) removal in plants and main effects of organic pollutants

In addition to the above observations on the influence of physico-chemical conditions on the decrease in dissolved metal and metalloid (MM) concentrations, plant uptake also plays an essential role in MM removal. As previously shown in several other studies (e.g. Collins et al., 2005), the MM that were most strongly concentrated in plants (Table 3 and Table 7SM) corresponded to the MM with higher concentrations in substrate (Table 8SM). On the whole, the order of element uptake in plants was Fe > Al > Mn > Cr, Ni, Zn, > Cu > As, Cd, Pb. Moreover, the uptake of MM in plants was generally higher in the MM pollutant mixture (MPM) and the organic and MM pollutant mixture (OMPM) conditions than in controls, which supports the fact that a higher concentration of MM in water usually induces a higher concentration in plants (Soda et al., 2012). In controls, where the main source of MM came from the natural content of pozzolan (Table 7SM), plants translocated rather high amounts of MM in their biomass. Obviously, pozzolan was not inert. This raises the problem of finding substrates for constructed wetland (CW) the physico-chemical composition of which bears sorption capacity for MM and organic contaminants, as is the case for pozzolan (Wang and Wu, 2006), but without constituting a potential source of additional MM release.

Qualitative and quantitative differences of MM uptake emerged between species and pollutant conditions. To sum up, the main effects of organic pollutants on plant MM uptake were i) an increase of Mn uptake and a decrease of Cd, Ni, Zn uptake in aboveground (AG) and belowground (BG) plant parts, as well as ii) an increase of Fe uptake and a decrease of Cu uptake in BG plant parts. With regard to the main plant species effects, *I. pseudacorus* appeared to be generally the weakest accumulator among the five species, whatever the condition (Fig. 3C—D; Table 3), and *C. cuprina* and *J. inflexus* were good accumulators of Al, Cr and Fe, whatever the condition. For the other species and MM, interaction effects were too strong to discern the main species or pollutant effects.

Finally, MM were generally retained in greater quantities in BG biomass than in AG biomass of plants, as previously reported by other authors (Cheng et al., 2002; Liu et al., 2010; Yadav et al., 2012). According to data provided by this experiment, if only the AG parts of the five helophytes were harvested, the proportions of MM removed by plants would be negligible, whatever the conditions (Fig. 6; Table 5).

4.3. Application to design and management of constructed wetlands (CWs)

When treating effluents containing metals and metalloids (MM) in constructed wetlands (CWs), the substrate should be excavated periodically to prevent the release of MM into aquatic receiving bodies, especially if the CW is fed with a variable flow of wastewater or susceptible of receiving salty waters (Eggleton and Thomas, 2004; Gambrell et al., 1991). Frequent plant harvesting (e.g. once a year, after the end of the growing period) can also maintain a higher vegetation uptake rate of pollutants, and thus, further reduction of pollutant concentrations in both effluent and CW substrate could be achieved (Tu et al., 2014).

The results of the present study show that for the CW treatment of industrial effluents containing mixtures of MM in ranges of concentrations ten times higher than the European regulatory limits for industrial releases (Directive 76/464/EEC, 1976), only whole plant biomass harvesting (including the aboveground (AG) and belowground (BG) parts) seems to be of significant interest. For this purpose, the use of floating-raft hydroponic systems (Tanner and Headley, 2011) could be appropriate both for facilitating the digging up of plants, and for reducing the use of MM enriched substrate. But from another point of view, for the treatment of industrial effluents containing mixtures of MM and organic pollutants, digging up plants is not recommended as the root system has to be maintained for purpose of enhancing microbial processes associated with organic pollutant biodegradation (Faulwetter et al., 2009). In this case, phytostimulation abilities of plant species (i.e. their contribution in enhancing microbial biodegradation processes in rhizosphere according to Pilon-Smits, 2005) would constitute a more appropriate criterion of selection than the ability to remove MM in biomass.

Therefore, compartmentalization of CW into different basins represents an intelligent strategy for the design of CW (Guittonny-Philippe et al., 2014): one basin with floating-rafts for a first round of MM removal, followed by one basin with plants anchored in substrate for organic pollutant biodegradation and MM retention both in substrate and plant roots. Among the five tested species, *C. cuprina* should be favored for MM removal. This species, in addition to showing very good MM accumulation abilities, exhibited an enhancement of BG biomass in presence of both MM and organic pollutants (Guittonny-Philippe et al., 2015), which is an allocation plasticity reaction that may be observed in plants in CW, depending on wastewater quality, climate or operational conditions changes (Audet and Charest, 2008; Galletti et al., 2010).

5. Conclusion

Qualitative and quantitative effects of plant species and organic pollutants on metal and metalloid (MM) removal in plant biomass were assessed in this study with the aim of improving the design and management of constructed

wetlands (CWs) for industrial effluents treatment. Results can be extrapolated to most European industrial contexts, since the studied helophytes are widely distributed in European countries and the relative concentrations of the artificial contaminating mixtures of pollutants used in the study were chosen in accordance to European regulation standards. The presence of organic pollutants altered, qualitatively and quantitatively, the MM removals, through both induction of MM speciation changes and variation of elements accumulation in plants. The main effect concerns the Mn, concentrations of which both in water and in plant biomasses were greatly enhanced in presence of organic pollutants. Moreover, statistical analyses showed that organic pollutants enhanced the retention of Fe in belowground (BG) parts of plants, while they reduced the accumulation of Cd, Ni, and Zn in BG and aboveground (AG) parts of plants and the retention of Cu in BG parts. For the other MM (Al, As, Cr, Pb), effects were variable depending on the plant species. The proportion of the MM loadings which can be removed from CW in plant biomass would be higher with *C. cuprina* than with other plant species, whether organic pollutants are present or not.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.10.014.

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