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

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Abstract

The effects of mercury (Hg) released from a chlor-alkali plant factory in aquatic plants along the Ebro River basin (NE Spain) were analysed considering the phytochelatins (PCs) and their isoforms content in these plants. These compounds were analyzed using HPLC with amperometric detection, and the macrophytes species *Ceratophyllum demersum* and *Myriopyllum spicatum* were collected in two sampling campaings, autumn and spring, respectively. To correlate the PC 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_2-Ala in extracts of *C. demersum* and PC_2-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.

1. Introduction

The effect that trace metal contamination has on terrestrial and 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 mammalians, plants, algae and some fungi have the capability to synthetize molecules to prevent the harmful effects produced by metals stress. In the case of plants, algae and some fungi, phytochelatin (PC_n) 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 cythosol and transporting the complexes formed to the vacuoles. The general structure of PC_n is $(\gamma\text{-Glu-Cys})_n$-Gly (n=2 to 5). Glutathione (GSH) serves as the substrate of PC_n biosynthesis through the transpeptidation of the γ-Glu-Cys moiety of GSH onto a second GSH to form PC_2 or onto a PC_n 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_n 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 2014b). This relationship of PC_n with trace metal contamination allows us to use PC_n content as an indicator of metal pollution. In the analysis of PC_n 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_n 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_n 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_n. 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_n that differ from them in one of their constituent aminoacids. Four families have been described, $(\gamma\text{-Glu-Cys})_n$-β-Ala, $(\gamma\text{-Glu-Cys})_n$-Ser, $(\gamma\text{-Glu-Cys})_n$ or $(\gamma\text{-Glu-Cys})_n$-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_2, 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.* $3.5\ 10^5$ t) of hazardous industrial waste (e.g. metals and organochlorine pollutants) containing high concentrations of Hg (up to 400 mg kg^{-1}) 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_n and related compounds synthetized 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_n 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; Keskinkan 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_n induced in *C. demersum* plants exposed to different levels of lead, and the presence of PC_2 and PC_3 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_n synthetized 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)$_n$-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_2, (γ-Glu-Cys)$_2$-Gly) and its isoforms (PC_2desGly ((γ-Glu-Cys)$_2$), PC_2Ala ((γ-Glu-Cys)$_2$-Ala), PC_2Glu ((γ-Glu-Cys)$_2$-Glu) and CysPC_2 (Cys-(γ-Glu-Cys)$_2$-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 ºC.

For plant extract preparation, cleaning the column and preparation of all solutions, ultrapure filtered water (18.2 MΩ cm^{-1}) 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

171 (TEMED) ReagentPlus, 99% (Sigma-Aldrich); and 3-mercaptopropyl-functionalized
172 silica gel (Aldrich). Whatman 0.45 µm pore size, 25 mm in diameter nylon membranes
173 were used as filters to protect the diffusive gel, and plastic DGT solution deployment
174 mouldings (3.14 cm^2 window) (DGT Research Ltd., UK) were used to support and
175 enclose all the layers.
176
177 ***2.2. Study area***
178 In order to cover the entire lower Ebro River (NE Spain) (Figure 1), from the Flix
179 reservoir to the Ebro Delta (approximately 125 km), 5 sampling sites were selected,
180 based on accessibility and abundance of aquatic plants. The entire monitored area has
181 vast ecological, agricultural and recreational values. Of special interest is the Ebro Delta
182 (320 km^2), which contains productive rice fields (210 km^2) and wetlands (80 km^2), and
183 is rich in waterfowl and fisheries. The Ebro Delta is on the List of Wetlands of
184 International Importance, designated under the Ramsar Convention. The strip of coastal
185 land on the delta plain has various designations of protection: it is a Natural Park, a
186 PEIN (Plan for Areas of Natural Interest) site, a Natura 2000 site, and it includes several
187 nature reserves and several wetlands included on the Wetland Inventory of Catalonia.
188 These natural values support important economic activities associated with tourism,
189 hunting, fishing and aquaculture.
190 Although it would be interesting to sampling inside the area limited by the retaining
191 wall, the entrance is prohibited and it was unmanageable to collect samples. The Flix
192 meander (FM) is the sample point nearer to the contamination focus, located
193 immediately downstream of the Flix dam; while Ascó (AS), Xerta (XT) and Deltebre
194 (DT) sites are in consecutive sections of the river, separated by different overflow dams,
195 located at 15, 65 and 110 km downstream of Flix, respectively. The Riba-roja dam
196 (RB), which is used as a reference site, is located 15 km upstream of Flix, and forms a
197 large water reservoir.
198

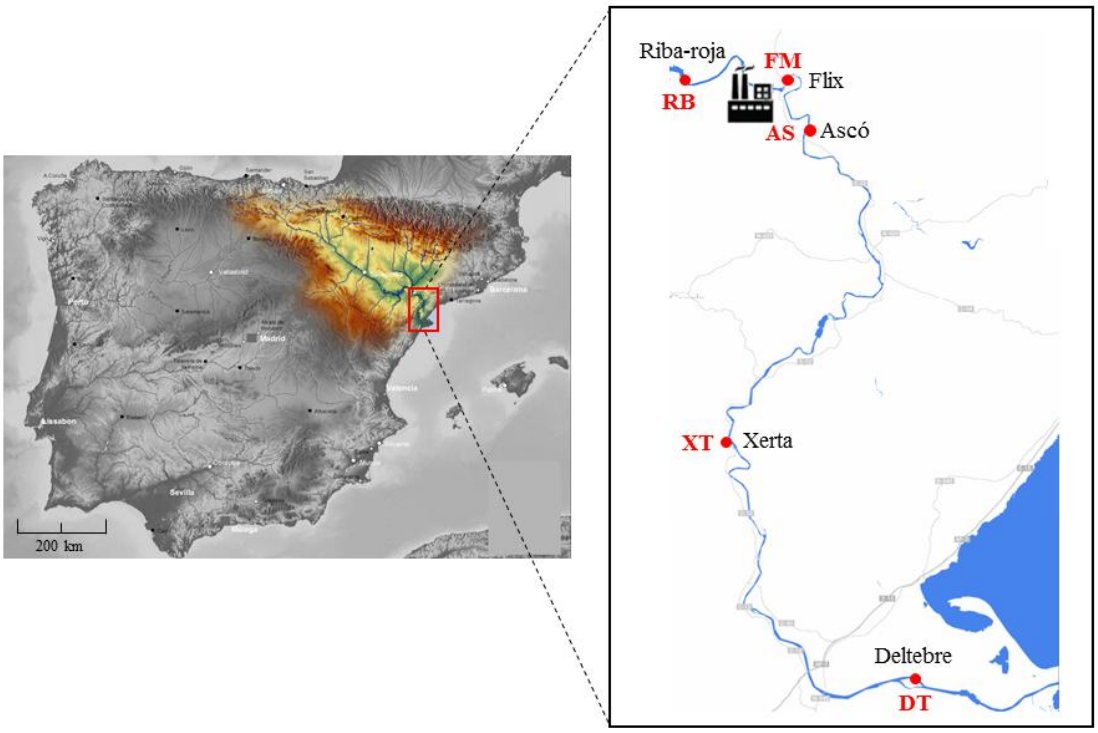

Figure 1. Sampling points in the Ebro River (Spain).

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 (Keskinkan 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 suspended 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.

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(µS cm^{-1})	1365	1335	1367	1348	1961	783	773	796	804	1530
TDS (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

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 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 °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 µm.

Suspended particulate matter (SPM) was obtained passing 1 L of water through a cellulose acetate membrane filter (pore size 0.45 µm, 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 µL of ultrapure filtered water at 1,500 rpm for 1 h in an Eppendorf MixMate (Hamburg, Germany) and filtered through 0.45 µm 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 Ascentis C18 5 µm particle size analytical column measuring 25 cm × 4.6 mm was provided by Supelco (Bellefonte, PA, USA). For the separation of phytochelatins (GSH, γ-Glu-Cys, and 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 (PC_2-desGly, PC_2-Ala, PC_2-Glu and Cys-PC_2), a mobile phase consisted of 1% of formic acid with 0.1 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 PC_n were not present, the method to determine PC_2 isoforms was modified to include the separation of 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 µL and the flow rate was 1.2 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 µAutolab Type III (Eco Chemie, Utrecht, The Netherlands). The flow cell consists on a glassy carbon working electrode (BASi) whose surface was daily polished, a stainless steel auxiliary electrode and a Ag/AgCl (NaCl 3 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 µm), 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

277 ligands, the time-averaged concentration of the metal in the solution, *C*, can be
278 calculated according to the Fick's first law of diffusion as:

$$C = \frac{M\Delta g}{DAt}$$

279 where *D* is the diffusion coefficient of the metal in the diffusive layer, *t* is the
280 deployment time, *A* the exposure surface area, and *Δg* the thickness of the diffusive
281 layer. The mass (*M*) of the analyte accumulated by the resin is experimentally measured
282 and provides the average labile metal concentration during the exposure time. The
283 preparation of the DGT gels and assembly are described in previous works (Fernandez-
284 Gomez et al., 2011; 2014).
285 In-house manufactured polyacrylamide DGT samplers were deployed in triplicate for 7
286 days, in all sampling sites, approximately 1–2 m above the sediment-water interface
287 (between 3 and 5.5 m of river depth). The sampling device used to hold and suspend the
288 samplers consisted of a homemade cylindrical basket made of a plastic net. Finally, the
289 basket was anchored to a rope with a weight on one end and a buoy on the other. After
290 retrieval, DGT units were rinsed with distilled water and kept in polyethylene bags for
291 the transport to the laboratory. Once in the laboratory, DGT units were dismantled and
292 the resin gel was extracted and analyzed for dissolved-bioavalable Hg content.
293

294 ***2.7. Analysis of total mercury concentration***

295 Samples of plants, sediments, suspended particulate matter, and the resin gel from
296 DGTs, were analyzed using an advanced mercury analyzer AMA-254 manufactured by
297 Altec (Prague, Czech Republic) and distributed by Leco (St. Joseph, MI, USA), which
298 is based on catalytic combustion of the sample, preconcentration by gold amalgamation,
299 thermal desorption and atomic absorption spectrometry (AAS). The entire analytical
300 procedure was validated by analyzing CRM from the National Research Council
301 Canada (NRCC; Ottawa) (DORM-2: 4.64 ± 0.26 μg g^{-1} and DORM-3: 0.382 ± 0.060 μg
302 g^{-1}) at the beginning and end of each set of samples, ensuring that the instrument
303 remained calibrated during the course of the study. The concentrations of Hg obtained
304 for repeated analyses (n=5) of both CRM were in good agreement to the certified
305 values. The absolute detection limit was 10 pg, and detection limit given as treble
306 standard deviation of Hg content in blank samples was 0.1 ng g^{-1}.

2.8. Statistical methods

The results for each sample were calculated as the mean ± 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 PC_n (PC_4 and 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 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 PC_2-Ala close to the hot spot in Flix and decreasing when moving away from the contaminant focus (Figure 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 PC_2-Ala in the samples as the peak increases when increasing the spiked concentration of standard (Figure 2b). The quantification of PC_2-Ala was done using external calibration curve and analyzing three independent replicates. The obtained results were 116 ± 6 nmol g^{-1}, 77 ± 2 nmol g^{-1} and 70.1 ± 0.5 nmol g^{-1} fresh weight, for the sampling sites FM, AS and XT, respectively. In RB and in the Ebro River mouth in DT, PC_2-Ala was non-quantifiable.

Only 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).

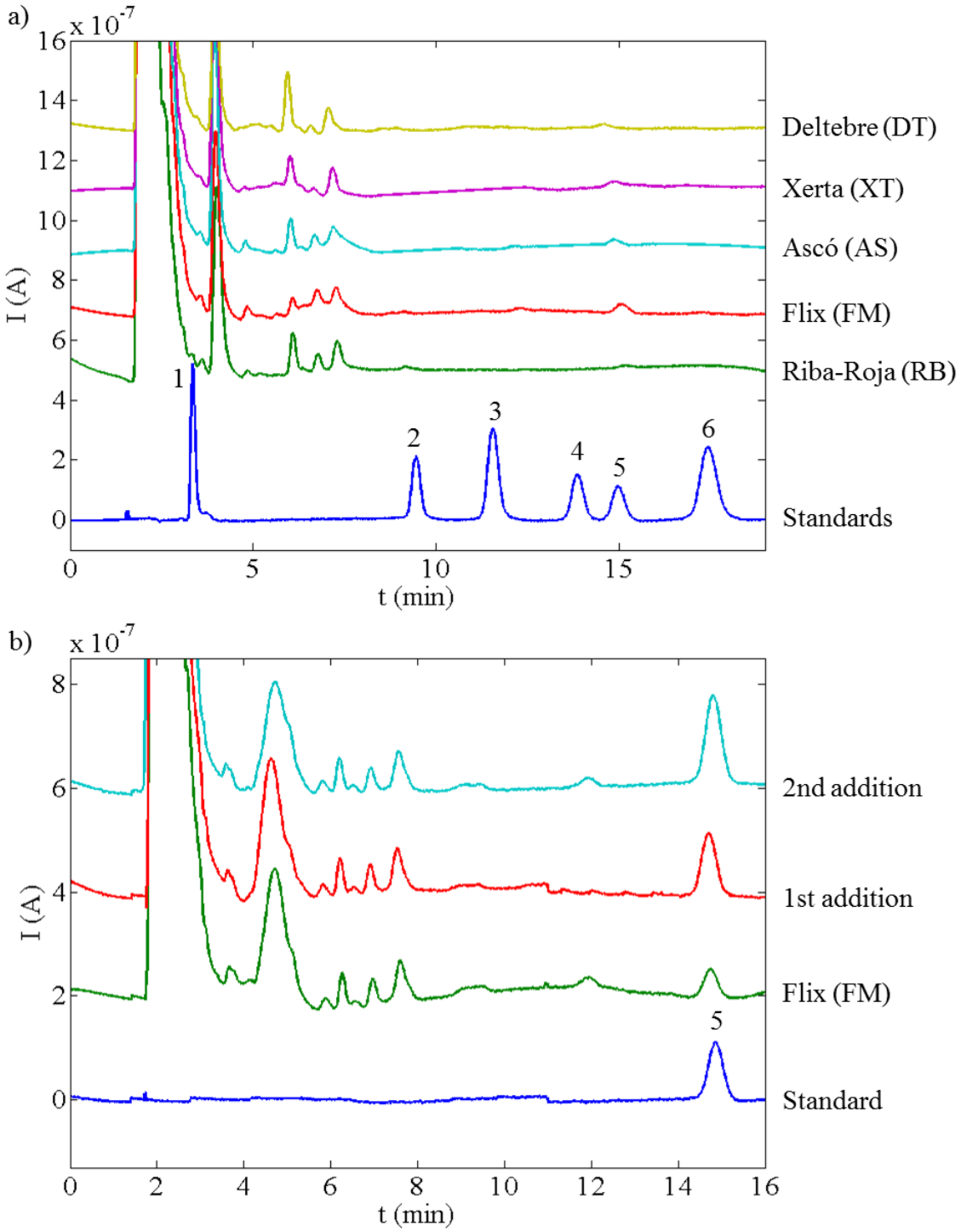

Figure 2. a) Chromatograms of extracts of *C. demersum* and standards of thiols (6 10^{-5} mol L^{-1}); 1: GSH, 2: PC_2, 3: PC_2-desGly, 4: PC_2-Glu, 5: PC_2-Ala and 6: Cys-PC_2. b) chromatograms from Flix (FM) with two additions of 10 and 25 µL, respectively, of PC_2-Ala 10^{-4} mol L^{-1}. Mobile phase of 1% formic acid and 0.1 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 (Figure 3a). The presence of PC_2-desGly could be clearly detected and confirmed by the addition of standard (Figure 3b). A small peak at the retention time of PC_2-Glu seemed to appear in the chromatogram obtained from FM samples, whose presence was also confirmed by spiking the sample with the standard (Figure 3b). While quantifying the thiols, only PC_2-desGly could be determined with values of 63 ± 3 nmol g^{-1}, 170 ± 3 nmol g^{-1}, 154 ± 2 nmol g^{-1} and 122 ± 2 nmol g^{-1} fresh weight, for the sampling sites RB, FM, AS and DT, respectively. PC_2-Glu could only be detected in the FM samples but it was non quantifiable.

These results show a low content in PCs 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 PCs (PC_2 and PC_3) 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_n.

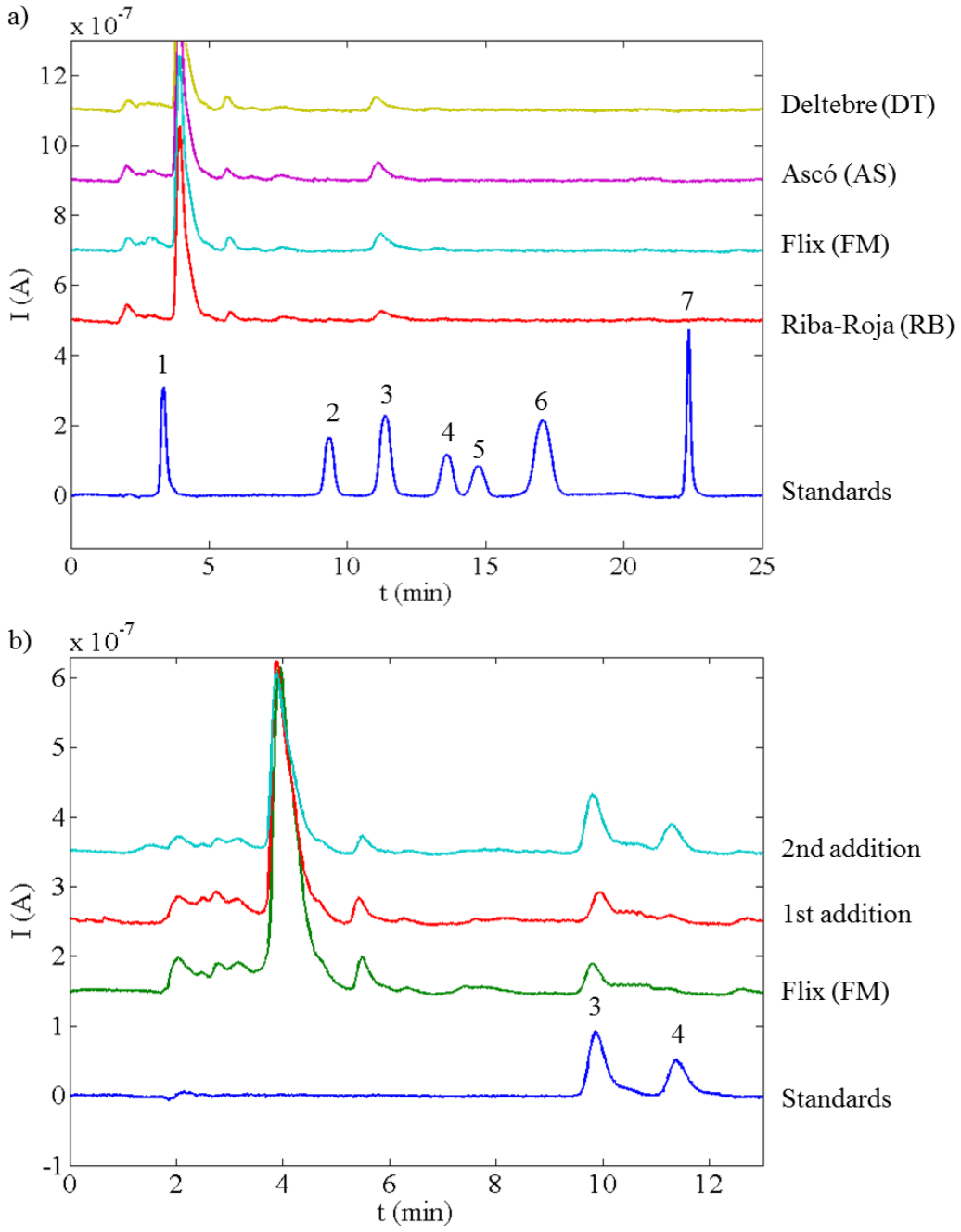

Figure 3. a) Chromatograms of extracts of *M. spicatum* and standards of thiols (6 10^{-5} mol L^{-1}); 1: GSH, 2: PC_2, 3: PC_2-desGly, 4: PC_2-Glu, 5: PC_2-Ala, 6: Cys-PC_2 and 7: PC_3. Mobile phase of 1% formic acid and 0.1 mol L^{-1} of NaCl at a pH of 2 and 1% of formic acid in acetonitrile with a gradient elution. b) Chromatograms of extracts of *M. spicatum* sampled in Flix (FM) with two additions of 120 and 320 µL, respectively, of a mixture of PC_2-desGly and PC_2-Glu 10^{-4} mol L^{-1}, and standards (6 10^{-5} mol L^{-1}) of 3: PC_2-desGly and 4: PC_2-Glu . Mobile phase of 1% formic acid and 0.1 mol L^{-1} of NaCl at a pH of 2 and 1% of formic acid in acetonitrile (96:4).

Regarding this aquatic plant species a relatively high concentration of PC_2-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_2-Glu could also be 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 Figure 4. In this figure the THg concentration in particles are expressed in contaminant mass per dry weight of particles (μg g^{-1}) and per volume of river water (ng L^{-1}).

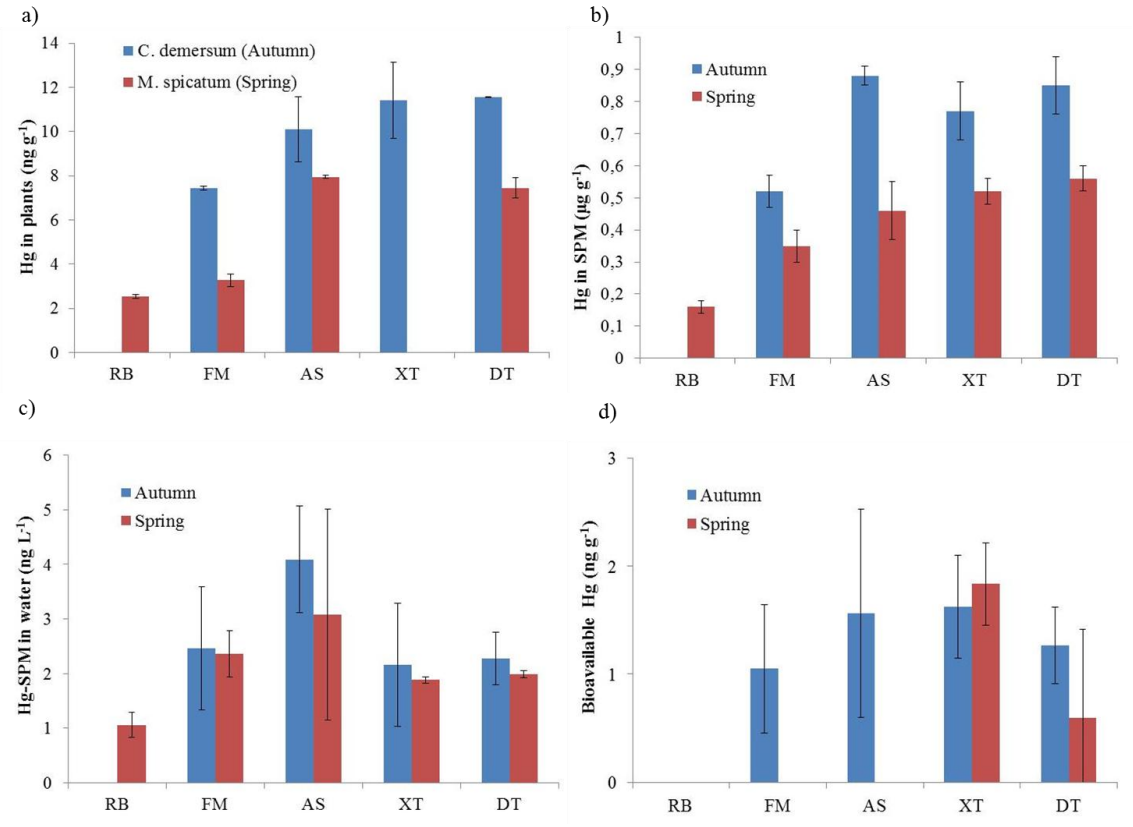

Figure 4.- Diagrams comparing THg in plants (ng g^{-1}) (a), THg in suspended particulate matter per mass weight of particles (μg g^{-1}) (b), THg in suspended particulate matter per volume of water (ng L^{-1}) (c) and dissolved-bioavailable Hg in water (pg g^{-1}) using DGT devices (c) for the two sampling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

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 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 RibaRoja 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 4d), 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 (Figure 4b) is consequence of the similar concentration of THg associated with the SPM flushing down after opening the gates of the dams. Figure 4d 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 ± 0.08 μg g^{-1} in FM and 0.67 ± 0.08 μg 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 (Figure 4c). 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_n 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-reducting 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_n, 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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