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Can PAHs influence Cu accumulation by salt marsh plants?

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1 Abstract

2 The presence of polycyclic aromatic hydrocarbons (PAHs) may change the mechanisms of
3 metal uptake, thus influencing kinetics and extent of metal phytoextraction. Studies on the
4 subject are scarce, particularly for salt marsh plants. The aim of this work was to investigate
5 the effect of PAHs on the uptake of Cu by *Halimione portulacoides*, a plant commonly found
6 in salt marshes. Experiments were carried out in the laboratory, either in hydroponics
7 (sediment elutriate) or in sediment soaked in elutriate, which were prepared with sediment
8 and water from a salt marsh of the Cavado river estuary (NW Portugal). Groups of *H.*
9 *portulacoides* (grown in a greenhouse) were exposed to those media during six days. Cu²⁺ (as
10 Cu(NO₃)₂), 10² and 10⁴ µg l⁻¹, was added to the media as well as 1.6 µg l⁻¹ of the sixteen EPA
11 priority PAHs (0.1 µg l⁻¹ of each PAHs). Cu was assayed in solutions, sediments and different
12 plant tissues before and after experiments. After exposure, photosynthetic efficiency and
13 levels of chlorophylls were also measured, indicating that plant stress indicators were
14 identical in all plants independently of the media to which the plants were exposed. PAHs
15 influenced both the soluble Cu fraction and Cu uptake by plants. The amounts of metal
16 accumulated in both roots and stems were significantly higher when the 10⁴ µg l⁻¹ of Cu
17 enriched elutriate was amended with PAHs. Thus, results suggest that PAHs may modify Cu
18 solubility, the Cu sorption by plants and / or the passive penetration of Cu into the root cells.
19 Therefore, the combined effects of different types of pollutants should be taken in
20 consideration when studying the remediation potential of plants, namely in terms of
21 phytoextraction.

22
23 **Keywords:** Mixture of pollutants; Salt marsh plant; Phytoextraction; Cu; PAHs; *Halimione*
24 *portulacoides*

1 **1. Introduction**

2
3 Estuaries are often considered sinks for pollutants, receiving important anthropogenic
4 inputs from upstream catchments as well as from metropolitan areas and industries located at
5 the vicinity of those areas. Many temperate estuaries possess salt marshes that are able to
6 perform important ecological roles not only because they are among the most productive
7 ecosystems on Earth (McLusky and Elliot, 2004), but also due to the ability to improve water
8 and sediment quality by removing nutrients (Magalhães et al., 2002) and pollutants
9 (Vernberg, 1993), including metals (Almeida et al., 2004; Reboreda and Caçador, 2007;
10 Williams et al., 1994) and petroleum compounds (Daane et al., 2001; Jackson and Pardue,
11 1999). Due to the importance of salt marsh ecosystems and their capability for degrading
12 pollutants, it is imperative to design remediation strategies to favour recovery of coastal
13 sediments.

14 Plants can take up chemical species from the environment, but the uptake is dependent not
15 only on the plant species (Burke et al., 2000; Fitzgerald et al., 2003; Weis et al., 2002;
16 Windham et al., 2003), but also on the sediment characteristics (Almeida et al., 2004). Metal
17 uptake by plants depends on the form in which metal is present in the sediment, particularly in
18 the rhizosphere (the region of soil surrounding a plant root). The metal form is determined by
19 the sediment constituents, the overlying and interstitial water chemistry, and the behaviour of
20 micro-organisms (Chapman and Wang, 2001).

21 The capability of salt marsh plants to accumulate trace metals is, in principle,
22 advantageous, as it reduces the levels of those metals in sediments and, therefore, the
23 potential toxicity to other benthic organisms is also reduced. Therefore, salt marsh plants can
24 be used in phytoremediation processes. These remediation techniques are considered an

1 effective, low cost, preferred cleanup option for moderately polluted areas (Weis and Weis,
2 2004), namely by Cu. This metal is an essential element for plant growth, which can be toxic
3 when present in higher levels.

4 Sites polluted with trace metals are also frequently affected by organic pollutants of
5 different types. Petroleum compounds, including polycyclic aromatic hydrocarbons (PAHs),
6 are among the most frequently found pollutants in coastal environment including estuaries.

7 PAHs are introduced from a number of sources including run-off and erosion, effluent
8 discharges, atmospheric deposition, naturally occurring biological processes, as well as from
9 shipping and oil spills (Zhang et al., 2004).

10 The combined presence of different pollutants might influence remediation processes (either
11 in bioremediation or in phytoremediation), because different compounds may interact among
12 themselves and/or with plants and their rhizosphere biota. The interactions might cause
13 antagonistic or synergetic effects on metal uptake. Some evidence of such phenomena already
14 exists, despite only a few studies having been carried out in medium contaminated with a
15 mixture of inorganic and organic pollutants, most of them on bioremediation (Amor et al.,
16 2001; Hoffman et al., 2005; Baldrian et al., 2000; Riis et al., 2002). For instance, studies of
17 the influence of trace metals on microbial degradation of aromatic and aliphatic
18 hydrocarbons, either in solution (Amor et al., 2001; Hoffman et al., 2005) or in soil (Baldrian
19 et al., 2000; Riis et al., 2002) have demonstrated biodegradation inhibition caused by
20 inhibition of the microbial activity as a result of metal toxicity (namely of Cd, Cr, Cu, Hg, Ni,
21 Pb and Zn) (Amor et al. 2001; Riis et al. 2002). In addition, Singer et al. (2007) have
22 examined the effects of PAHs on the ability of the hyperaccumulator plant *Alyssum lesbiacum*
23 to phytoextract Ni from co-contaminated soil. They observed that plant growth was

1 negatively affected by PAHs, notwithstanding the lack of significant effect on the
2 phytoextraction of Ni per unit of biomass of shoot.

3 A very recent study has examined the depth profiles of PAHs in salt marsh sediments and
4 observed higher concentration of PAHs in sediments colonised by the salt marsh plant
5 *Sarcocornia fruticosa* than in sediments non-colonised (Martins et al., 2007). Thus, authors
6 have hypothesised that the elevated concentrations of PAHs in colonised sediments might be
7 attributed to the transfer of dissolved PAH compounds towards the roots, and subsequent
8 sequestration onto organically rich particles, as the plant takes up water. Relatively high
9 concentrations of non-polar organic compounds (with hydrophobic characteristics) around
10 roots might influence the extent of interactions between ionic and polar metal ions
11 compounds with roots and, consequently, metal uptake. The literature on this topic is scarce,
12 not only *in situ* and *ex situ* but also *in vitro*. However, it has been shown that, the pH buffer
13 HEPES (N-hydroxyethylpiperazine-N'-ethanesulfonic acid), often used in biological and
14 biochemical studies, increased Cu toxicity for the marine dinoflagellate *Amphidinium*
15 *carterae*, yet HEPES was not toxic (Lage et al., 1996). Surfactant activity of HEPES has been
16 demonstrated (Vasconcelos et al., 1996) and considered as a possible explanation for the
17 influence of the pH buffer on Cu toxicity to *A. carterae*. In addition, several studies have
18 reported the benefits of chelate-assisted phytoextraction, i.e., the use of metal chelators, such
19 as EDTA or histidine, to enhance metal phytoextraction capacity of plants (e.g., Vassil et al.
20 1998; Kramer et al., 1996). For instance, Vassil et al. (1998) observed a significant increase in
21 the accumulation of Pb by the plant *Brassica juncea* due to the direct uptake of the metal
22 complex Pb-EDTA.

23 Therefore, in principle, the presence of organic pollutants may change the mechanisms of
24 metal uptake by plants, thus, changing metal phytoextraction potential.

1 So far, few studies have investigated the efficiency of metal phytoextraction in soils
2 contaminated with mixture of inorganic and organic pollutants (Singer et al., 2007) and
3 studies concerning salt marsh plants are, to our knowledge, non-existent.

4 This study aimed to investigate the effect of PAHs on the uptake of Cu by the salt marsh
5 plant *Halimione portulacoides*. Laboratory experiments were carried out using media
6 prepared with sediment and water retrieved from a salt marsh. Controlled conditions were
7 chosen to facilitate the interpretation of the results. *H. portulacoides* was the plant selected
8 because it is commonly found in Portuguese temperate salt marshes and is capable of
9 accumulating several metals, including Cu, which can be useful for metal phytoextraction
10 (Caçador et al., 2000).

11

12 **2. Experimental Section**

13

14 *2.1. Materials and Reagents*

15

16 To prevent contamination, all sampling and labware materials were soaked in 20 % (v/v)
17 HNO₃ solution for at least 24 h, rinsed several times with bi-deionised water (conductivity <
18 0.1 µS cm⁻¹) and dried in a Class 100 laminar flow hood. Sample manipulation was carried
19 out in a clean room with Class 100 filtered air. Suprapure concentrated HNO₃ and
20 concentrated HCl, both from Merck, were used without further purification. Methanol used
21 was of chromatographic grade from Panreac. All other reagents used were pro analysis grade
22 or equivalent. The stock standard solution containing a mixture of the sixteen EPA priority
23 PAHs (each PAHs at 2 g L⁻¹) in dichloromethane-benzene (1:1 (v:v)) and the stock standard
24 mixture solution of deuterated PAHs containing acenaphthene-d10, chrysene-d12,

1 naphthalene-d8, perylene-d12 and phenanthrene-d10 (each at 2 g L⁻¹) in dichloromethane
2 were obtained from Supelco. The stock Cu standard solution was obtained from BDH
3 (Spectrosol).

4

5 2.2. Samples

6

7 *H. portulacoides* used in the experiments was grown in a greenhouse. For that, the plant was
8 collected at the salt marsh, then the roots and a small part of the stem were cut and the
9 remaining part was placed in dark-walled vases filled with Light Expanded Clay Aggregate
10 (LECA, supplied by MaxitGroup Portugal) and supplied with one quarter strength modified
11 Hoagland nutrient solution (Hoagland and Arnon, 1950) for approximately 2 months to allow
12 new root biomass growth. All plants used in the experiments were of the same age and of
13 similar size. Plants total weight (dry weight) ranged from 1.1 to 3.2 g. Plant roots, stems and
14 leaves represented, respectively, 8-17 %, 52-67 % and 17-35 % of total plant weight.

15 Sediment was collected in the salt marsh area of the Cavado river estuary (41.5228 N;
16 8.7846 W) during the winter season (January) 2007 at low tide in an area colonized by *H.*
17 *portulacoides*. Sediments were retrieved by means of a plastic shovel, placed in individual
18 sterile bags and immediately refrigerated in an ice chest. Simultaneously, estuarine water was
19 also collected in plastic bottles and refrigerated.

20

21 2.3. Experiments

22

1 Experimental design is shown in Fig. 1. Two sets of experiments were carried in January
2 2007: one with sediment elutriate (*set I*), and another with sediment soaked in the respective
3 elutriate (*set II*).

4 Sediment elutriate was obtained by mixing 50 g of wet sediment with 250 ml estuarine
5 water, being vigorously stirred for 30 min with a magnetic stirrer (complemented by manual
6 agitation every 10 min to ensure complete mixing), according to EPA protocols (USEPA,
7 1991). Afterwards, the mixture was left to settle for 12 hours. Then, for *set I*, elutriate was
8 separated from the sediment by decantating and filtering through 0.45 μm pore size filters
9 (cellulose nitrate membrane, Millipore), to remove particulate suspended matter (except
10 colloids) and to reduce the presence of micro-organisms. For *set II* the medium consisted of
11 sediment soaked in the elutriate, as obtained after settling, without further manipulation. In
12 the preparation of elutriates, sediment was used as collected, i.e. with below-ground biomass
13 incorporated, because the sediment was collected in a vegetated area. The
14 separation/elimination of below-ground biomass, mostly plant roots, from the sediment was
15 only possibly through sediment washing which could alter metal fractionation in the
16 sediment. A change in the forms of the metals in the sediment may alter the exchange
17 between water and sediment and influence the obtained results.

18 In each set, media were amended with 0, 10^2 and $10^4 \mu\text{g l}^{-1} \text{Cu}^{2+}$ (as $\text{Cu}(\text{NO}_3)_2$), as well
19 as with 0 and $0.1 \mu\text{g l}^{-1}$ of each of the sixteen EPA priority PAHs (naphthalene,
20 acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene,
21 benz[*a*]anthracene, chrysene, benz[*b*]fluoranthene, benz[*k*]fluoranthene, benz[*a*]pyrene,
22 indeno[1,2,3-*cd*]pyrene, dibenz[*a,h*]anthracene and benz[*ghi*]perylene). As NO_3^- is a nutrient
23 of plants, the addition of NO_3^- to the media may influence plants activity. However, NO_3^-
24 concentration was the same with or without addition of PAHs to the media for each added Cu

1 concentration. Therefore, the added NO_3^- is not expected to influence the results of the present
2 study, which were obtained by comparison between media with the same Cu (and NO_3^-)
3 concentration and with or without addition of PAHs. Cu was added to the medium solution
4 only at the beginning of the experiments, whereas PAHs were added every day throughout the
5 experiment to overcome a possible PAHs removal from solution (e.g. by adsorption and/or
6 degradation). PAHs were added by pipetting a small volume (10 μL) of a working standard
7 solution prepared by dilution of the stock standard solution. The total amount of PAHs added
8 per day was $1.6 \mu\text{g l}^{-1}$. After the addition of pollutants, the solution was manually stirred for
9 homogenous distribution within the medium. Groups of *H. portulacoides* (five plants per
10 experiment) were exposed to the media for six days. Control experiments without plants were
11 also carried out in parallel.

12 Experiments carried out with sediment soaked in the respective elutriate (*set II*) allowed
13 simulation of nutrients and contaminants exchange among plants, solution and sediment, as in
14 the natural environment. Experiments carried out with elutriate alone (*set I*) is a much
15 simplified medium which facilitated the interpretation of results. Solutions are more
16 homogenous media than sediments. In this case there is only exchange of nutrients and
17 contaminants between solution and plant. Sediment elutriates are used in EPA protocols for
18 elutriate sediment toxicity tests in sediment quality trials (USEPA, 1991) as they can give an
19 estimation of the amount of metal that is exchanged between sediment and aqueous phase
20 during re-suspension processes, such as floods, strong tides, regional sea level rise or
21 dredging operations, which frequently occur in estuaries.

22 The $10^2 \mu\text{g l}^{-1}$ Cu concentration corresponded to the higher Cu concentration observed in
23 sediment interstitial water at a site contaminated with Cu (Mucha et al., 2007), i.e. the
24 concentration that could be bioavailable in a site contaminated with Cu. The $10^4 \mu\text{g l}^{-1}$ Cu

1 concentration was chosen because this would be the Cu concentration in solution if all Cu
2 present in the mass of sediment used could be bioavailable during the experiments carried out
3 in elutriates. Although this concentration may not be realistic in terms of environmental
4 pollution, it would probably cause a more marked effect on plants facilitating the attainment
5 of measurable results. In addition, Cu concentration in the sediment used was similar to the
6 ERL (Effect Range Low, which is $35 \mu\text{g/g}_{\text{dry sediment}}$ for Cu), the sediment quality guideline
7 that indicates the concentration above which adverse biological effects may occasionally
8 occur (Long et al., 1995). So, the $10^4 \mu\text{g l}^{-1}$ Cu concentration may be of environmental
9 concern. The PAHs concentration selected (each PAHs at $0.1 \mu\text{g l}^{-1}$) has been set as the
10 Environmental Quality Standard for several organic compounds being of environmental and
11 regulatory concern and of relevance in the Drinking Water Directive and for Water Treatment
12 Plants (Lacorte et al., 2001).

13 During the experiments, plants were subjected to natural day:night regime with natural
14 sunlight. For each experiment each container was wrapped in aluminium foil to prevent light
15 influencing the results, because in the natural medium plant roots are not exposed to direct
16 light. Containers were in a randomized position and were randomly rotated each day. To
17 maintain solution levels during experiments, estuarine water, identical to that used in the
18 preparation of elutriates, was added to the media for compensation of losses, due to
19 evaporation and absorption.

21 *2.4. Samples treatment and chemical analysis*

22
23 Cu and PAHs concentrations were measured in solutions at the beginning and at the end
24 of the experiments, as well as the pH. The initial pH was ca. 6.5 in all media before addition

1 of any of the pollutants. In the media that were amended with $10^4 \mu\text{g l}^{-1}$ Cu (and only in these
2 media), after the addition of Cu, the pH decrease and it was adjusted (with 400 μL of a
3 saturated solution of CaCO_3) to ca. 6.5 at the beginning of the experiments. As the initial Ca
4 concentration in the media was already relatively high (water salinity 2.3) the addition of
5 CaCO_3 did not have significance for nutrient availability. Cu concentrations were also
6 measured in the different tissues (root, stems and leaves) of plants not exposed (initial
7 conditions) and exposed to the different media (final conditions). Cu concentrations in
8 sediment used for the elutriate preparation (initial conditions) and in sediments of the media at
9 the end of the experiments were also measured.

10 For determination of pH, Cu and PAHs in the soluble phase, all solutions were previously
11 filtered through 0.45 μm pore size filters of cellulose nitrate and glass fibre filters,
12 respectively. In *set II*, solutions were firstly separated from sediments by decantation.

13 For determination of Cu in sediments and in the different parts of the plant (which were
14 thoroughly rinsed with bi-deionised water), samples were dried at 30 °C to a constant weight
15 in trays covered with filter paper. Sediments were fractionated and homogenized by sieving
16 through nylon nets of 2 mm. Plants were separated into stems, leaves and roots, which were
17 separately crushed and homogenized. Portions of each dry sample (for plants it was a
18 combination of several plants per experiment) were, afterwards, digested with suitable
19 amounts of concentrated HNO_3 (and 30 % H_2O_2 , only for plant tissues) in closed PTFE
20 vessels using a high-pressure microwave system (MLS-1200 Mega, Milestone) as described
21 before (Almeida et al., 2004). The procedure for sediments only provides total-recoverable
22 metal contents, because it does not allow a complete dissolution of the sample, particularly of
23 silicates. Nevertheless, it has the advantage of being a strong acid digestion that dissolves all
24 elements that could become “environmentally available”.

1 Atomic absorption spectrometry with flame atomisation (AAAnalyst 200, Perkin Elmer)
2 was used for determination of total-recoverable levels of Cu in sediment and plant samples
3 using the procedures described elsewhere (Almeida et al., 2004).

4 Cu concentration in solutions was determined by anodic stripping voltammetry using
5 procedures and conditions described elsewhere (Vasconcelos and Leal, 2001). Voltammetric
6 equipment consisted of an Autolab voltammeter (Ecochemie) connected to a Metrohm 663-
7 VA electrode stand provided with a hanging mercury drop electrode.

8 PAHs in solutions were determined by using a method developed by Evtuygina et al.
9 (2007), which was adapted from the literature (e.g. King et al 2004). Determinations were
10 carried out in a Varian CP-3800 GC provided with a split/splitless injector and a Saturn 2200
11 ion trap mass spectrometric (MS) detection system, after extraction with a solid-phase micro-
12 extraction (SPME) device with fibres coated with a 100 µm thickness polydimethylsiloxane
13 film. The GC-MS determination was carried out in selected ion monitoring mode. All samples
14 were dosed with a known concentration of deuterated PAHs, before extraction. Quantification
15 of PAHs was carried out through response factors obtained from PAHs standards with
16 deuterated PAHs. At least one deuterated PAHs per class of aromaticity was used to
17 determine accurate concentrations for all PAHs. The accuracy of the analytical procedure was
18 checked by spiking aliquots of all filtered solutions from the different media with known
19 amounts of PAHs and determining recovery percentages, which were between 80 and 120 %.

20 Blank solutions were prepared for each type of sample following the respective sample
21 treatment. Three independent replicates of each sample were prepared and analysed and, after
22 blank subtraction, the mean and respective standard deviations were calculated.

23 Statistically significant differences among samples were evaluated through ANOVA
24 tests using SPSS software and Tukey pairwise comparisons were used.

1

2 *2.5. Biological parameters*

3

4 At the end of the experiments, the photosynthetic efficiency and levels of chlorophylls in
5 plants were also determined as plant stress indicators. Photosynthetic parameters were
6 measured fluorometrically using a Diving-PAM (Pulse Amplitude Modulation) device (Walz,
7 Germany). Each pulse was given immediately after putting the leaf under the fibre optic light
8 guide of the Diving-PAM. Photochemical efficiency (as Genty's parameter) was determined
9 as in (Genty et al., 1989). The chlorophyll *a* and *b* and carotenoid contents were determined
10 according to Lichtenthaler and Wellburn (1983). For that, leafs of each sample were taken and
11 placed in covered tubes with 100% methanol over-night at -4°C, macerated in low luminosity
12 conditions, and supernatant samples were subjected to spectrophotometrical absorbance
13 determination at 470, 644.8 and 661.6 nm (Lichtenthaler and Wellburn, 1983).

14 Due to the relatively low biomass available (chemical analysis required most of the
15 available material), biological parameters were only determined in one plant per experiment.

16

17 **3. Results and Discussion**

18

19 *3.1. Biological parameters*

20

21 Although plants have the capacity to degrade or sequester many toxic compounds, they
22 are sensitive to many of them (Davis et al., 2002). The stress response of plants to the
23 presence of pollutants can alter the plants capacity to control the uptake of those pollutants,
24 increasing the uptake and sometimes causing serious problems to the viability of the plant.

1 However, these effects depend on the pollutant, on the plant species and on the concentration
2 and time of exposure to the pollutant (Davis et al., 2002).

3 In a previous study carried out *in vitro* with seawater amended with $5 \times 10^3 \mu\text{g l}^{-1}$ Cu and
4 *H. portulacoides*, during an eight weeks trial, no apparent damage (with no decrease in the
5 *chlorophyll a* and *b* content) in the plant was observed (Reboredo, 1991).

6 As for PAHs, several plants have been shown to tolerate crude oil pollutants in soil.
7 However, the mechanisms of the PAH toxicity to plants are poorly understood, and the
8 phytotoxicity appears to vary depending on the particular PAH and plant species and
9 presently, it is not clear to what extent PAH exposure triggers stress signalling pathways
10 common to other abiotic or biotic stresses in plants, and whether signalling components
11 specific to PAH stress exist (Alkio et al., 2005 and references therein).

12 In the present study, photosynthetic efficiency and levels of chlorophylls were measured
13 (Fig. 2), to be used as plant stress/damage indicators, as plants were in the same mature state
14 (see experimental section). In general, plants were not significantly affected by the addition of
15 pollutants to the medium. The photochemical efficiency of the plants was identical either with
16 or without addition of pollutants (either alone or combined) to the media. Genty's parameter
17 was also identical for plants exposed to the different media. The only exception was for 10^4
18 $\mu\text{g l}^{-1}$ Cu added together with PAHs to the medium with sediment, where photosynthetic
19 efficiency was reduced to nil and photosynthetic pigments decreased significantly, apparently
20 due to serious damages to the plant caused by the treatment. However, this result may be
21 accidental because (a) Cu levels observed in plants of this experiment were similar to those
22 found when Cu was added in the absence of PAHs; and (b) plants exposed to higher amounts
23 of soluble Cu when PAHs were added to the medium (*set I*) did not revealed appreciable
24 damage. Therefore, no significant stress/damage to plants, caused by the addition of

1 pollutants to the medium, was observed after the 6 day exposure period. So, the effects
2 observed in the present work (see below) may not be related to plant damage.

3

4 *3.2. pH in the medium*

5

6 The initial pH was ca. 6.5 in all media. At the end of the experiments pH ranged from 6.0
7 to 7.0, except in *set I* experiments carried out with plants in elutriates not amended with any
8 pollutant and in elutriates amended only with PAHs, where the pH was ca. 4. Plants can
9 exude a variety of compounds such as organic acid anions, phytosiderophores, sugars,
10 vitamins, amino acids, purines, nucleosides and inorganic ions (e.g. HCO_3^- , OH^- , H^+) (Jones,
11 1998). The lower pH values observed in the two mentioned cases were probably caused by
12 root exudation. The fact that pH values did not vary significantly when media were amended
13 with Cu suggests that, in these cases, chemical exchanges among media and roots were of a
14 different nature. Root exudation may be a response to nutrients deficiency or to stress induced
15 by pollutants, being highly variable and dependent on physiochemical environment besides
16 other factors (Jones, 1998).

17

18 *3.3. Cu in medium vs. Cu in plants*

19

20 *3.3.1. Absence of added PAHs*

21 Cu levels were measured at the beginning and after six days in sediments (when present),
22 in solution (subsequently called soluble fraction, although it included also the colloid fraction)
23 and in plant tissues.

1 *Levels of Cu in sediments* (Fig. 3) did not vary significantly ($P>0.05$) with the addition to
2 the medium of $10^2 \mu\text{g l}^{-1}$ of Cu, because the amount of Cu added to the medium ($0.5 \mu\text{g/g}$) did
3 not significantly increase the Cu above the initial level in sediment (ca. $50 \mu\text{g/g}$). However,
4 when the medium was amended with $10^4 \mu\text{g l}^{-1}$, Cu concentration in sediment increased
5 significantly (Fig. 3; $P<0.01$). In this case, exposure of plants, which could accumulate Cu
6 (see below), caused a significant decrease of Cu in the sediment.

7 *Cu concentrations in solution* increased significantly and markedly in all media amended
8 with both $10^2 \mu\text{g l}^{-1}$ and $10^4 \mu\text{g l}^{-1}$ Cu ($P<0.01$). Fig. 4 shows that after the experiments carried
9 out in the absence of plants the soluble Cu was much higher in elutriate (*set I*) than in solution
10 in contact with the sediments (*set II*). This difference is explained by the fact that sediment
11 metals are removed from solution through various processes, such as precipitation as
12 hydroxides, adsorption to Fe and Mn oxides or complexed/adsorbed by a variety of organic
13 (e.g. humic substances) or inorganic (e.g. carbonates) ligands from the sediment (Almeida et
14 al., 2004). Cu sulphide probably did not played a role in the removal of Cu for the following
15 reasons: (a) sediment was collected in a vegetated area (colonized by *H. portulacoides*) and
16 previous studies have indicated that salt marsh plant roots (including *H. portulacoides*,
17 unpublished data) could oxidize the medium, resulting in undetectable levels of acid volatile
18 sulphide (Almeida et al. 2006); (b) in the laboratory at the onset of the experiment, sediment
19 was intensively stirred with estuarine water under aerobic conditions for elutriate preparation,
20 which promoted oxidation of the sulphide that might remain in the sediment. In elutriate
21 experiments (*set I*) only the solubility product of Cu hydroxide and metal
22 adsorption/absorption to colloid particles in suspension could control removal of Cu from
23 solution. Nevertheless, it must be noted that the elutriate (*set I*) amended initially with $10^4 \mu\text{g}$
24 l^{-1} Cu had at the end of the experiments only 6 % of the initially added metal (only ca. $600 \mu\text{g}$

1 I^{-1} Cu was measured in the soluble form, see Fig. 4). This large decrease of Cu concentration
2 in solution can be a disadvantage in hydroponics experiments investigating the effects caused
3 by high concentrations.

4 *Levels of Cu in the tissues of plants* pre-exposed (controls) and after six days exposure are
5 shown in Fig. 5. *H. portulacoides* could accumulate Cu in roots either when it was exposed to
6 elutriate contaminated with Cu (*set I*) or when it was exposed to media of *set II*, in which the
7 sediment contained a relatively high initial concentration of Cu (ca. 50 $\mu\text{g/g}$) before any Cu
8 was added. In addition, the levels of Cu in roots increased with the degree of Cu
9 contamination. Accumulation of Cu in stems was also observed (Fig. 5), with a pattern similar
10 to that found for roots. These results indicate that at least a fraction of the Cu fixed by the
11 plant was absorbed into the roots and translocated to stems in a short period of time (six
12 days). The levels of Cu in leaves did not vary significantly during the experiments, indicating
13 that translocation to the leaves would require a longer exposure period. These results are
14 compatible with those obtained by Reboredo (1991) in hydroponics experiments, carried out
15 in seawater, which indicated that *H. portulacoides* could accumulate Cu not only in its roots
16 and stems but also in its leaves after a period of two weeks exposure. The amount of Cu
17 accumulated per gram of plant tissue was much higher in roots than in stems (ratio root/stem
18 up to 14), confirming that metal in halophyte plants are mainly accumulated in the roots with
19 small quantities translocated to stems and leaves (Almeida et al., 2006; Weis et al., 2002;
20 Windham et al., 2003), except in the case of more mobile elements like Cd, Mn or Zn
21 (Almeida et al., 2006; Caçador et al., 2000). The capability of *H. portulacoides* to accumulate
22 Cu mostly in its roots has been also observed before (Caçador et al., 2000).

23

24 3.3.2. PAH effects

1 PAHs ($0.1 \mu\text{g l}^{-1}$ of each of the sixteen EPA priority PAHs) were added to the medium
2 every day during the six days experiments. Levels of PAHs were measured in elutriate before
3 any addition (control levels) and in all solutions after the experiments.

4 *Levels of PAHs* were only measurable in amended elutriates (*set I*) without plants (Table
5 1), the remaining media displaying values below the limits of detection (included in Table 1).
6 The marked reduction of PAHs in the solution in contact with sediment (*set II*) may result
7 from either adsorption on organic matter present in the sediment or degradation by micro-
8 organisms. In *set II* experiments micro-organisms were present, whereas in *set I* experiments
9 micro-organisms presence was markedly reduced (results not shown). Therefore, the elutriates
10 with measurable PAHs levels had a much lower microbial population than the other
11 conditions, but even in these cases PAH concentrations were much lower than the dosed
12 values indicating either sorption to the container walls or compound degradation by the
13 remaining micro-organisms. In the presence of plants, even in elutriate experiments (*set I*),
14 extensive removal of PAHs from the solution also occurred. For instance, a faster degradation
15 of PAHs has been reported in vegetated soils than in non vegetated soil, which has been
16 mediated by root-associated micro-organisms (Chaudhry et al., 2005). In addition, PAHs
17 could also be adsorbed on the root surface or absorbed into root tissues (Lin et al., 2007)).
18 However, in the current study it is not possible to determine the exact causes contributing to
19 the removal of PAHs from solution.

20 *PAHs influenced the levels of Cu in solution*, when plants were present (Fig 6).
21 Experiments carried out in the presence and absence of PAHs showed that the levels of
22 soluble Cu (normalized for the mass of roots) were significantly lower in the PAH exposed
23 compared with the unexposed when experiments were amended with $0 \mu\text{g l}^{-1}$ and $10^2 \mu\text{g l}^{-1}$
24 added Cu. However, soluble Cu levels were markedly higher ($P < 0.05$) in those amended with

1 $10^4 \mu\text{g l}^{-1}$ Cu. Variations in the pH of solutions, which can influence Cu solubility, were small
2 (maximum difference in pH of solutions with and without addition of PAHs was 0.5, see Fig.
3 6) and, probably, did not influenced the observed differences in Cu concentrations. The
4 effects caused in the soluble fraction by the addition of PAHs were much smaller in the
5 experiments carried out in the presence of sediment (*set II*) than in *set I*, which might result
6 from a higher removal of PAHs from solution, namely due to the adsorption or faster
7 degradation of PAHs in the sediments.

8 In contrast, in the absence of plants, the Cu levels in solution were, in most cases,
9 statistically identical in the media amended and not amended with PAHs (Fig. 4), which
10 indicated that PAHs by themselves did not control Cu solubility. Thus, the observed effects
11 were the result of unknown interactions among PAHs, plants and probably Cu (as the PAHs
12 effects depended on the levels of Cu in the medium).

13 *PAHs did not significantly influence the levels of Cu in sediment* (Fig. 3), probably
14 because the levels of Cu were already relatively high, masking possible small variations
15 caused by PAHs in the presence of plants.

16 *Influence of PAHs on Cu uptake.* A comparison of Fig. 5 and 6 shows that accumulation
17 of Cu by plants in elutriate experiments (*set I*) reflected the pattern of variation of Cu levels
18 observed in solution. Particularly for $10^4 \mu\text{g l}^{-1}$ Cu added, the amounts of metal accumulated
19 either in roots or in stems were significantly and markedly higher when the medium was
20 amended with PAHs, that is, when the soluble Cu was much higher ($P < 0.01$). However, the
21 level of Cu in leaves did not increase in experiments using media with and without PAHs,
22 possibly because of the short exposure time.

23 Together, these results suggest that PAHs can alter the Cu sorption by plants or modify
24 the Cu solubility. For instance, (Mucha et al., 2005) has shown that plants can exude organic

1 compounds capable of complexing metals, which can change metal availability. It is possible
2 that PAHs might control exudation of Cu ligands capable of forming soluble and bioavailable
3 Cu complexes. Dissolved metals in waters and pore waters of soils and sediments are present
4 as free ions (aqua-complexes), or as complexes with organic or inorganic ligands. Increases in
5 metal availability, particularly an enhanced uptake by plants, in the presence of metal
6 complexes have been found (Degryse et al., 2006a and references therein), including Cu
7 (Degryse et al., 2006b). Therefore, a possible role of PAHs in changing exudation deserves
8 investigation. Alternatively, PAHs, may passively penetrate the root cells membranes without
9 any carrier (Alkio et al., 2005), which can then facilitate metal (or a metal complex)
10 penetration into the cell.

11 In the experiments carried out in the presence of sediments, the influence of PAHs in Cu
12 accumulation by plants was not significant (Fig. 5), which is consistent with the small
13 differences in soluble Cu observed among media amended and not amended with PAHs (Fig.
14 6).

15 The present results are novel and indicate that PAHs may influence Cu accumulation by
16 *H. portulacoides*, which can alter the metal phytoextraction potential of this plant. Therefore,
17 combination of different types of pollutants should be taken into consideration when studying
18 the remediation potential of plants, namely in terms of phytoextraction.

19 While the results of *in vitro* experiments, like those of the present study, may help
20 predicting the effects of pollutant mixtures, such as Cu and PAHs, on estuarine salt marshes,
21 it must be recognised that these are short-term experiments and in the natural ecosystem
22 exposure the accumulation of pollutants takes places over much longer periods of time. As a
23 result, validation of the results obtained in *in vitro* studies, like these, should be carried out in
24 the field.

1

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3

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1 **Caption for figures:**

2

3 **Fig. 1.** Experimental design of the work with each experimental treatment carried out in
4 triplicate.

5

6 **Fig. 2.** Photosynthetic pigments (chlorophyll *a* (Chl *a*) and *b* (Chl *b*) and carotenoids (C + X))
7 and photosynthetic efficiency (Genty's parameter) after plant exposure both to elutriate (*set I*)
8 and sediment soaked in elutriate (*set II*), amended with 0, 10² and 10⁴ µg l⁻¹ Cu, as well as
9 with 0 and 1.6 µg l⁻¹ of total PAHs concentration. Lighter bars: 0 µg l⁻¹ PAHs; Darker bars:
10 1.6 µg l⁻¹ total PAHs concentration. Mean and standard deviation are shown.

11

12 **Fig. 3.** Cu concentration (mean and standard deviation) observed in sediments used for
13 elutriate preparation (white bar) and in sediments at the end of the experiments (grey and
14 black bars) amended with 0, 10² and 10⁴ µg l⁻¹ Cu, as well as with 0 µg l⁻¹ (grey bars) and 1.6
15 µg l⁻¹ (dark bars) total PAHs concentration, in experiments without or with plants.

16

17 **Fig. 4.** Cu concentration (mean and standard deviation) observed in solution after the
18 experiments without plants. Experiments were carried out both in elutriate (*set I*) and
19 sediment soaked in elutriate (*set II*), amended with 0, 10² and 10⁴ µg l⁻¹ Cu, as well as with 0
20 µg l⁻¹ (grey bars) and 1.6 µg l⁻¹ (dark bars) total PAHs concentration.

21

22 **Fig. 5.** Cu concentrations (mean and standard deviation) observed in the different tissues of
23 plants in the experiments carried out both in elutriate (*set I*) and sediment soaked in elutriate
24 (*set II*), amended with 0, 10² and 10⁴ µg l⁻¹ Cu, as well as with 0 µg l⁻¹ (grey bars) and 1.6 µg

1 l^{-1} (dark bars) total PAHs concentration. Determinations were carried out in pre-exposed
2 plants (white bars) and after six days exposure (grey and black bars).

3

4 **Fig. 6.** Cu concentration (mean and standard deviation (error propagation)) observed in
5 solution after plant exposure, normalized for the mass of plant's roots. Experiments were
6 carried out both in elutriate (*set I*) and sediment soaked in elutriate (*set II*), amended with 0,
7 10^2 and $10^4 \mu\text{g l}^{-1}$ Cu, as well as with $0 \mu\text{g l}^{-1}$ (grey bars) and $1.6 \mu\text{g l}^{-1}$ (dark bars) total PAHs
8 concentration. The pH of solutions are also included.

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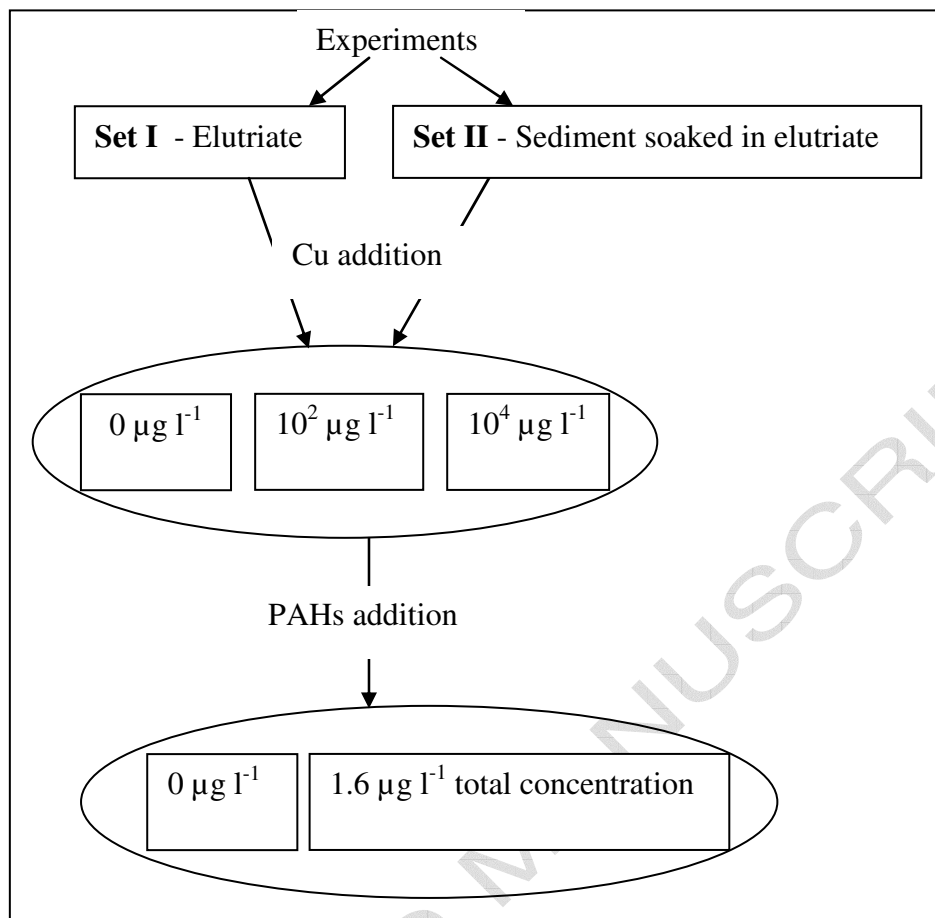


Fig. 1.

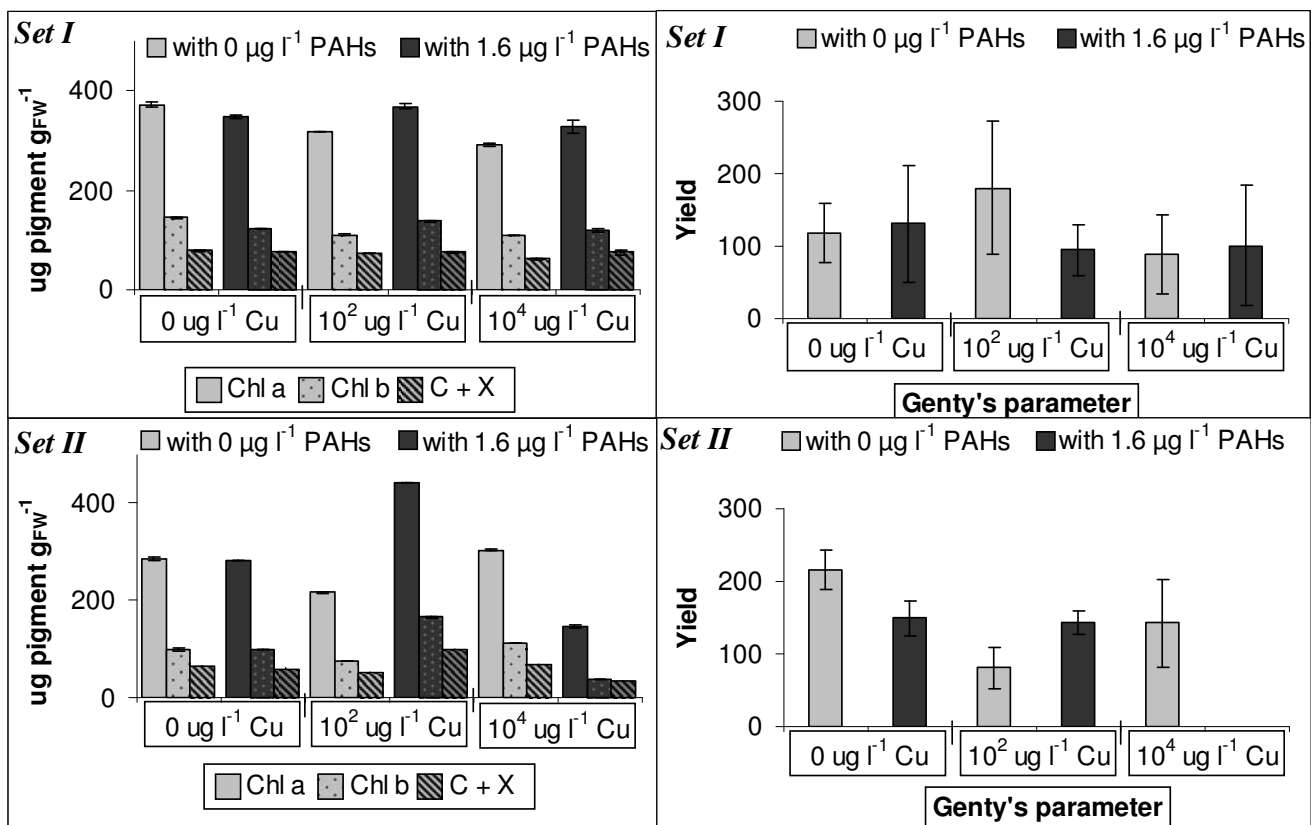


Fig. 2.

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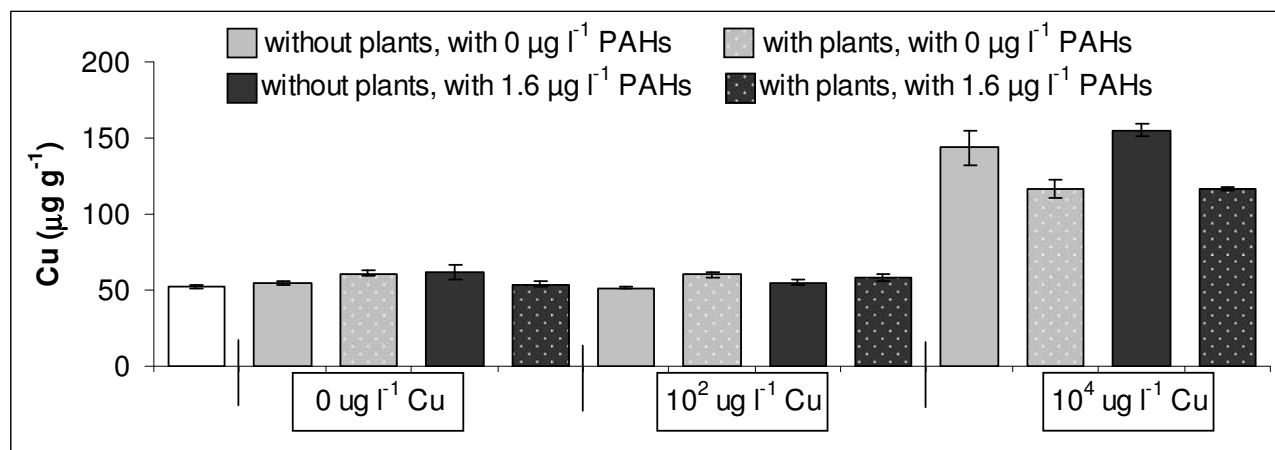


Fig. 3.

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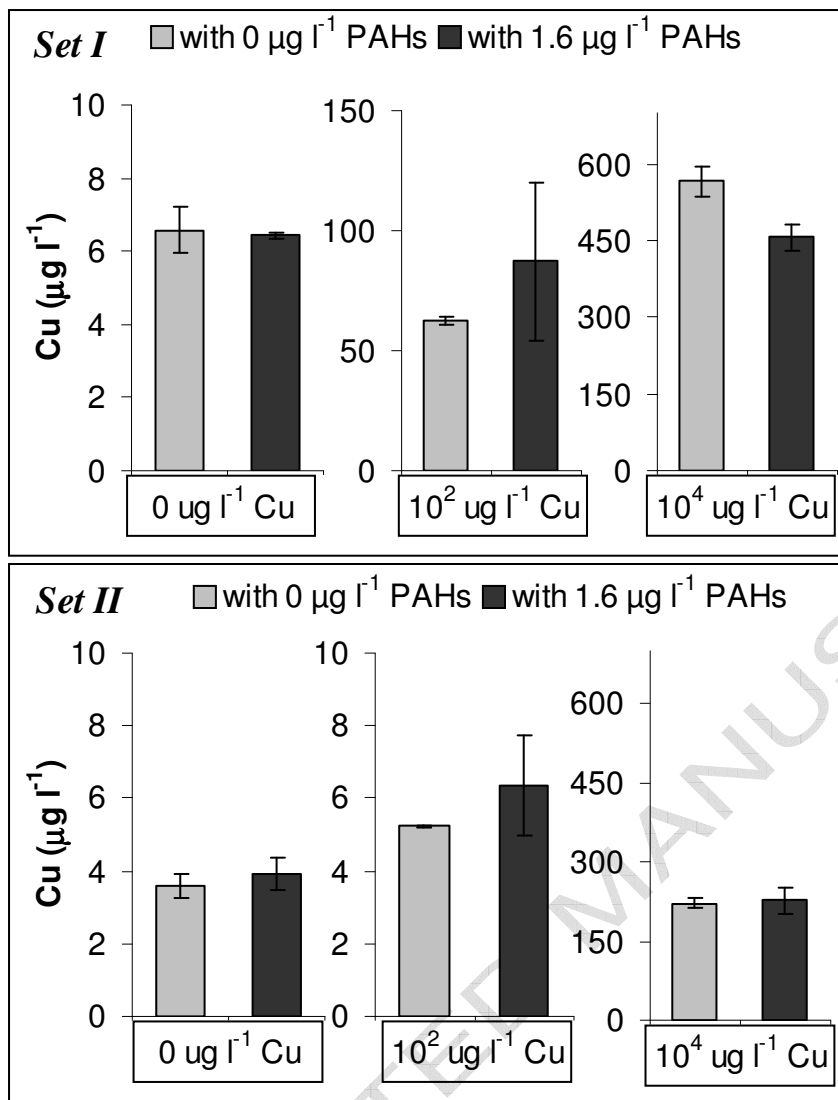


Fig. 4.

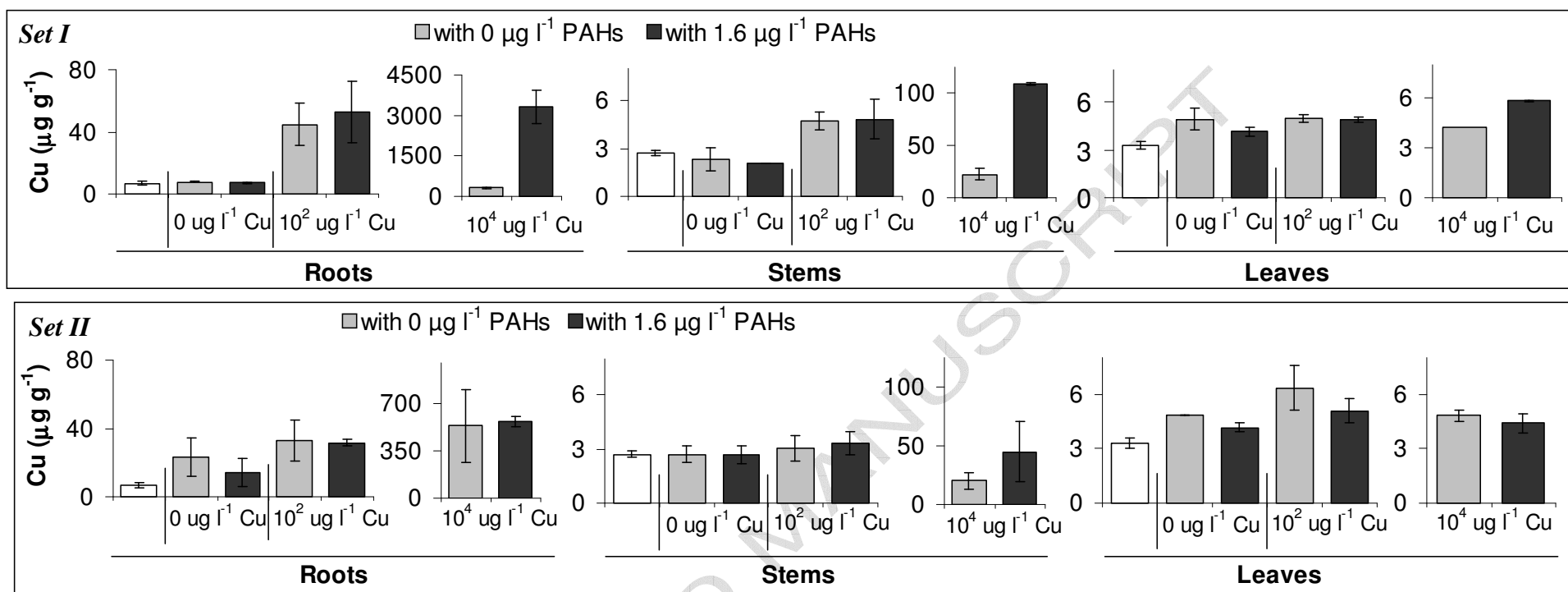


Fig. 5.

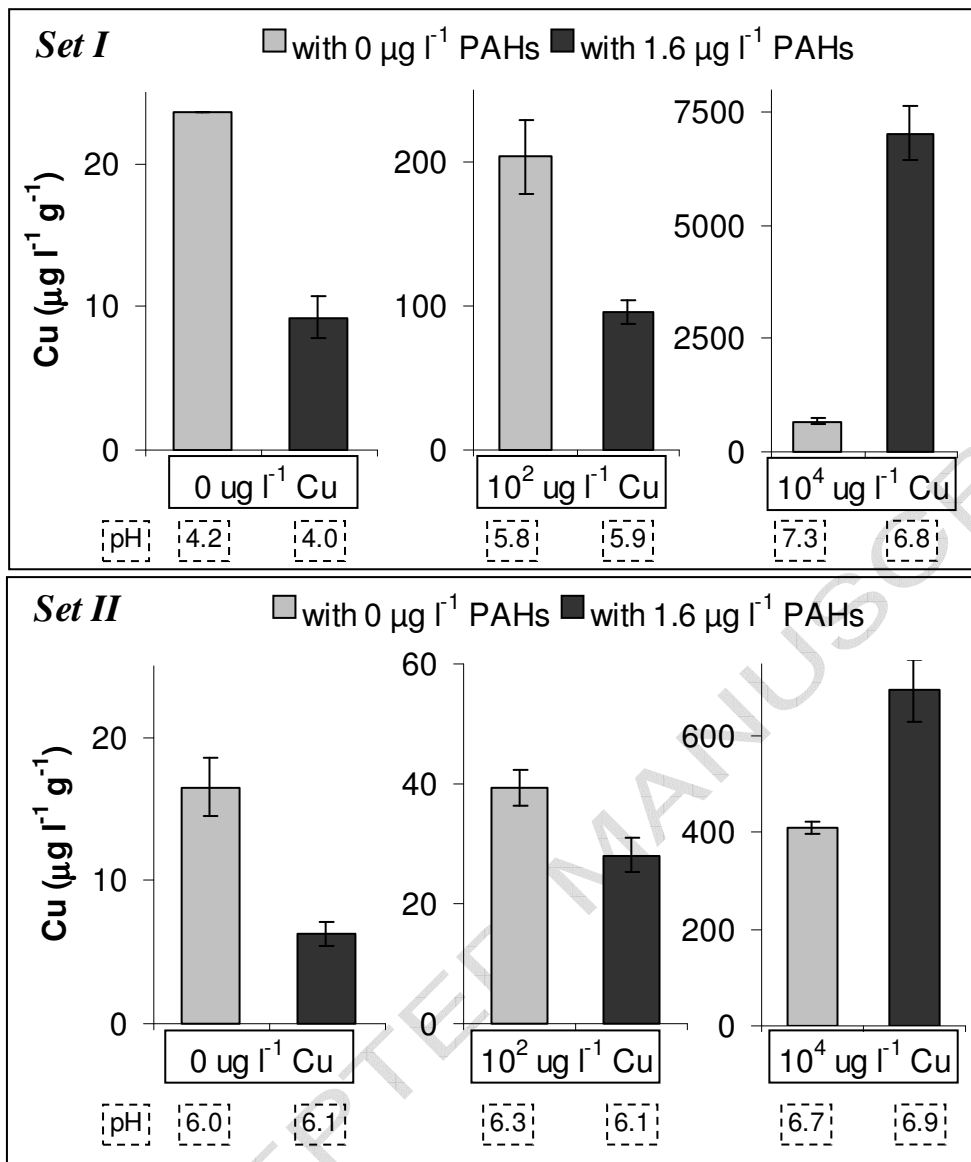


Fig. 6.

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Table 1

PAHs concentrations (ng l^{-1} , mean and standard deviation (in parenthesis, $n=3$)) measured in elutriate without plants and without sediments (*set I*, Fig. 1). After all the experiments with plants and after the experiments with sediments all sixteen EPA priority PAHs were below limits of detection (LD, included in the Table)

	LD	Added Cu concentration		
		$0 \mu\text{g l}^{-1}$	$10^2 \mu\text{g l}^{-1}$	$10^4 \mu\text{g l}^{-1}$
naphthalene	52	< LD	< LD	< LD
acenaphthylene	29	< LD	31 (2)	< LD
acenaphthene	9	16 (2)	17 (2)	< LD
fluorene	10	23 (1)	27 (2)	15.2 (0.2)
phenanthrene	19	32 (1)	39 (2)	32.3 (0.6)
anthracene	30	< LD	< LD	< LD
fluoranthene	7	45 (6)	43 (2)	35 (3)
pyrene	5	45 (7)	40 (2)	36.9 (0.4)
chrysene	20	28 (6)	27 (7)	33 (10)
benz[a]anthracene	12	40 (4)	31 (1)	29 (2)
benz[k]fluoranthene	16	28.7 (03.)	28 (3)	22.5 (0.5)
benz[b]fluoranthene	26	26.4 (0.9)	26 (4)	< LD
benz[a]pyrene	44	< LD	< LD	< LD
Indeno[1,2,3- <i>cd</i>]pyrene	28	< LD	35 (4)	< LD
dibenz[a,h] anthracene	24	< LD	24 (3)	< LD
benz[ghi]perylene	13	29.0 (0.2)	38 (3)	< LD