



# Phytochelatin synthesis in response to Hg uptake in aquatic plants near a chlor-alkali factory



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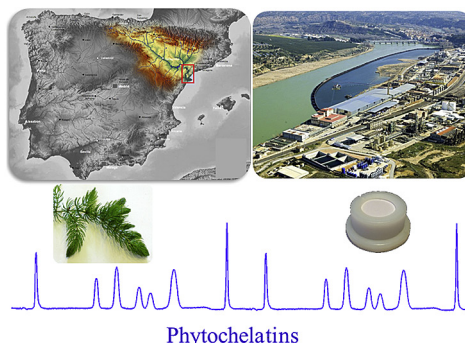
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## HIGHLIGHTS

- The effect of a Hg-rich industrial waste in Ebro River macrophytes was studied.
- Total Hg was analyzed in plants, sediments and suspended matter.
- The use of DGT allowed the estimation of the bioavailable Hg.
- PCs were detected at low levels with decreasing content downstream from the hot spot.
- PCs production by macrophytes is an indicator of the bioavailable Hg in the river.

## GRAPHICAL ABSTRACT



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## ABSTRACT

The effects of mercury (Hg) released from a chlor-alkali factory in aquatic plants along the Ebro River basin (NE Spain) were analysed considering the phytochelatin (PC<sub>n</sub>) and their isoforms content in these plants. These compounds were analyzed using HPLC with amperometric detection, and the macrophytes species *Ceratophyllum demersum* and *Myriophyllum spicatum* were collected in two sampling campaigns, autumn and spring, respectively. To correlate the PC<sub>n</sub> content in macrophytes with the Hg contamination, analysis of total Hg (THg) content in plants and suspended particulate matter, as well as the dissolved-bioavailable fraction of Hg in water measured by the diffusive gradient in thin film (DGT) technique were done. The results confirm the presence of PC<sub>2</sub>-Ala in extracts of *C. demersum* and PC<sub>2</sub>-desGly in *M. spicatum*, and the concentration of these thiol compounds depends clearly on the distance between the hot spot and the downstream sites: the higher the levels are, the closer the hot spot is. Since most of the Hg is hypothesized to be associated with SPM and transported downstream, our results of the DGT suggest that trace amounts of Hg in water can be released as free metal ions yielding a certain accumulation in plants (reaching the ppb level) that are enough for activation of induction of PCs. A few PCs species have been determined, at different seasons, indicating that they can be used as good indicators of the presence of bioavailable Hg in aquatic media throughout the year.

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## 1. Introduction

The effect that trace metal contamination has on terrestrial and

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aquatic ecosystems is a matter of major concern. Some trace toxic metals, like mercury (Hg), are capable of increasing in concentration upward through the food chain, reaching high levels in top predators (Morel et al., 1998). Then, an interesting point to be considered in contamination studies is the negative effect that pollutants have on living organisms. It is well known that some mammals, plants, algae and some fungi have the capability to synthesize molecules to prevent the harmful effects produced by metals stress. In the case of plants, algae and some fungi, phytochelatin (PC<sub>n</sub>) synthesis is considered necessary to tightly regulate the distribution of metal and to minimize damage under excess metal supply conditions. Phytochelatins are small cysteine-rich peptides, which play an essential role in heavy metal detoxification by chelating metals through thiol groups in the cytosol and transporting the complexes formed to the vacuoles. The general structure of PC<sub>n</sub> is (γ-Glu-Cys)<sub>n</sub>-Gly (n = 2 to 5). Glutathione (GSH) serves as the substrate of PC<sub>n</sub> biosynthesis through the transpeptidation of the γ-Glu-Cys moiety of GSH onto a second GSH to form PC<sub>2</sub> or onto a PC<sub>n</sub> molecule to produce an n+1 oligomer (Stillman et al., 1992; Bordin, 2000; Cobbett, 2000; Cobbett and Goldsbrough, 2002; Riordan and Vallee, 1991; Suzuki et al., 1993). By other hand, PC<sub>n</sub> synthesis depends not only on the plant species but also of the metal stressor, being Cd the best activator followed by Ag, Bi, Pb, Zn, Cu, Hg and Au cations (Bundy et al., 2014; Dago et al., 2014b). This relationship of PC<sub>n</sub> with trace metal contamination allows us to use PC<sub>n</sub> content as an indicator of metal pollution. In the analysis of PC<sub>n</sub> and their Hg complexes different methodologies have been applied. Among them the use of MS-HPLC is the most common, although detection by fluorescence or absorption spectroscopy is also considered (Serrano et al., 2015). In a previous work of our research group, a method to determine glutathione, PC<sub>n</sub> and their Hg complexes using amperometric detection in a glassy carbon electrode has been developed (Dago et al., 2009, 2011). This method has been applied to study the effect that Hg and other metal ions have in the synthesis of PCs in *Hordeum vulgare* plants cultured in controlled conditions in the lab in the presence of these toxic substances (Dago et al., 2014a, 2014b) or in *Asparagus acutifolius* plants growing naturally in the mining district of Almadén (Dago et al., 2014c). These studies determined PC<sub>n</sub> and their Hg-PC complexes in plants and established a correlation between the metal content in the growing media and the level of synthesized PC<sub>n</sub>. On the other hand, some PC related compounds can also be synthesized simultaneously by plants; these compounds, also called iso-phytochelatins, are structural variants of PC<sub>n</sub> that differ from them in one of their constituent aminoacids. Four families have been described, (γ-Glu-Cys)<sub>n</sub>-β-Ala, (γ-Glu-Cys)<sub>n</sub>-Ser, (γ-Glu-Cys)<sub>n</sub> or (γ-Glu-Cys)<sub>n</sub>-Glu, that together with the phytochelatins described above form the metallothioneins class III (Grill et al., 1987; Rauser, 1995; Zenk, 1996). Another methodology, also based in HPLC with amperometric detection, has been proposed in a previous work of our research group for the determination of the isoforms of PC<sub>2</sub>, which, in principle, are the most abundant in natural samples (Dago et al., 2015).

In the present work, a case of residue disposal from a chlor-alkali electrochemical plant sited in one bank of the Flix reservoir on the lower Ebro River (NE Spain) is considered. For more than 100 years, large amounts (ca. 3.5 10<sup>5</sup> t) of hazardous industrial waste (e.g. metals and organochlorine pollutants) containing high concentrations of Hg (up to 400 mg kg<sup>-1</sup>) were dumped in front of the dam riverbank causing a strong contamination (Palanques et al., 2014; Esbrí et al., 2015; Carrasco et al., 2011a; Navarro et al., 2009). In order to control the contaminant sludge effects and to remove the sludges, a retaining wall was built in 2012 around the sludge deposit. Nowadays, sludges are still being removed and transported to a controlled dumping area. A way to evaluate their impact on living

organisms of the Ebro River could be through the analysis of PC<sub>n</sub> and related compounds synthesized by macrophytes growing in the river, since they are good biomarkers for heavy metal stress. It is interesting to remark that most of the environmental studies related with this factory were devoted to the effects of Hg exposure in the aquatic environment (e.g. fish species, crayfish, molluscs) (Carrasco et al., 2008, 2011a; 2011b; Navarro et al., 2009), or focused on atmospheric Hg and its incorporation in soils and lichens (Esbrí et al., 2015), however, determination of PC<sub>n</sub> and related compounds in aquatic plants has never been reported.

Recently, there is a growing interest in the use of aquatic macrophytes in the abatement of heavy metal pollution and as sentinel organisms of pollution in aquatic ecosystems (Rezania et al., 2016). Among them, the submerged species are particularly useful in the monitoring of heavy metals (Rai, 2009). In this sense, since *Ceratophyllum demersum* and *Myriophyllum spicatum* are invasive submerged aquatic plants and the dominant species growing in the Ebro River basin, they were selected for investigation in this work. Both have a large capacity to adsorb metal ions and by this reason both can potentially be used to remove metals from the aquatic media (Abdallah, 2012; Keskinan et al., 2007; Milojkovic et al., 2014). However, as far as we know, studies of PC synthesis by these plants are scarce, only Mishra et al. (2006) determine the PC<sub>n</sub> induced in *C. demersum* plants exposed to different levels of lead, and the presence of PC<sub>2</sub> and PC<sub>3</sub> was reported.

Thus, the aim of this work is to evaluate the toxic effect that upstream Hg contamination by the industrial waste and the wall built have on downstream macrophytes of this basin. In order to accomplish this, we carried out a field study in the low Ebro River basin, where two submerged macrophytes were collected and phytochelatin production was reported as a measure of the metal stress response. In order to correlate the PC<sub>n</sub> synthesized with Hg concentration in the media, total Hg in aquatic plants, in suspended particulate matter and in sediment samples, as well as the dissolved-bioavailable fraction of Hg have also been reported.

## 2. Materials and methods

### 2.1. Chemicals

Glutathione (GSH) was obtained from Merck (Darmstadt, Germany). Phytochelatins (γ-Glu-Cys)<sub>n</sub>-Gly (n = 3–5), as trifluoroacetate salts, were provided by DiverDrugs S.L. (Barcelona, Spain) with a purity ranging from 86.2% to 99.0%. Phytochelatin 2 (PC<sub>2</sub>, (γ-Glu-Cys)<sub>2</sub>-Gly) and its isoforms (PC<sub>2</sub>desGly ((γ-Glu-Cys)<sub>2</sub>), PC<sub>2</sub>Ala ((γ-Glu-Cys)<sub>2</sub>-Ala), PC<sub>2</sub>Glu ((γ-Glu-Cys)<sub>2</sub>-Glu) and CysPC<sub>2</sub> (Cys-(γ-Glu-Cys)<sub>2</sub>-Gly)) were provided by Genosphere Biotechnologies (Paris, France) with a purity of 95%.

For preparing the mobile phase, acetonitrile from Panreac (Barcelona, Spain), NaCl, formic acid and KOH from Merck and trifluoroacetic acid (TFA) from Sigma-Aldrich (St. Louis, MO, USA) were used. Methanol from Merck was used for cleaning the column, and ethanol (96% purity) from Panreac was used for cleaning the glassy carbon electrode. EDTA (from Merck) was used for cleaning plants before storing at –80 °C.

For plant extract preparation, cleaning the column and preparation of all solutions, ultrapure filtered water (18.2 MΩ cm<sup>-1</sup>) obtained from a Synergy UV equipment from Merck Millipore (Darmstadt, Germany) was used.

The materials and reagents employed for the preparation of the in-house manufactured DGT gels were acrylamide solution (40%), electrophoresis grade (Fisher Scientific); DGT gel cross-linker, 2% aqueous solution (DGT Research Ltd., UK); ammonium peroxydisulfate, certified A.C.S., 99% (Fisher); N,N,N',N'-tetramethylethylenediamine (TEMED) ReagentPlus, 99% (Sigma-

Aldrich); and 3-mercaptopropyl-functionalized silica gel (Aldrich). Whatman 0.45  $\mu\text{m}$  pore size, 25 mm in diameter nylon membranes were used as filters to protect the diffusive gel, and plastic DGT solution deployment mouldings (3.14  $\text{cm}^2$  window) (DGT Research Ltd., UK) were used to support and enclose all the layers.

## 2.2. Study area

In order to cover the entire lower Ebro River (NE Spain) (Fig. 1), from the Flix reservoir to the Ebro Delta (approximately 125 km), 5 sampling sites were selected, based on accessibility and abundance of aquatic plants. The entire monitored area has vast ecological, agricultural and recreational values. Of special interest is the Ebro Delta (320  $\text{km}^2$ ), which contains productive rice fields (210  $\text{km}^2$ ) and wetlands (80  $\text{km}^2$ ), and is rich in waterfowl and fisheries. The Ebro Delta is on the List of Wetlands of International Importance, designated under the Ramsar Convention. The strip of coastal land on the delta plain has various designations of protection: it is a Natural Park, a PEIN (Plan for Areas of Natural Interest) site, a Natura 2000 site, and it includes several nature reserves and several wetlands included on the Wetland Inventory of Catalonia. These natural values support important economic activities associated with tourism, hunting, fishing and aquaculture.

Although it would be interesting to sampling inside the area limited by the retaining wall, the entrance is prohibited and it was unmanageable to collect samples. The Flix meander (FM) is the sample point nearer to the contamination focus, located immediately downstream of the Flix dam; while Ascó (AS), Xerta (XT) and Deltebre (DT) sites are in consecutive sections of the river, separated by different overflow dams, located at 15, 65 and 110 km downstream of Flix, respectively. The Riba-roja dam (RB), which is used as a reference site, is located 15 km upstream of Flix, and forms a large water reservoir.

## 2.3. Plant, sediment and water sampling

Submerged aquatic plants (i.e. *C. demersum* and *M. spicatum*) are cosmopolitan species, colonising mainly eutrophic stagnant and flowing waters (Germ et al., 2006). They exhibit a similar life form, having similar “mesh-like” architecture (i.e. branched stems and finely dissected leaves arranged in whorls around the main axis) (Rovira et al., 2016), and previous studies also showed similar adsorption capabilities for metals (Keskinan et al., 2007). Their proliferation is different during the year, while *C. demersum* dominates in autumn and *M. spicatum* in spring. Since both macrophyte

species grow in the same habitat (i.e. sampling point) and have similar plant architecture, we consider that comparisons between seasonal surveys during both the autumn and spring are feasible, because we would expect similar responses. Thus, in October 2014 (autumn sampling campaign), aquatic plants of the species *C. demersum* and water samples were sampled, and where it was possible river sediments were also collected. Plant species *M. spicatum* and water samples were collected in April 2015 (spring sampling campaign). Several physico-chemical parameters of sampling media (i.e. temperature, conductivity, total dissolved solids (TDS) and pH) were measured at each sampling point with a Hydrolab DS5 multisensor probe (Hach Environmental; Loveland, CO, USA). Obtained results are shown in Table 1. The analysis of the variance of these values shows that in the case of temperature and pH values there is not a significant difference between values measured at the different sampling points but a significant difference between both campaigns exists. Related with conductivity and TDS a similar behaviour is observed, except for DT point where the salinity increases as a consequence of the proximity of this point to the sea. Collected plants and water samples were preserved in a fridge and transported to the laboratory along the same day.

## 2.4. Sample preparation

Once plant samples were in the laboratory, they were cleaned with ultrapure filtered water and with a solution of EDTA 0.1  $\text{mol L}^{-1}$  to remove superficial adsorbed metals, afterward they were accurately dried with filter paper and cut into small pieces. Samples were ground with liquid nitrogen in a mortar and stored at  $-80\text{ }^\circ\text{C}$  until analysis.

Sediments were ground and homogenized with an agate mortar and pestle and sieved through mesh to obtain a particle size lower than 200  $\mu\text{m}$ .

Suspended particulate matter (SPM) was obtained passing 1 L of water through a cellulose acetate membrane filter (pore size 0.45  $\mu\text{m}$ , Albet, Sant Joan Despí, Spain).

## 2.5. Analysis of phytochelatins by HPLC-ED

Prior to HPLC analysis, 100 mg of fresh plant sample were mixed with 500  $\mu\text{L}$  of ultrapure filtered water at 1500 rpm for 1 h in an Eppendorf MixMate (Hamburg, Germany) and filtered through 0.45  $\mu\text{m}$  Nylon filter discs (Osmonics, Minnetonka, MN, USA).

An Agilent 1200 chromatographic system (Agilent, Santa Clara, CA, USA) equipped with a quaternary pump, an automatic injector and a vacuum degasser were used. An Ascensis C18 5  $\mu\text{m}$  particle size analytical column measuring 25  $\text{cm} \times 4.6\text{ mm}$  was provided by Supelco (Bellefonte, PA, USA). For the separation of phytochelatins (GSH,  $\gamma$ -Glu-Cys, and  $\text{PC}_{2-5}$ ), a mobile phase consisted of 0.1% TFA in ultrapure filtered water pH = 2.00 and 0.1% TFA in acetonitrile was used. Separation was performed with gradient elution, as described in Dago et al., 2011. For the analysis of PC isoforms ( $\text{PC}_2$ -desGly,  $\text{PC}_2$ -Ala,  $\text{PC}_2$ -Glu and Cys- $\text{PC}_2$ ), a mobile phase consisted of 1% of formic acid with 0.1  $\text{mol L}^{-1}$  of NaCl in ultrapure filtered water pH = 2.00 and 1% of formic acid in acetonitrile was used with isocratic elution (96:4, aqueous solution:organic solution) (Dago et al., 2015). Because larger  $\text{PC}_n$  were not present, the method to determine  $\text{PC}_2$  isoforms was modified to include the separation of  $\text{PC}_3$  by using gradient elution starting at 4% of organic solvent for 16 min then increasing to 20% during 6 min and keeping it constant until a total analysis time of 25 min. The injected volume was 20  $\mu\text{L}$  and the flow rate was 1.2  $\text{mL min}^{-1}$ . Amperometric detection was performed in an electrochemical flow cell from Bioanalytical Systems, Inc. (BASi, West Lafayette, IN, USA) controlled by a potentiostat  $\mu\text{Autolab Type III}$  (Eco Chemie, Utrecht, The Netherlands).

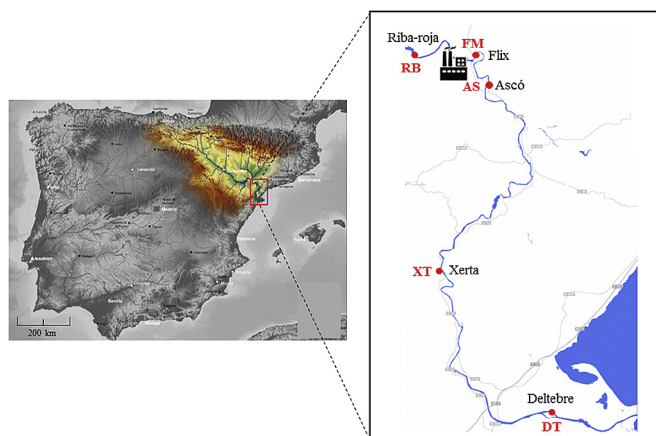


Fig. 1. Sampling points in the Ebro River (Spain).

**Table 1**

Physico-chemical parameters of river water in the different sampling points and sampling campaigns. Sites: RB Riba-roja dam; FM Flix meander; AS Ascó; XT Xerta; and DT Deltebre.

|  | October 2014 |      |      |      |      | April 2015 |      |      |      |      |
|--|--------------|------|------|------|------|------------|------|------|------|------|
|  | RB           | FM   | AS   | XT   | DT   | RB         | FM   | AS   | XT   | DT   |
| Temperature (°C)                       | 19.7         | 19.7 | 21.8 | 22.2 | 22.2 | 15.7       | 17.9 | 16.6 | 18.3 | 18.2 |
| Conductivity ( $\mu\text{S cm}^{-1}$ ) | 1365         | 1335 | 1367 | 1348 | 1961 | 783        | 773  | 796  | 804  | 1530 |
| TDS ( $\text{g L}^{-1}$ )              | 0.88         | 0.85 | 0.87 | 0.86 | 1.25 | 0.50       | 0.49 | 0.51 | 0.51 | 0.98 |
| pH                                     | 7.74         | 7.83 | 7.88 | 7.92 | 7.96 | 8.22       | 8.47 | 8.26 | 8.22 | 8.58 |

The flow cell consists on a glassy carbon working electrode (BASi) whose surface was daily polished, a stainless steel auxiliary electrode and an Ag/AgCl ( $\text{NaCl } 3 \text{ mol L}^{-1}$ ) reference electrode. The optimized potential for the working electrode was 1.2 V.

## 2.6. Dissolved Hg in water

For the determination of the dissolved-bioavailable fraction of Hg in water, the diffusive gradient in thin film (DGT) technique was used as a passive sampling technique. The principle of the DGT technique is based on the diffusion of the dissolved species through a membrane-diffusive layer and their accumulation in an ion-exchange resin (binding phase). These two layers are separated from the solution to be analysed by a filter membrane (usually  $0.45 \mu\text{m}$ ), and are enclosed and sealed in a small plastic device, so that only the filter is exposed to the deployment solution. In solutions with no ligands, the time-averaged concentration of the metal in the solution,  $C$ , can be calculated according to the Fick's first law of diffusion as:

$$C = \frac{M\Delta g}{DA t}$$

where  $D$  ( $D = 1.60 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ ) is the diffusion coefficient of the metal in the diffusive layer,  $t$  is the deployment time,  $A$  the exposure surface area, and  $\Delta g$  the thickness of the diffusive layer. The mass ( $M$ ) of the analyte accumulated by the resin is experimentally measured and provides the average labile metal concentration during the exposure time. The preparation of the DGT gels and assembly are described in previous works (Fernández-Gómez et al., 2011, 2014).

In-house manufactured polyacrylamide DGT samplers were deployed in triplicate for 7 days, in all sampling sites, approximately 1–2 m above the sediment-water interface (between 3 and 5.5 m of river depth). The sampling device used to hold and suspend the samplers consisted of a homemade cylindrical basket made of a plastic net. Finally, the basket was anchored to a rope with a weight on one end and a buoy on the other. After retrieval, DGT units were rinsed with distilled water and kept in polyethylene bags for the transport to the laboratory. Once in the laboratory, DGT units were dismantled and the resin gel was extracted and analyzed for dissolved-bioavailable Hg content.

## 2.7. Analysis of total mercury concentration

Samples of plants, sediments, suspended particulate matter, and the resin gel from DGTs, were analyzed using an advanced mercury analyzer AMA-254 manufactured by Altec (Prague, Czech Republic) and distributed by Leco (St. Joseph, MI, USA), which is based on catalytic combustion of the sample, preconcentration by gold amalgamation, thermal desorption and atomic absorption spectrometry (AAS). The entire analytical procedure was validated by analyzing CRM from the National Research Council Canada (NRCC; Ottawa) (DORM-2:  $4.64 \pm 0.26 \mu\text{g g}^{-1}$  and DORM-3:

$0.382 \pm 0.060 \mu\text{g g}^{-1}$ ) at the beginning and end of each set of samples, ensuring that the instrument remained calibrated during the course of the study. The concentrations of Hg obtained for repeated analyses ( $n = 5$ ) of both CRM were in good agreement to the certified values. The absolute detection limit was 10 pg, and detection limit given as treble standard deviation of Hg content in blank samples was  $0.1 \text{ ng g}^{-1}$ .

## 2.8. Statistical methods

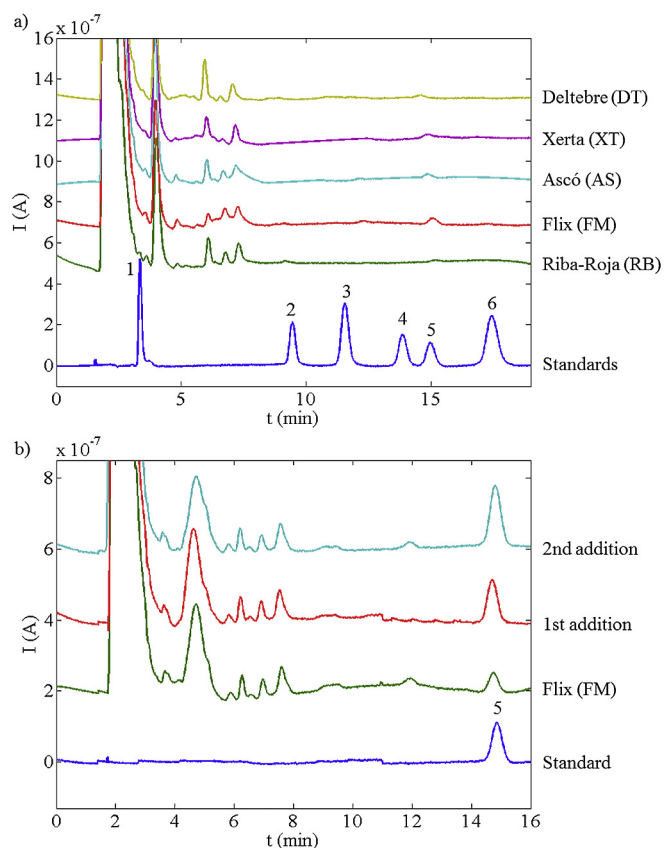
The results for each sample were calculated as the mean  $\pm$  the standard deviation from triplicate determinations. Normal distributions were obtained based on the Kolmogorov–Smirnov test for THg concentrations in aquatic plants and SPM, whereas data in DGT were not normally distributed. The student's t-test or the analysis of the variance were used to compare parametric data, and Mann-Whitney U and Kruskal-Wallis tests to compare non-parametric data. Pearson's linear correlation and regression analysis were used to establish the relationships between the variables (THg in plants, THg in SPM and THg in DGTs). Statistical significance was defined as  $p \leq 0.05$ . The experimental results were statistically evaluated using the IBM SPSS version 23 (Chicago, IL).

## 3. Results and discussion

### 3.1. Phytochelatin analysis

As it has been said above, a way to follow heavy metal stress response in plants is to analyse the synthesized phytochelatins. The analysis of *C. demersum* extracts corresponding to the autumn sampling campaign was done first using as mobile phase 0.1% TFA in ultrapure filtered water pH = 2.00 and 0.1% TFA in acetonitrile applying a gradient elution. Regarding the obtained chromatograms (results not shown) the presence of longer  $\text{PC}_n$  ( $\text{PC}_4$  and  $\text{PC}_5$ ) was discarded. By this reason, we decided to focus on the analysis of smaller thiols using as mobile phase formic acid with  $0.1 \text{ mol L}^{-1}$  of NaCl in ultrapure filtered water pH = 2.00 and 1% of formic acid in acetonitrile applying the elution profile described above. Using this methodology, chromatograms indicated the highest concentration of  $\text{PC}_2$ -Ala close to the hot spot in Flix and decreasing when moving away from the contaminant focus (Fig. 2a). To better ascertain the presence of this thiol compound, samples were spiked with different concentrations of standard and analysed. Data confirmed the presence of  $\text{PC}_2$ -Ala in the samples as the peak increases when increasing the spiked concentration of standard (Fig. 2b). The quantification of  $\text{PC}_2$ -Ala was done using external calibration curve and analyzing three independent replicates. The obtained results were  $116 \pm 6 \text{ nmol g}^{-1}$ ,  $77 \pm 2 \text{ nmol g}^{-1}$  and  $70.1 \pm 0.5 \text{ nmol g}^{-1}$  fresh weight, for the sampling sites FM, AS and XT, respectively. In RB and in the Ebro River mouth in DT,  $\text{PC}_2$ -Ala was non-quantifiable.

Only  $\text{PC}_2$ -Ala could be detected and determined, and its concentration decreases from the source of contamination (FM, highest value) to the estuary (DT, non-quantifiable).

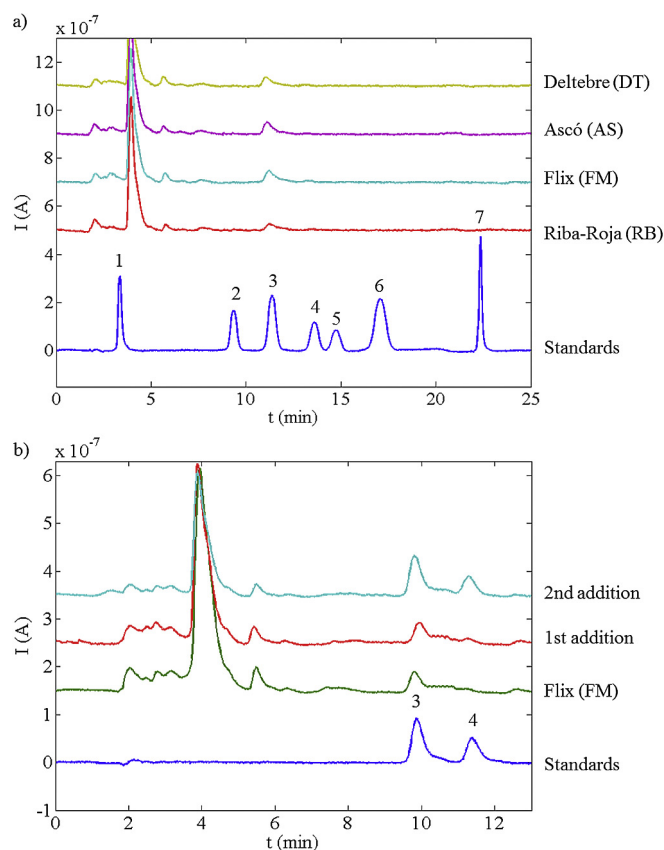


**Fig. 2.** a) Chromatograms of extracts of *C. demersum* and standards of thiols ( $6 \times 10^{-5} \text{ mol L}^{-1}$ ): 1: GSH, 2: PC<sub>2</sub>, 3: PC<sub>2</sub>-desGly, 4: PC<sub>2</sub>-Glu, 5: PC<sub>2</sub>-Ala and 6: Cys-PC<sub>2</sub>. b) chromatograms from Flix (FM) with two additions of 10 and 25  $\mu\text{L}$ , respectively, of PC<sub>2</sub>-Ala  $10^{-4} \text{ mol L}^{-1}$ . Mobile phase of 1% formic acid and 0.1  $\text{mol L}^{-1}$  of NaCl at a pH of 2 and 1% of formic acid in acetonitrile (96:4).

The second sampling campaign was done in spring and the sampled aquatic plant species was *M. spicatum*. No plants were found in XT site, so only four sampling points were considered. In this case, we proceed directly to the analysis of samples using formic acid-NaCl-water (pH = 2.00)/formic acid – acetonitrile as mobile phase (Fig. 3a). The presence of PC<sub>2</sub>-desGly could be clearly detected and confirmed by the addition of standard (Fig. 3b). A small peak at the retention time of PC<sub>2</sub>-Glu seemed to appear in the chromatogram obtained from FM samples, whose presence was also confirmed by spiking the sample with the standard (Fig. 3b). While quantifying the thiols, only PC<sub>2</sub>-desGly could be determined with values of  $63 \pm 3 \text{ nmol g}^{-1}$ ,  $170 \pm 3 \text{ nmol g}^{-1}$ ,  $154 \pm 2 \text{ nmol g}^{-1}$  and  $122 \pm 2 \text{ nmol g}^{-1}$  fresh weight, for the sampling sites RB, FM, AS and DT, respectively. PC<sub>2</sub>-Glu could only be detected in the FM samples but it was non quantifiable.

These results show a low content in PC<sub>n</sub> in comparison with those obtained in other studies of the research group (Dago et al., 2014a, 2014c) in which plants grown in media with very high levels of Hg. Nonetheless, some PC<sub>n</sub> (PC<sub>2</sub> and PC<sub>3</sub>) were found in *C. demersum*, growing in the lab and stressed with lead, at levels of the same order as those found in this work (Mishra et al., 2006). We can conclude that the available Hg uptake by the macrophytes may be low as compared to media with higher content of available metal but still sufficient to promote a noticeable synthesis of PC<sub>n</sub>.

Regarding this aquatic plant species a relatively high concentration of PC<sub>2</sub>-desGly could be found in all sampling stations with the highest concentration at the hot spot and decreasing downstream until the estuary. The presence of PC<sub>2</sub>-Glu could also be



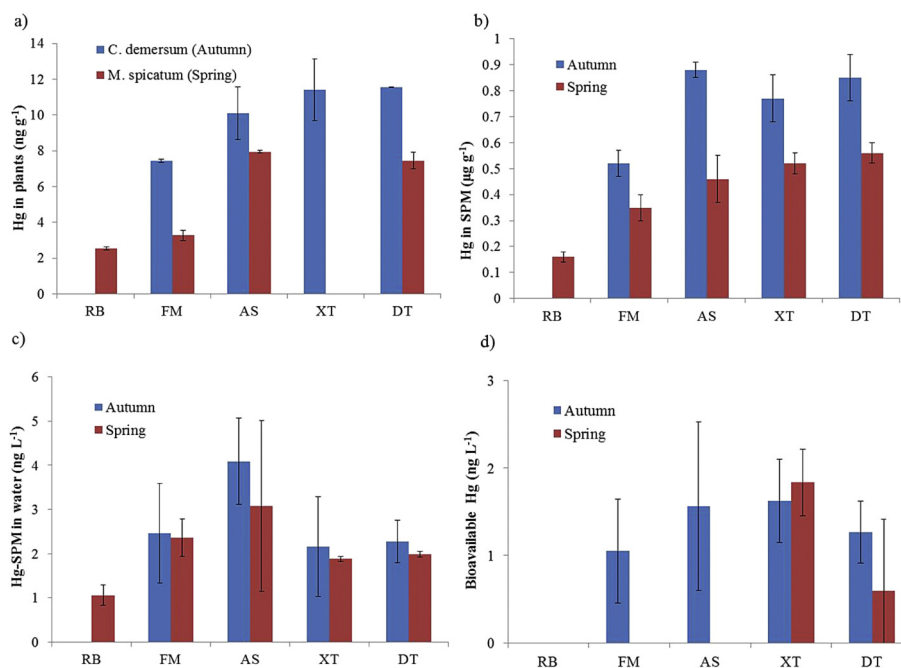
**Fig. 3.** a) Chromatograms of extracts of *M. spicatum* and standards of thiols ( $6 \times 10^{-5} \text{ mol L}^{-1}$ ): 1: GSH, 2: PC<sub>2</sub>, 3: PC<sub>2</sub>-desGly, 4: PC<sub>2</sub>-Glu, 5: PC<sub>2</sub>-Ala, 6: Cys-PC<sub>2</sub> and 7: PC<sub>3</sub>. Mobile phase of 1% formic acid and 0.1  $\text{mol L}^{-1}$  of NaCl at a pH of 2 and 1% of formic acid in acetonitrile with a gradient elution. b) Chromatograms of extracts of *M. spicatum* sampled in Flix (FM) with two additions of 120 and 320  $\mu\text{L}$ , respectively, of a mixture of PC<sub>2</sub>-desGly and PC<sub>2</sub>-Glu  $10^{-4} \text{ mol L}^{-1}$ , and standards ( $6 \times 10^{-5} \text{ mol L}^{-1}$ ) of 3: PC<sub>2</sub>-desGly and 4: PC<sub>2</sub>-Glu. Mobile phase of 1% formic acid and 0.1  $\text{mol L}^{-1}$  of NaCl at a pH of 2 and 1% of formic acid in acetonitrile (96:4).

detected, but not quantified because signals are below LOQ.

### 3.2. Hg concentrations in river samples

Total concentration of Hg in plants, THg in suspended particulate matter and dissolved-bioavailable Hg in water using DGTs are presented in Fig. 4. In this figure the THg concentration in particles are expressed in contaminant mass per dry weight of particles ( $\mu\text{g g}^{-1}$ ) and per volume of river water ( $\text{ng L}^{-1}$ ).

The values of Hg in SPM are 2 and 5 orders of magnitude higher than Hg in aquatic plants and in DGTs respectively. These data show that THg is significantly higher ( $P < 0.05$ ) in plants collected in autumn (*C. demersum*) than in spring surveys (*M. spicatum*) (Fig. 4a). These values are in good agreement with the significantly ( $P < 0.05$ ) different content of Hg also observed between seasons for SPM (Fig. 4b). Moreover, a clear trend was observed for Hg levels found in SPM with values increasing with distance from the hot spot (FM) to downstream sites (AS, XT and DT) in both seasons. Considering THg in plants (Fig. 4a), a similar behaviour is also observed. Indeed, statistically significant differences ( $P < 0.05$ ) in Hg concentrations were observed for *M. spicatum* between site FM and all the downstream sites, whereas no statistical differences were found for *C. demersum*. Some of these facts can be related to the two flood events occurred just before the autumn sampling campaign, when *C. demersum* was collected. Dams in the upper part



**Fig. 4.** Diagrams comparing THg in plants ( $\text{ng g}^{-1}$ ) (a), THg in suspended particulate matter per mass weight of particles ( $\mu\text{g g}^{-1}$ ) (b), THg in suspended particulate matter per volume of water ( $\text{ng L}^{-1}$ ) (c) and dissolved-bioavailable Hg in water ( $\text{ng L}^{-1}$ ) using DGT devices (d) for the two sampling campaigns at different sampling points: Riba-roja (RB); Flix meander (FM); Ascó (AS); Xerta (XT) and Deltebre (DT).

of the lower Ebro River alter the downstream flow regime, since during the scouring out of deposited sediments, a downstream impact is expected. Accordingly, accumulated contaminants in sediments could be mobilized downstream during flushing operations (Kirchner et al., 2000). This occurs when an excess of rain made it necessary to open the dams of Riba-roja and Flix reservoirs. The opening of both reservoir dams dramatically increased the water flow of Ebro River downstream and consequently the amount of re-suspended particle matter as well as Hg residues associated with it. Therefore, suspended solids present in the water column varied across sampling periods and sites, decreasing during the periods of lower water flow (i.e. April 2015) and increasing during the autumn. So, observed high levels of suspended solids in autumn are related to the high flow regimen in comparison to that of the Ebro River in spring (normal/low flow). Furthermore, after the opening of dams, the concentration of THg in SPM is similar during the course of the river and toward the river downstream (Fig. 4b, AS, XT and DT), because the Hg associated with sediments coming from upstream are flooding downstream. On the other hand, it could be observed that the amount of Hg per volume of water is decreasing (Fig. 4c), because the amount of SPM that goes further is lower at far away sites.

These results are in the same line as those obtained previously (Carrasco et al., 2011a; Navarro et al., 2009). Maximum levels of THg in liver, kidney and muscle of feral carp (*Cyprinus carpio*), as well as the highest biological impact (i.e. increased concentration of reduced glutathione in liver and on mRNA expression of two metallothionein genes, MT1 and MT2), did not occur at the discharge sites, but several kilometres downstream.

These data are influenced by the concentration of SPM in the river water in each sampling point. As stated above, the quasi-stationary plateau reached by Hg in SPM (Fig. 4b) is consequence of the similar concentration of THg associated with the SPM flushing down after opening the gates of the dams. Fig. 4c shows the levels of THg in SPM per liter of water, indicating a maximum at the AS sampling point in both campaigns, and a decrease in

particulate matter in the water at the XT and DT sites.

Related with sediments, they were only sampled and analysed in two sampling points (FM and AS) in the first campaign. Values of Hg in sediments ( $0.32 \pm 0.08 \mu\text{g g}^{-1}$  in FM and  $0.67 \pm 0.08 \mu\text{g g}^{-1}$  in AS) were of similar order of magnitude than those found in SPM. On the other hand, values of the dissolved-bioavailable fraction of Hg were in the ppt range in both campaigns (Fig. 4d). During the spring campaign, DGT devices were lost (theft or vandalized) in several stations; therefore, unfortunately, only two sampler devices (at XT and DT) were measured during this season. Although direct comparison of the dissolved-bioavailable Hg by DGT with the bioavailable Hg incorporated by the plant is not possible, the low levels of Hg measured by DGT match very well with the scarce varieties and low levels of PC<sub>n</sub> found in plants. It must be pointed out that according to typical distribution of Hg species in river waters (Morel et al., 1998; Boszke et al., 2002), Hg mostly forms insoluble hydroxides, which are surely incorporated to SPM and sediments justifying the very low levels of bioavailable Hg found in water. The significant pH difference between both campaigns (around 0.5 pH units) does not change this distribution (Morel et al., 1998). Thus, during periods of low stream flow, sediment accumulates in the bed load of the river or the reservoir, and adsorbs Hg that becomes highly enriched in sediment. During high-flow events, this Hg enriched sediment (e.g. iron hydroxide sediment) (Rytuba, 2000) is transported downstream producing a high flux of Hg that can be a significant source of bioavailable Hg depending on site-specific conditions (availability of sulphate-reducing bacteria, electron donors, organic carbon, pH, and salinity).

Considering both sampling periods, the THg content in plants had only statistically significant correlations with THg in SPM ( $r = 0.828$ ;  $p = 0.008$ ). When exploring correlations at the different seasons, it was found, that in autumn, the THg content in plants correlates with THg in SPM ( $r = 0.576$ ) and THg in DGT ( $r = 0.471$ ), although these correlations were no significant ( $P > 0.05$ ). On the other hand, in spring, due to the loss of devices, the sample size

disables to give reliable statistical results for DGT. Moreover, the rest of variables do not correlate at all between them.

These results suggest that the THg in plants were influenced by the content in SPM, and the bioavailable Hg measured with DGT is very similar in all cases.

#### 4. Conclusions

In 2012, a retaining wall was built to contain large amounts of Hg-rich industrial waste in Ebro River. Due to the restricted access to the dumping area to obtain samples for Hg determination, the present study focused on the presence of Hg outside the wall to assess the efficiency of this barrier to prevent the release of Hg to sites downstream during removal operations. Results suggest a non-negligible Hg discharge, since relatively high levels of this metal are present in SPM (at the ppm level), which is adsorbed and transported downstream onto particulate phases.

As for the evolution of the Hg-SPM content, it increases downstream while the amount of Hg per volume of water is decreasing, because SPM can be transported away (especially during high-flow events), but tends to settle before reaching longer distances.

Measurements of low values (at the ppt level) of dissolved-bioavailable Hg in water (Hg-DGT), indicate that trace amounts of Hg adsorbed onto SPM can be released as free metal ions yielding a certain accumulation in plants (reaching the ppb level). These trace amounts of dissolved-bioavailable Hg are enough for activation of PC<sub>n</sub>, at different seasons, indicating that PCs can be used as good indicators of the presence of bioavailable metal in aquatic media throughout the year.

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#### References

- Abdallah, M.A.M., 2012. Phytoremediation of heavy metals from aqueous solutions by two aquatic macrophytes, *Ceratophyllum demersum* and *Lemna gibba* L. *Environ. Technol.* 33(1), 609–1614.
- Bordin, G. Guest (Ed.), 2000. *Cell. Mol. Biol.*, vol. 46 (Special Issue on Metallothioneins).
- Boszke, L., Glosinska, G., Siepak, J., 2002. Some aspects of speciation of mercury in a water environment. *Pol. J. Environ. Stud.* 11, 285–298.
- Bundy, J.G., Kille, P., Liebeke, M., Spurgeon, D.J., 2014. Metallothioneins may not be enough – the role of phytochelatin in invertebrate metal detoxification. *Environ. Sci. Technol.* 48, 885–886.
- Carrasco, L., Barata, C., Garcia-Berthou, E., Tobias, A., Bayona, J.M., Díez, S., 2011a. Patterns of mercury and methylmercury bioaccumulation in fish species downstream of a long-term mercury-contaminated site in the lower Ebro River (NE Spain). *Chemosphere* 84, 1642–1649.
- Carrasco, L., Benjam, L., Benito, J., Bayona, J.M., Díez, S., 2011b. Methylmercury levels and bioaccumulation in the aquatic food web of a highly mercury-contaminated reservoir. *Environ. Int.* 37, 1213–1218.
- Carrasco, L., Díez, S., Soto, D.X., Catalan, J., Bayona, J.M., 2008. Assessment of mercury and methylmercury pollution with zebra mussel (*Dreissena polymorpha*) in the Ebro River (NE Spain) impacted by industrial hazardous dumps. *Sci. Total Environ.* 407, 178–184.
- Cobbett, C.S., 2000. Phytochelatin and their roles in heavy metal detoxification. *Plant Physiol.* 123, 825–832.
- Cobbett, C., Goldsbrough, P., 2002. Phytochelatin and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol.* 53, 159–182.
- Dago, A., González-García, O., Ariño, C., Díaz-Cruz, J.M., Esteban, M., 2009. Liquid chromatographic analysis of Hg(II) binding by thiol-rich peptides using both UV-vis and electrochemical detection. *J. Chromatogr. A* 1216, 6752–6757.
- Dago, A., González-García, O., Ariño, C., Díaz-Cruz, J.M., Esteban, M., 2011. Characterization of Hg(II) binding with different length phytochelatin using liquid chromatography and amperometric detection. *Anal. Chim. Acta* 695, 51–57.
- Dago, A., Ariño, C., Díaz-Cruz, J.M., Esteban, M., 2014a. Analysis of phytochelatin and Hg-phytochelatin complexes in *Hordeum vulgare* plants stressed with Hg and Cd: HPLC study with amperometric detection. *Intern. J. Environ. Anal. Chem.* 94, 668–678.
- Dago, A., González, I., Ariño, C., Díaz-Cruz, J.M., Esteban, M., 2014b. Chemometrics applied to the analysis of induced phytochelatin in *Hordeum vulgare* plants stressed with various toxic non-essential metals and metalloids. *Talanta* 118, 201–209.
- Dago, A., González, I., Ariño, C., Martínez-Coronado, A., Higuera, P., Díaz-Cruz, J.M., Esteban, M., 2014c. Evaluation of mercury stress in plants from the Almadén mining district by analysis of phytochelatin and their Hg complexes. *Environ. Sci. Technol.* 48, 6256–6263.
- Dago, A., Navarro, J., Ariño, C., Díaz-Cruz, J.M., Esteban, M., 2015. Carbon nanotubes and graphene modified screen-printed carbon electrodes as sensitive sensors for the determination of phytochelatin in plants using liquid chromatography with amperometric detection. *J. Chromatogr. A* 1409, 210–217.
- Esbrí, J.M., López-Berdonces, M.A., Fernández-Calderón, S., Higuera, P., Díez, S., 2015. Atmospheric mercury pollution around a chlor-alkali plant in Flix (NE Spain): an integrated analysis. *Environ. Sci. Pollut. Res.* 22, 4842–4850.
- Fernández-Gómez, C., Dimock, B., Hintelmann, H., Díez, S., 2011. Development of the DGT technique for Hg measurement in water: comparison of three different types of DGT samplers in laboratory assays. *Chemosphere* 85, 1452–1457.
- Fernández-Gómez, C., Bayona, J.M., Díez, S., 2014. Comparison of different types of diffusive gradients in thin films samplers for measurement of dissolved methylmercury in freshwaters. *Talanta* 129, 486–490.
- Germ, M., Mazej, Z., Gaberščik, A., Trošt Sedej, T., 2006. The response of *Ceratophyllum demersum* L. and *Myriophyllum spicatum* L. to reduced, ambient, and enhanced ultraviolet-B radiation. *Hydrobiologia* 570, 47–51.
- Grill, E., Winnacker, E.-L., Zenk, M.H., 1987. Phytochelatin, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. *Proc. Natl. Acad. Sci. U. S. A.* 84, 439–443.
- Keskinkan, O., Goksu, M.Z.L., Yuceer, A., Basibuyuk, M., 2007. Comparison of the absorption of *Myriophyllum spicatum* and *Ceratophyllum demersum* for zinc, copper and lead. *Eng. Life Sci.* 7, 192–196.
- Kirchner, J.W., Feng, X., Neal, C., 2000. Fractal stream chemistry and its implications for contaminant transport in catchments. *Nature* 403, 524–527.
- Milojkovic, J.A., Mihajlovic, M.L., Lopovic, Z.R., Petrovic, M.S., Sostaric, T.D., Ristic, M.D., 2014. Pb(II) removal from aqueous solution by *Myriophyllum spicatum* and its compost: equilibrium, Kinetic and thermodynamic study. *J. Chem. Tech. Biotech.* 89, 662–670.
- Mishra, S., Srivastava, S., Tripathi, R.D., Kumar, R., Seth, C.S., Gupta, D.K., 2006. Lead detoxification by coontail (*Ceratophyllum demersum* L.) involves induction of phytochelatin and antioxidant system in response to its accumulation. *Chemosphere* 65, 1027–1039.
- Morel, F.M.M., Kraepiel, A.M.L., Amyot, M., 1998. The chemical cycle and bioaccumulation of mercury. *Annu. Rev. Ecol. Syst.* 29, 543–566.
- Navarro, A., Quiros, L., Casado, M., Faria, M., Carrasco, L., Benjam, L., Benito, J., Díez, S., Raldua, D., Barata, C., Bayona, J.M., Pina, B., 2009. Physiological responses to mercury in feral carp populations inhabiting the low Ebro River (NE Spain), a historically contaminated site. *Aquat. Toxicol.* 93, 150–157.
- Palanques, A., Grimalt, J., Belzunces, M., Estrada, F., Puig, P., Guillén, J., 2014. Massive accumulation of highly polluted sedimentary deposits by river damming. *Sci. Total Environ.* 497–498, 369–381.
- Rai, P.K., 2009. Heavy metal phytoremediation from aquatic ecosystems with special reference to macrophytes. *Crit. Rev. Env. Sci. Tec.* 39, 697–753.
- Rausser, W.E., 1995. Phytochelatin and related peptides. *Plant. Physiol.* 109, 1141–1149.
- Rezania, S., Taib, S.M., Md Din, M.F., Dahalan, F.A., Kamyab, H., 2016. Comprehensive review on phytotechnology: heavy metals removal by diverse aquatic plants species from wastewater. *J. Hazard. Mater.* 318, 587–599.
- Riordan, J.F., Vallee, B.L., 1991. Metallobiochemistry Part B. Metallothionein and related molecules. In: *Methods in Enzymology*, vol. 205. Academic Press: New York, New York.
- Rovira, A., Alcaraz, C., Trobajo, R., 2016. Effects of plant architecture and water velocity on sediment retention by submerged macrophytes. *Freshw. Biol.* 61, 758–768.
- Rytuba, J.J., 2000. Mercury mine drainage and processes that control its environmental impact. *Sci. Total Environ.* 260, 57–71.
- Serrano, N., Díaz-Cruz, J.M., Ariño, C., Esteban, M., 2015. Recent contributions to the study of phytochelatin with an analytical approach. *Trends Anal. Chem.* 73, 129–145.
- Stillman, M.J., Shaw III, C.F., Suzuki, K.T., 1992. *Metallothioneins, Synthesis, Structure and Properties of Metallothioneins, Phytochelatin and Metal-thiolate Complexes*. VCH, New York.
- Suzuki, K.T., Imura, N., Kimura, M., 1993. *Metallothionein III*. Birkhauser, Basel.
- Zenk, M.H., 1996. Heavy metal detoxification in higher plants—a review. *Gene* 179, 21–30.