Ecotoxicity of sediments in rivers: Invertebrate community, toxicity bioassays and the toxic unit approach as complementary assessment tools

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HIGHLIGHTS

- We performed a sediment toxicity risk assessment in four rivers of the Iberian Peninsula.
- The risk assessment included chemical, toxicological and ecological descriptors.
- Acute toxicity for unicellular organisms was detected in most of the samples.
- Lethal and sub-lethal effects were detected with the *C. riparius* long-term test.
- Organophosphate insecticides and metals were the main contributors to the toxicity.

GRAPHICAL ABSTRACT
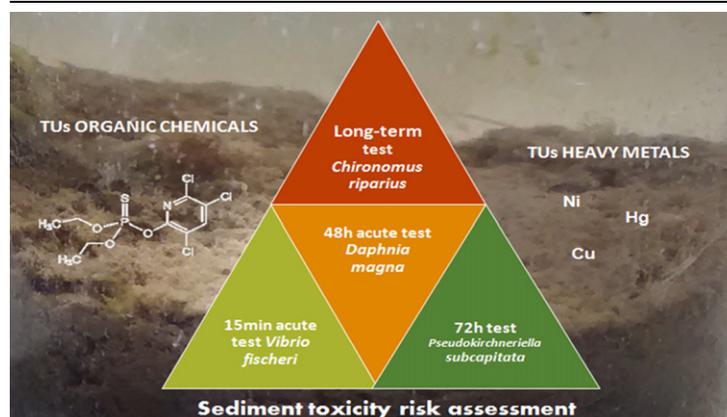
ABSTRACT

The determination of the real toxicity of sediments in aquatic ecosystems is challenging and necessary for an appropriate risk assessment. Different approaches have been developed and applied over the last several decades. Currently, the joint implementation of chemical, ecological and toxicological tools is recommended for an appropriate and successful toxicity risk assessment. We chose the combination of the toxic unit approach with acute pore water tests (*Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Daphnia magna*) and whole-sediment exposure tests (*V. fischeri*, *Chironomus riparius*), together with invertebrate community composition (multivariate analyses) to detect short and long-term responses of the organisms in four rivers of the Iberian Peninsula. High toxicity was detected in three sites (the downstream sites of the Llobregat and the Júcar, and the most upstream site of the Ebro). We identified organophosphate insecticides and metals as the main variables responsible for this toxicity, particularly in the whole-sediment tests. In particular, chlorpyrifos was mostly responsible for the toxicity (TUs) of *D. magna*, coinciding with the *C. riparius* mortality (long-term toxicity) in the mentioned sites, and copper was the main pollutant responsible for the short-term toxicity of *P. subcapitata*. The combination of the different approaches allowed us to detect

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ecotoxicological effects in organisms and identify the main contributors to the toxicity in these multi-stressed rivers.

1. Introduction

Sediment quality is crucial to the health of an aquatic ecosystem (Davoren et al., 2005). Many pollutants (e.g., metals and POPs) can bind physically and chemically with sediments and persist for long periods of time to become bioavailable depending under certain hydrological conditions and exert adverse effects on aquatic organisms (Winkels et al., 1998; Zoppini et al., 2014). Therefore, sediments may act both as a sink and as a source of pollution (Hollert et al., 2000).

The determination of emerging compounds (ECs) (e.g., endocrine disrupting compounds, pharmaceuticals and personal care products) with target methodologies based on the combination of efficient extraction technologies and liquid chromatography mass spectrometry (LC–MS) (e.g., Llorca et al., 2011; Masiá et al., 2013; Köck-Schulmeyer and Olmos, 2013; Gorga et al., 2014) has allowed for the precise and accurate quantification of the organic pollution. However, chemical concentrations alone are insufficient to demonstrate adverse environmental effects because they do not provide any evidence of toxicity (US EPA, 1994; Zhou et al., 2008). Sediment toxicity is difficult to address because of the interaction of the chemicals with the sediment, which determines their bioavailability. The sorption strength of sediments may vary depending on the composition of sediments and the organic matter content (Cornelissen and Gustafsson, 2005). In addition, this composition may vary with the hydrological dynamics of the river (e.g., flow fluctuations, changes in physicochemical variables).

Suitable and ecologically relevant tools must be used to properly assess the toxicological impacts of sediment pollution. Various risk assessment methods are used to evaluate the potential toxicity of the sediment (e.g., toxic equivalent factor approach, toxic unit summation, hazard index) (Scholze et al., 2014). The toxic unit (TU) approach, which was first proposed by Sprague and Ramsay (1965), was developed by Höss et al. (2011) to evaluate the toxicity of complex mixtures in sediments for different organisms. Because it is based on the concentration addition model for mixture toxicity (Norwood et al., 2003 and Sprague, 1970), and is valid for the application to organic chemicals and metals, TU allows for the estimation of the cumulative toxicity in sediment for key test organisms (e.g., *Daphnia magna*, *Pseudokirchneriella subcapitata*).

Different exposure routes, modes of chemical action and different sensitivities may exist for benthic organisms (Rodriguez and Reynoldson, 1999; Ingersoll et al., 2015). From an ecotoxicological perspective, various approaches (e.g., interstitial water quality, spiked sediment toxicity, tissue residue) were developed to detect the specific effects of chemicals on organisms living in sediment, but only whole-sediment tests using benthic organisms are suitable for a realistic risk assessment of the sediment compartment (OECD, 1992; Vandegehuchte et al., 2013). It is possible to address adequately all routes of exposure using these tests (EC, 2003). Additionally, pore water tests may complement the whole-sediment toxicity, because benthic organisms are exposed both to interstitial water and sediment.

Different species have different sensitivities to chemical stress; therefore, the combination of a battery of contact tests with organisms from different organizational and trophic levels allows a better sediment ecotoxicity assessment (Maltby et al., 2005; Höss et al., 2010; Tuikka et al., 2011). However, whole-sediment and pore water laboratory tests cannot capture effects that could be observed at the community level in the natural system. The direct and indirect effects of toxic pollutants on individuals and species may alter community structure (e.g., biodiversity decrease, change from sensitive to more tolerant species). The additional study of the whole community changes along the

river basins might allow for the detection of responses at this level, thereby improving the ecological realism of the laboratory ecotoxicity assessment.

In fact, the guidance documents of the WFD (EC, 2010) emphasize the need for new monitoring tools that help to understand the link between chemical and ecological status and provide indications for the use of ecotoxicity methods in a Triad approach, combining the three assessment methods mentioned above: chemical, laboratory bioassays, and ecology. Therefore, the aims of this study were to (i) study the sediment pollution of four Iberian rivers and determine their associated toxicity using the TU approach; (ii) analyze the composition and density changes of the benthic invertebrates' community; (iii) detect the specific effects of polluted sediment on organisms using a battery of toxicity bioassays with organisms of different organization levels, covering different trophic levels; and (iv) find common trends among the results of the bioassays and the community changes regarding the sediment ecotoxicity risk.

2. Materials and methods

2.1. Study area and sampling

The study was performed in four representative rivers of the Iberian Peninsula: the Llobregat, the Ebro, the Júcar, and the Guadalquivir. The selected basins cover a substantial area of the Mediterranean region, as well as a rich set of socio-ecological conditions. The climate is Mediterranean, with mild and moderately moist winters, warm and dry summers and irregular rainfall concentrated in the spring and autumn. Only the upper section of the Ebro basin has a more continental climate. The four basins flow through areas with high population, particularly the middle and lower courses of the rivers. Important agricultural areas and industrial clusters are located in the watersheds, which depend on the surface and groundwater resources and water transfers (De Castro-Català et al., 2015).

A total of 17 sites were sampled in early autumn of 2011, under conditions of base flow, in the main channel of the rivers: 4 in the Ebro (E1, E2, E3 and E5), 4 in the Llobregat (L3, L4, L5 and L7), 5 in the Júcar (J2, J4, J5, J6 and J7) and 4 in the Guadalquivir (G1, G2, G3 and G4) (Fig. 1). The sites were selected along the rivers to represent the pollution gradients. At each site, a sediment sample (jointed sample of the uppermost 10 cm layer from the two river banks) was taken for chemical analyses. Five randomly distributed sediment samples were taken to determine community composition. To perform the bioassays, sediment from the surface (10 cm) of the riverbed was collected and stored in two containers (~3 kg). Samples were mixed up before the toxicity assays.

2.2. Chemical analyses

2.2.1. Sediment characterization

Sediment samples for grain size characterization and organic matter content quantification were taken in all of the sampling sites. The humidity of the sediments was measured using the UNE 77311 procedure and porosity, according to DiToro (2001) (Table S1).

2.2.2. Organic pollutants

A total of 21 perfluorinated compounds (PFCs) were determined by solvent extraction using acetic acid and methanol followed by liquid chromatography tandem mass spectrometry (LC–MS/MS)
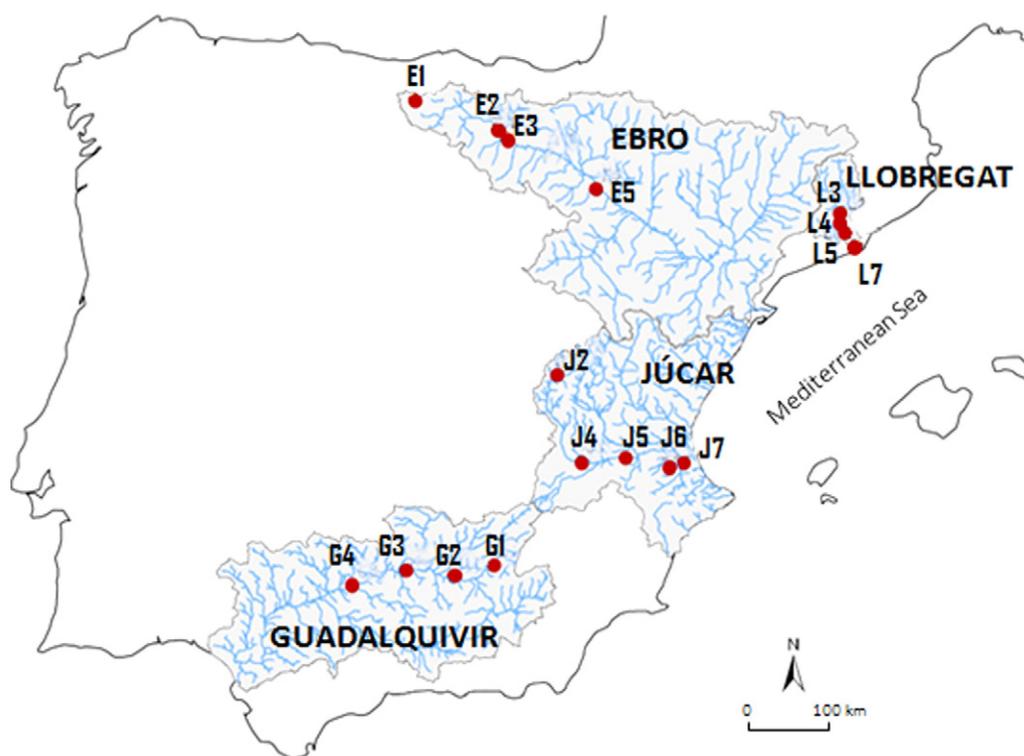
Fig. 1. Study sites in the Ebro, Llobregat, Júcar and Guadalquivir River basins (Iberian Peninsula).

according to already reported procedures (Campo et al., 2015) and 50 pesticides (Pests), namely triazines, benzimidazoles, carbamates and organophosphates, were extracted by a modification of the QuEChERS approach and also determined by LC–MS/MS (Masià et al., 2015) (Table S2A).

A total of 31 endocrine disrupting compounds (EDCs), including estrogens (natural and synthetic), alkyphenolics, benzotriazoles (corrosion inhibitors), parabens, antimicrobials and flame retardants were analyzed using a method based on dual column switching using turbulent flow chromatography followed by liquid chromatography coupled to tandem mass spectrometry (TFC–LC–MS/MS) (Gorga et al., 2014).

The concentrations of 73 pharmaceutical active compounds (PhACs) belonging to different pharmaceutical families, namely analgesics and antiinflammatories, antibiotics, lipid regulators, antihypertensives, antihistamines, psychiatric drugs, diuretics and beta-blockers, were determined in sediments using multi-residue analytical method based on LC–MS/MS after an extraction with the combination of accelerated solvent extraction and solid phase extraction (Osorio et al., 2012).

Persistent organic pollutants (POPs), which comprised 16 polycyclic aromatic hydrocarbons (PAHs) and 7 polychlorinated biphenyls (PCBs), were analyzed by GC/MS following Quesada et al. (2014).

2.2.3. Metals

To calculate the metal bioavailability in sediments, a sequential extraction was performed according to the Community Bureau of Reference (BCR) method (Mossop and Davidson, 2003), as adapted by Roig et al. (2013). The BCR sequential extraction method provides information about in which fractions of sediment are associated with the trace metals. Therefore, the metals bound by ionic exchange or forming carbonate salts correspond to the most bioavailable fraction, followed by metals bound to iron and manganese oxyhydroxides, organic matter and sulfides and the residual fraction.

The concentrations of some potentially toxic elements (PTEs) (arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb), and zinc (Zn)) were analyzed in the pore water of

sediment, total sediment, BCR fractions and SEM by inductively coupled plasma-mass spectrometry (ICP-MS) (Roig et al., 2013). The metal concentrations in the total sediment were compared with the corresponding US EPA benchmarks in order to determine the potential risk of ecological damage (US EPA, 2007).

Additionally, the acid-volatile sulfide (AVS) and simultaneously extracted metal (SEM) analyses were performed according to Allen et al. (1993) with some modifications, to distinguish the potentially cationic toxic metals associated to sulfides in sediments. If the SEM/AVS ratio is higher than one, toxicity due to bioavailable metals in sediment may be occurring.

2.2.4. Toxic units

The TU approach was used (Sprague, 1970) to evaluate the toxicity of sediments. Sediment toxic units were defined as the ratio of the estimated (for organic compounds) or measured (for metals) pore water concentration of a contaminant and the water exposure based on toxicity values for *D. magna* (48-hour acute EC50) and *P. subcapitata* (72-hour EC50) (Eq. (1)):

$$TU_i = \frac{C_{ipw}}{EC50i_w} \tag{1}$$

where TU_i is the toxic unit of an individual compound detected in sediment; C_{ipw} estimated (for organics, see below) or directly measured (for metals) pore water concentration (μg/l) of the compound; and water only exposure based EC50$_i$ (μg/l) effective concentration for 50% of the individuals when exposed to the substance concerned. Following the equilibrium-partitioning approach (DiToro, 2001), the measured bulk sediment concentrations were used to estimate the bioavailable fraction of organic contaminants in the pore water, as suggested by several authors (Schäfer et al., 2011; Wetzel et al., 2013). Partitioning coefficients between sediment and water were (K_d) used to calculate

the following (Eq. (2)):

$$C_S = C_{PW} \times K_d \qquad (2)$$

where, K_d is the partitioning coefficient between the water and sediment of the chemical, C_S is the bulk sediment concentration and C_{PW} the is the pore water concentration of the contaminant.

For non-ionic organic chemicals, the organic matter was assumed to be the major binding phase in sediments (DiToro, 2001). Therefore, the fraction of organic carbon in sediment (f_{oc}) and the partitioning coefficient between organic carbon and water (K_{oc}) were used to calculate the pore water concentration (Eq. (3)):

$$C_{PW} = \frac{C_s}{f_{oc} \times K_{oc}}. \qquad (3)$$

The total risk of the sediment to aquatic life was assessed by summing the toxic units for all of the contaminants detected in the sample following the concentration addition concept (CA). Metals and organic chemicals were considered separately. For organic chemicals TUs were calculated for families of compounds and also for groups (PFCs, Pests, EDCs, PhACs and POPs) (Table S2A).

2.3. Invertebrate metrics

2.3.1. Community composition

Five sediment samples were randomly collected with a polyvinyl sand corer (24 cm² area). Samples were sieved through a 500-μm mesh to isolate the macroinvertebrates, which were fixed with 4% formaldehyde. The invertebrates were sorted, counted, measured and identified in the laboratory under a dissecting microscope (Leica Stereomicroscope). The identification was at the species level for most taxa, – including Oligochaeta – with the exception of the Chironomids, which were identified to the genus level, and the Phylum Nematoda. The identification was performed according to Tachet et al. (2000) for macroinvertebrates, Brinkhurst and Jamieson (1971) for Oligochaetes and Wilderholm (1983) for Chironomidae.

2.3.2. Bioassays

Ecotoxicity tests were performed in pore water and whole sediment samples. Pore waters were obtained by sterile vacuum filtration through 0.22 μm pore size. The ecotoxicity was evaluated using the following test organisms: the bacterium *Vibrio fischeri*, the freshwater green algae *P. subcapitata* and the crustacean *D. magna*. The whole sediment ecotoxicity was evaluated with *V. fischeri* and the midge *Chironomus riparius*. All these species are used worldwide as representative test species in ecotoxicology and toxicity data are available in literature for different compounds.

The acute toxicity of the pore water and whole sediment was tested on the luminescent bacteria *V. fischeri* using the Microtox® M500 analyzer (Azur Environmental Ltd.). The inhibition luminescence test was performed following the Basic Test for 90% aqueous extracts and the Solid Phase Test in pore water and whole sediment, respectively, according to the Microtox® supplier protocols and the norm ISO 11348-1:2007. The EC50 at 15 min was calculated in both tests. The units of EC50 were expressed as percentage (v:v) and mg sediment/ml test medium in the pore water and whole sediment tests, respectively.

The *D. magna* acute immobilization test with pore water was performed using the microbiotest Daphtoxkit F™ (MicroBioTests Inc., Belgium) according to the corresponding supplier protocol and the OECD 202 norm.

The toxicity of pore water for *P. subcapitata* was performed as required by the OECD 201. The EC50 at 72 h was calculated, and growth inhibition was considered the endpoint. Units of EC50 were expressed as percentage (v:v) of pore water dilution.

The *C. riparius* long-term toxicity test was performed with whole sediment according to the ASTM E 1706-05 method (ASTM, 2005) considering survival, growth (biomass) and development (head width) as endpoints, following Agra and Soares (2009).

Toxicity acute effects were classified into different levels of toxicity following the ranges established by Roig et al. (2015), which went from marginally toxic to highly toxic (Table 1). For *C. riparius*, the effects were considered when values were equal or higher than 30% mortality or when significant differences in growth or development with respect to the control were observed.

2.4. Statistical analyses

The normality and homoscedasticity of the chemical and environmental dataset were tested with Kolmogorov–Smirnov tests. The chemical and sediment-related variables were log-transformed. Multivariate analyses were performed using CANOCO™ v4.56 software (Microcomputer Power, Ithaca, NY, USA). Rare invertebrate taxa (present in <5% of the samples) were omitted. The compounds were grouped into different families, taking into account their mode of action (Table S2A). A detrended canonical correspondence analysis (DCA) was initially performed on the benthic invertebrate data to determine whether unimodal (canonical correspondence analysis, CCA) method or linear ordination (redundancy analysis, RDA) method was the most appropriate (Lešp and Šmilauer, 2003). Finally, a CCA was used to explore the relationship among the chemical data (independent variables) and invertebrate abundances (dependent variables). Forward selection of significant variables was performed using Monte Carlo permutations test (n = 1000).

An ANOVA followed by Dunnet's post-hoc tests was used to compare the sub-lethal responses of *C. riparius* with the controls. Spearman rank correlations between *D. magna* TUs and long-term effects, and between *P. subcapitata* TUs and short-term effects (*P. subcapitata* and *V. fischeri* tests) were performed using SPSS software, version PASW Statistics 18 (SPSS Inc., 2009).

3. Results

3.1. Pollution

3.1.1. Metals

The BCR sequential extraction method showed that metals were the compounds with the highest loads that accumulated in the sediments of the four river basins. These high levels were mainly due to Zn. Sites J4 and L7 exceeded the reference values for river sediments for the total concentrations (Fig. 2). The most exchangeable fraction of Zn ranged from 1.5 to 24.5 μg/g, with E3 presenting the highest concentration. In addition to Zn, Ni was another metal with high concentrations of the exchangeable fraction in the sediments of the Guadalquivir River (1.8 to 4.1 μg/g), and in the most downstream site of the Llobregat River (1.7 μg/g). These levels of Ni showed that it was relatively available, especially in L7, G2, G3, and G4, where the benchmarks for the total levels were exceeded. For Cu, J4, J7, G3 and G4 exceeded the benchmarks. Cu binds primarily to the organic matter of the sediment, because it forms the strongest metal–humic acid complexes out of all of the divalent cations. In this case, the most bioavailable fraction was above 0.5 μg/g in E1, L5 and J4. The most potentially bioavailable fraction of was below 0.4 μg/g in most of the sediments, and only L5 and L7 exceeded the benchmarks. L7, G3 and G4 exceeded the benchmarks for Cr, although the bioavailable fractions were insignificant. This result may indicate that Cr III is the predominant form of Cr (insoluble and mildly toxic). Hg was the element whose benchmarks were exceeded at many points in the four basins. However, the residual fraction was very high and close to the benchmark in most sites, which indicated that the geological background levels were already high in these rivers. No benchmarks were exceeded for Pb, Co and Cd. However, Cd was the

Table 1

A) Results of the short-term toxicity assays in pore water (PW) and whole sediment (WS). Toxicity ranges: in blue, non-toxic; in green, slightly toxic; in yellow, marginally toxic; in orange, moderately toxic; in red, highly toxic. B) Results of the long-term toxicity assay in whole-sediment (WS) expressed in absolute values and in percentage with respect to control. HW: head capsule width; DW: dry weight. In purple: toxic effects.

| | Short-term (15 min to 72 h) | | | | Long-term (10 days) | | | | |
| | *V. fischeri* (WS) | *V. fischeri* (PW) | *P. subcapitata* (PW) | *D. magna* (PW) | *C. riparius* (WS) | | | | |
Sample	EC50 (mg/l)	EC50 (%vol:vol)	EC50 (%vol:vol)	EC50 (%vol:vol)	Survival %	Development HW (mm)	%control	Biomass DW (mg)	%control
E1	5348	43.4	183	>100	50	0.56	103	0.46	98
E2	1488	47.4	229	>100	20	0.54	100	0.42	90
E3	1421	72.8	101	>100	0	0.53	97	0.24	51
E5	5087	55	121	>100	0	0.57	104	0.53	112
G1	814	>100	131	>100	10	0.55	101	0.34	73
G2	664	>100	129	>100	0	0.54	100	0.17*	36*
G3	31.1	>100	76	>100	0	0.55	102	0.30	63
G4	87.7	51.5	89	>100	0	0.50	92	0.29	61
J2	2157	70.6	66	>100	20	0.58	107	0.90**	192**
J4	5127	>100	101	>100	0	0.56	104	0.43	91
J5	586	>100	147	>100	30	0.57	105	0.50	107
J6	902	>100	172	>100	0	0.56	104	0.45	96
J7	3092	>100	116	>100	10	0.56	104	0.49	104
L3	3244	>100	227	>100	10	0.56	103	0.47	100
L4	11682	>100	321	>100	20	0.55	102	0.28	59
L5	3076	>100	28	>100	30	0.45*	83*	0.23	49
L7	1729	>100	50	>100	0	0.51	95	0.30	64

*p-value < 0.05, **p-value < 0.01.

element that presented higher percentages of bioavailability (50% in many cases) and, although the total values were low, cadmium bioaccumulation cannot be discarded. The SEM/AVS analysis confirmed that J4, G4 and E2 presented high bioavailability of metals, and thus potential effects on organisms may be occurring in these sites (Table S3).

The concentrations of metals in pore waters were very low compared with the total concentrations in the sediment. This fraction corresponds to metals that are more readily available. Cu at some sites (J6, G2, E2, E3 and E5), and Hg and As in all sediments (except for G3 for As and G1 for Hg) exceeded the benchmarks for surface waters (US EPA, 2006)
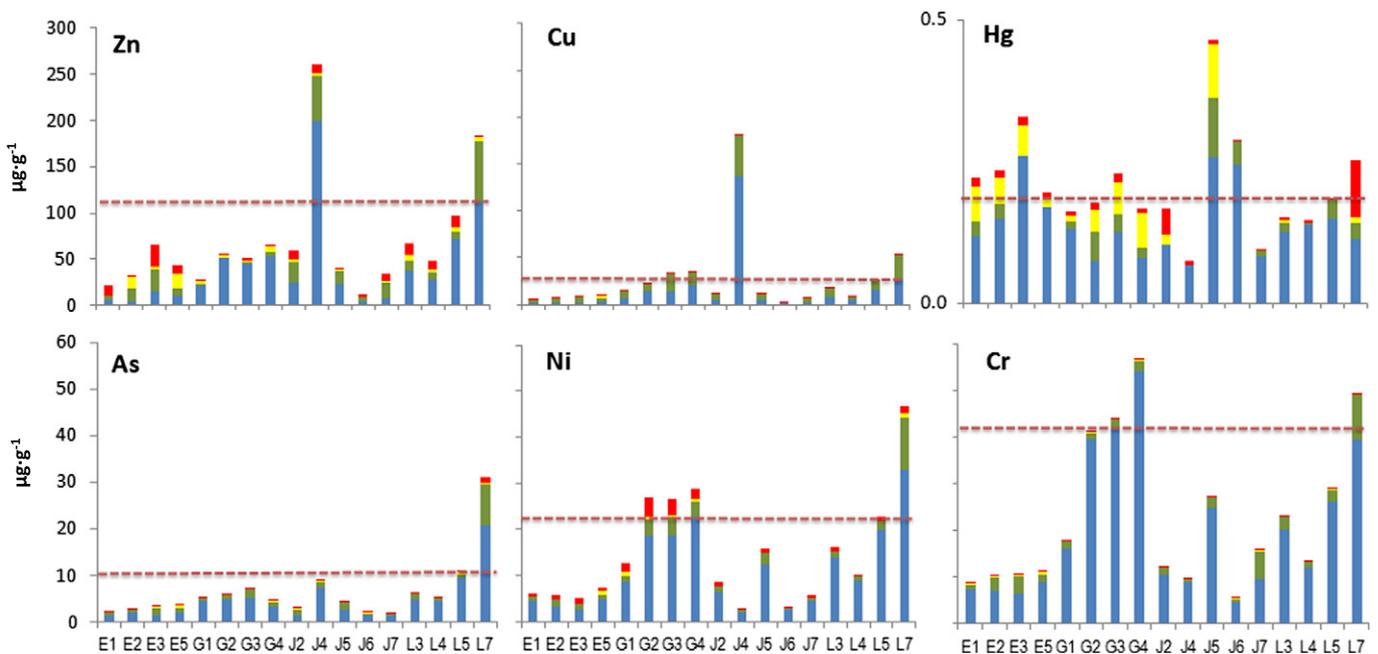

Fig. 2. Total metal concentrations in sediments (µg/g). Colors show the different sediment fractions, in blue: residual metal fraction; in green: metal fraction bound to organic matter and sulfides; in yellow: metal fraction bound to Fe and Mn oxyhydroxides; in red: metal fraction exchangeable and/or associated to carbonates (the most bioavailable). The red lines show the US EPA Benchmarks (only those metals that exceeded the benchmarks in at least one sampling site are shown).

(Table S2B). Generally, pore waters tend to be more concentrated than surface waters, but it is important to emphasize that benthic organisms are highly exposed to them.

The sites with highest levels of metals were J4 and L7, and being the latter site presented the highest diversity of metals, with levels above the reference thresholds for Zn, Cu, Hg, As, Cr and Ni.

3.1.2. Organic chemicals

POPs were the group of organic compounds with the highest levels detected, particularly in L4 and E1 where some PAHs (e.g., chrysene, pyrene, phenanthrene) were detected at the μg/g level (Fig. 3). The EDC nonylphenol reached concentrations of 100 ng/g in almost all the basins, with a peak of 200 ng/g in E3. Within the EDCs group, methylparaben and the flame retardant tris (chloroisopropyl) phosphate also reached high levels in the Júcar and Llobregat Rivers, respectively. One site in the Ebro River (E3) presented high values of both compounds. Among the pesticides, the organophosphate chlorpyrifos reached 40 ng/g in the Llobregat. Some pesticides from the family of the azoles presented concentrations above 10 ng/g in the Júcar and the Llobregat Rivers. Almost all of the PhACs presented levels below 2 ng/g with the exception of ketoprofen, which presented values above 4 ng/g in almost all the sites and above 10 ng/g in the J2 and L4. Metronidazole reached a peak of 8 ng/g in G4 and acridone (a transformation product of carbamazepine) reached levels above 5 ng/g in L4, J7 and E3.

3.2. Toxic units

3.2.1. TUs for D. magna

Total organic chemical TUs ranged from 0.01 to 5.3 (Fig. 4A). The Llobregat River presented the highest values, with the exception of L7. E1 and G1 also presented high levels of toxicity, above 2. Organophosphate pesticides, specifically chlorpyrifos, were responsible for the high toxicity values in almost all of the sites were. The highest values (TU > 2) for this insecticide were detected upstream of the Ebro River and midstream of the Llobregat River. Chlorfenvinphos and malathion (also organophosphates) presented high TUs in G1 (2.7) and J5 (1.3), respectively. Other pesticides that contributed to these very high levels of toxicity in the sediment were diazinon in the Guadalquivir River and the carbamate methiocarb in J1.

The values for the total TUs for metals ranged from 0.05 to 1.65 (Fig. 4B). Two sites, J6 and E5, presented values higher than 1. Although Zn was the element with the highest measured concentrations, Cu had the highest toxicity. The EC50 values for D. magna (and P. subcapitata), are very low for the latter, compared with those of the other metals (Table S4). Therefore, the TUs for this metal were higher than 0.1 in all of the sites, which indicated a risk of acute toxicity.

The sums of POPs' TUs were the highest in E4 (0.04), L4 (0.06) and L7 (0.035). The individual TUs were all below 0.03. PAHs (anthracene, benzo-a-pyrene and fluoranthene) dominated in all of the sites with the exception of L7, where two PCBs were detected. Other sites that presented lower values that were still above 0.001 were the rest of the sites of the Ebro and the Llobregat Rivers, J5 and G4. The TUs of EDCs increased towards downstream and were approximately 0.04 in G4, L6 and L7. The compounds with the highest TUs were methylparaben in G3, G4, J4, J5, J7 and L7, and some alkylphenols (nonylphenol and octylphenol) in the most downstream sites of the Ebro, Guadalquivir and Llobregat Rivers. The toxicity associated with POPs and EDCs was due to the high levels detected in the sediment, not to the high potential of toxicity of the compounds. The Guadalquivir River presented values higher than 0.002 for PhACs' TUs in all the sites. G1 and G4 were the sites with the highest PhACs' TUs (0.008 and 0.006, respectively), which were mainly related to two antibiotics (ofloxacin and ciprofloxacin). The Llobregat River presented a high diversity of PhACs, but it did not exceed the TUs of 0.001 in any of the sites. The TUs of total PFCs were below 0.0005 in all of the rivers. In summary, the sites with the highest toxicity for the crustacean were L5, L3, L4, G1 and E1, mainly due to the toxicity of the insecticide chlorpyrifos.

3.2.2. TUs for P. subcapitata

Metals were the main contributors to toxicity for P. subcapitata (Fig. 4B). Metals' TUs ranged from 0.2 to 3.6. J6 was the site with the highest levels of toxicity. The Ebro River presented levels above one in all the sites, and the levels were higher than 2.5 in E5 and in J6. All of the sites had levels above 0.1 TU. Cu was the main contributor, followed by Hg in the Júcar, and Ni and Zn in the Ebro River. Another metal with low EC50 values, apart from Cu, is Hg, which was the main responsible of metal toxicity upstream of the Júcar (J2, J4 and J5). The other metals contributing to the toxicity were Ni, Mg, and As. Nonetheless, the individual TUs were below 0.05.

Total organic TUs ranged from 0.01 to 0.1 (Fig. 4A). POPs' TUs were approximately 0.08 in E1, E3 and L4. In J5 and E5, the values were approximately 0.03 and were due to anthracene. PhACs were higher than 0.03 in G2, G3 and G4 due to the toxicity of ciprofloxacin. EDCs' TUs were below 0.02 in all of the sites, with the exception of J5 and downstream sites of the Guadalquivir and Llobregat Rivers, following the same trends as the D. magna TUs. Pesticides' TUs were below 0.01 in all of the sites with the exception of J5, which presented a slim peak of prochloraz. Thus, green algae seem to be more sensitive to metals than cladocerans. Sites J6, E5, E2 and E3 presented the highest toxicity mainly due to the toxicity of copper.

An underestimation of the real toxicological risk could have occurred to a certain degree due to the absence of toxicological data for some compounds such as some PCBs and PFCs.

3.3. Benthic community of invertebrates

The benthic community was primarily composed of Chironomidae and Oligochaeta. Some of the sites located upstream of the studied rivers presented some distinctive species not present in the respectively downstream sites, such as the oligochaeta Potamothrix hammoniensis in the Ebro River and the snail Potampyrgus antypodarum in the Júcar River. The taxonomical richness decreased downstream in all of the rivers. The taxa present in these downstream sites were more tolerant to pollution and included Polypedium sp., Branchiura sowerbyi and Limnodrilus hoffmeisteri.

The canonical correspondence analysis (CCA) between invertebrates' densities and chemical and environmental variables explained 12% of the total inertia. The significant variables explaining the invertebrate density variation were the proportion of fine sediment (3.4%), POPs (3.4%), Pests (2.7%) and PFCs (2.5%) (Fig. 5).
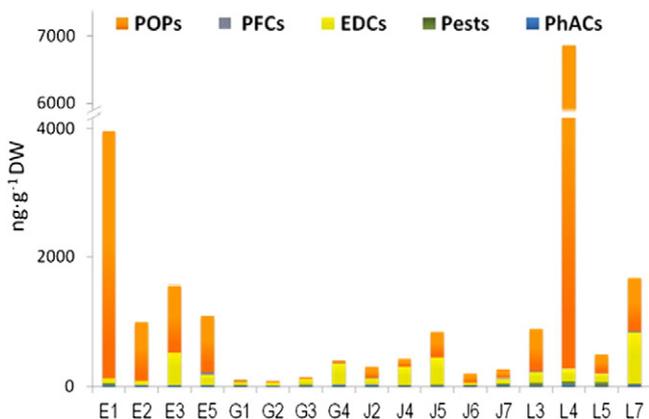

Fig. 3. Concentrations of the main groups of organic chemicals analyzed in sediment (ng/g).
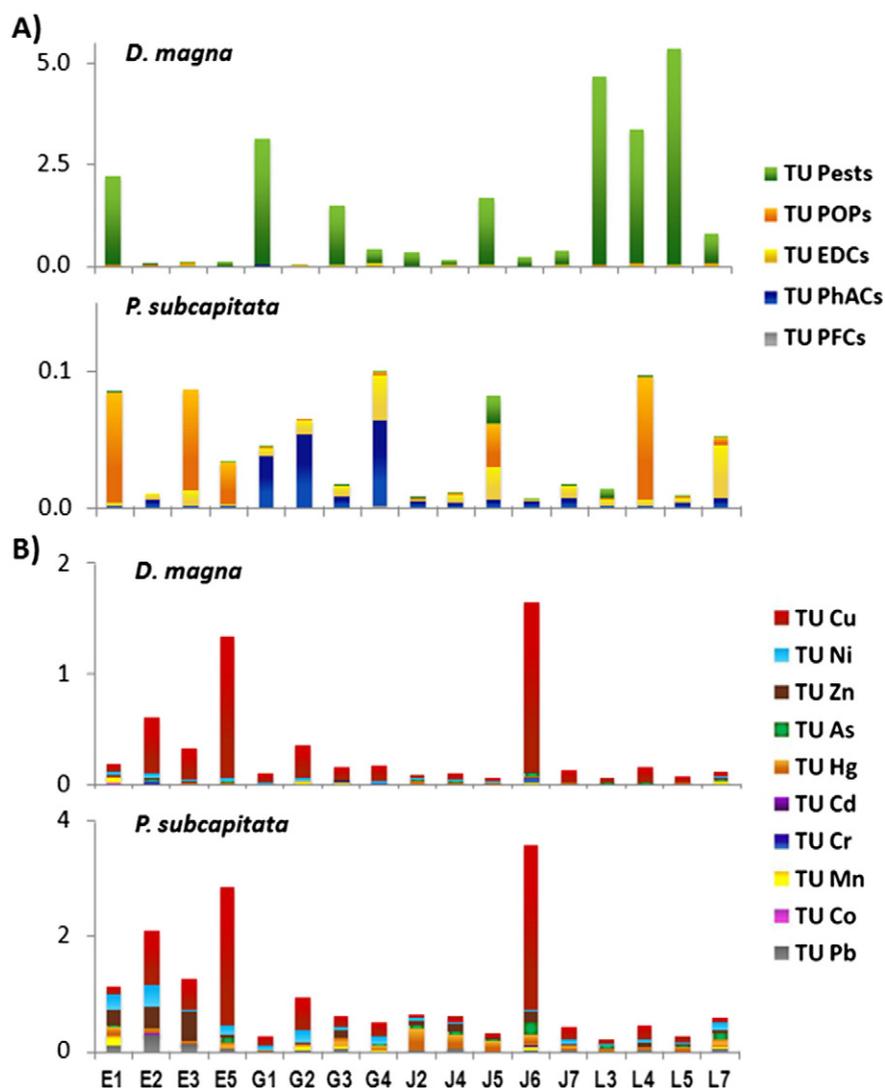
Fig. 4. Toxic units for *D. magna* and *P. subcapitata*. A) Toxicity of organic compounds. B) Toxicity of metals.

3.4. Ecotoxicity bioassays

All sediments presented ecotoxicity for at least one bioassay with the exception of L4. Toxicity results obtained in solid phase tests presented higher ecotoxicity than those obtained on pore water tests (Table 1), although fine textured materials may interfere, especially for *V. fischeri* test. This species was the most sensitive according to the pore water tests. Four samples showed marginally ecotoxicity (E1, E2, E5, G4) and two others presented slightly ecotoxicity (E3 and J2) according to the ecotoxicity ranges (Roig et al., 2015). Samples L5 and L7 were classified as marginally toxic for *P. subcapitata*, while samples G3, G4 and J2 were classified as slightly toxic. Any of the samples presented ecotoxicity for *D. magna*. Whole-sediment tests performed with *V. fischeri* presented ecotoxicity in almost all of the sediments, especially in the Guadalquivir River and in J5 and J6. The mortality of *C. riparius* was of 50% in E1, and 30% in L5 and J5. Sub-lethal long-term effects were also observed in the ecotoxicity test with *C. riparius*. Individuals from J2 showed lower biomass with respect to the control (Dunnett's test, p < 0.01) and, on the other hand, individuals from G2 presented higher biomass (Dunnett's test, p < 0.05). Development seemed to be delayed in L5 (Dunnett's test, p < 0.05). According to these results, sediments that presented the highest ecotoxicity for the different tests were E1 and L5, followed by J5, J2, G4, E3 and G2.

Significant correlations among the results of the *C. riparius* test and *D. magna* TUs, and the results of *V. fischeri* tests and *P. subcapitata* TUs were found. The mortality of *C. riparius* was correlated with the TUs of chlorpyrifos (r = 0.48, p < 0.05). Significant correlations were also detected between the TUs of PhACs and changes in development (r = −0.62, p < 0.01) and biomass of *C. riparius* (r = −0.51, p < 0.05). The TUs of PhACs were also correlated with the EC50 values of the *V. fischeri* whole-sediment test (r = −0.76, p < 0.01). Regarding pore water tests, the EC50 values of *V. fischeri* were correlated with the TUs of metals (r = −0.53, p < 0.05), particularly with the TUs of Ni (r = −0.57, p < 0.05), and also with pore water concentrations of Hg (r = −0.57, p < 0.05).

4. Discussion

Using the TU approach we evaluated the potential toxicity of the sediments. All the sediments of the Ebro River exceeded the value of expected acute toxicity for metals, and also for pesticides in site E1. TU values of the Guadalquivir and Llobregat Rivers' sediments were high due to the presence of organic compounds. The sediments of the Júcar River presented high values of TUs for metals in some sites (e.g., J6) and high toxicity values for organic pollutants in other sites (e.g., J5).
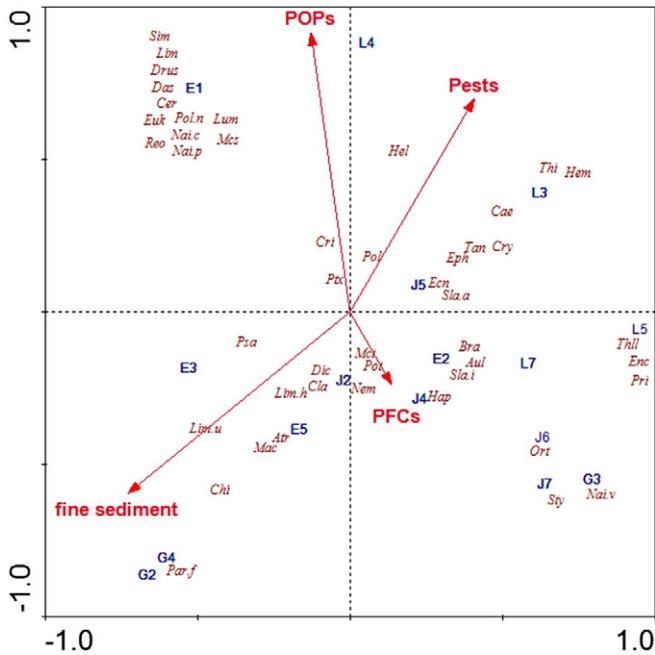
Fig. 5. CCA between invertebrate community and concentrations of pollutants in sediment. Abbreviations: Atr — *Atrichops* sp., Cae — *Caenis luctuosa*, Eph — *Ephemera danica*, Lim — sF. Limoniinae, Pot — *Potamopyrgus antipodarum*, Drus — sF. Drusinae, Das — sF. Dasyheleinae, Cer — sF. Ceratopogoninae, Hel — *Helobdella stagnalis*, Pol — *Polycelis nigra-tennis*, Nem — P. Nematoda, Sim — Tr. Simuliini, Hem — sF. Hemerodromiinae, Cry — *Cryptochironomus* sp., Chi — *Chironomus* sp., Euk — *Eukiefferiella* sp., Reo — *Reocricotopus* sp., Dic — *Dicrotendipes* sp., Mic — *Microtendipes* sp., Pol — *Polypedilum* sp., Cla — *Cladotanytarsus* sp., Sti — *Stictochironomus* sp., Mcs — *Micropsectra* sp., Nan — *Nanocladius* sp., Pen — Tr. Pentaneurinii, Cri — *Cricotopus* sp., Ort — *Orthocladius* sp., Thi — *Thienemannimyia* sp., Thll — *Thienemanniella* sp., Par — *Parametriocnemus* sp., Mac — Tr. Macropelopinii, Tan — *Tanytarsus* sp., Bra — *Branchiura sowerbyi*, Lim.h — *Limnodrilus hoffmeisteri*, Lim.u — *Limnodrilus udekemianus*, Ptx — *Potamothrix hammoniensis*, Lum — *Lumbriculus variegatus*, Enc — F. Enchytraeidae, Nai.c — *Nais communits*, Nais.p — *Nais pseudobtusa*, Nais.v — *Nais vairabilis*, Psa — *Psammoryctides barbatus*, Pri — *Pristina leidyi*, Par.f — *Paranais frici*, Aul — *Aulodrilus pigueti*, Slav.i — *Slavina isochaeta*, Sla.a — *Slavina appendiculata*, Sty — *Stylaria lacustris*, Hap — *Haplotaxis gordioides*.

Combining the TU approach with the results of the bioassays, we could identify sites L5, E1 (3 different tests showed toxicity) and J5 (2 different tests showed toxicity, including *C. riparius* mortality) as those presenting higher ecotoxicity. The results of the bioassays matched with the results of the TU approach in these sites. L5 presented the highest TUs for *D. magna*, mainly due to chlorpyrifos. A reduction of 30% of the survival was observed for the midge *C. riparius* in this site, as was a delay in development. Additionally, the As levels in the sediment were above the benchmarks, and toxicity was detected for *V. fischeri* and *P. subcapitata*. The same trend was observed in E1, the most upstream site of the Ebro River, and although the toxicity due to chlorpyrifos was not as elevated as in L5, TUs for metals exceeded one and the mortality of *C. riparius* was the highest in this site. E1 has been previously reported as a highly toxic site by Roig et al. (2015). In J5, TUs were high due to another organophosphate insecticide: malathion. Reduction of 30% of the *C. riparius* survival was detected, and the toxicity for *V. fischeri* was severe. The levels of toxicity in these sites exceeded the threshold for expected acute effects (Schäfer et al., 2012).

Organophosphorus (OPs) insecticides are widely used because they are highly effective and exhibit relatively nonpersistent characteristics. Moreover, these pesticides lack specificity, and it has been demonstrated that they are also highly toxic to nontarget species, including other aquatic organisms different than insects (Mise Yonar et al., 2014). In particular, chlorpyrifos has been categorized as "very toxic to aquatic life" and "very toxic to aquatic life with long lasting effects" (Watts, 2012). Lethal and sub-lethal effects have been detected in different invertebrate species, including *C. riparius*, at environmentally relevant

concentrations (e.g., Arambourou and Stoks, 2015, 1971; Rakondravelo et al., 2006; Pérez et al., 2013). Bioaccumulation of chlorpyrifos in fish tissues has been found by Masiá et al. (2015) in a study performed in the Llobregat basin. Chlorfenvinphos, which has been banned in many countries because of its severe neurotoxic effects, presented very high toxic levels in G1, and, to a lesser extent, in J2. Malathion has also been reported as a compound highly toxic for invertebrates, causing acute and chronic effects even at the ng/l level, affecting the reproduction and the development of species such as *D. magna* (Barata et al., 2004; Ren et al., 2007; Toumi et al., 2015). Acute and chronic effects have also been detected for chironomids at the ng/l level (Rebechi et al., 2014). Another pesticide worthy of note is diazinon, which has been described as one of the most stable organophosphates (Albanis et al., 1998; Aronzon et al., 2014) and presented certain toxicity in three of the four basins, particularly in the Guadalquivir River. The toxic effects of diazinon to non-target organism have been reported for several groups of aquatic organisms, including cladocerans (Fernández-Casalderrey et al., 1995; Sánchez et al., 2000; Bailey et al., 2001; Jemec et al., 2007) and chironomids (Schuler et al., 2005).

In general, the solid phase tests showed higher ecotoxicity levels than did the pore water tests. This observation was in accordance with the results of the study performed by Roig et al. (2015). Despite no effects were detected with the *D. magna* pore water short-term assay in any of the sampling sites, long-term effects were detected with the *C. riparius* whole sediment test in some sites, including lethality. These results allowed for the detection of relevant effects after chronic exposure, and support the importance of combining a battery of tests for a correct ecotoxicological assessment. Although it has recently been detected that species with high metabolic rates (e.g., *D. magna*) are more sensitive to pollutants than species with lower metabolic rates (Baas and Kooijman, 2015), the toxicity data collected for some insects by the same authors provided evidence of their high sensitivity to the OPs pesticides. That is, the toxicity of a pollutant is also determined by its mode of action in relation to the test organism. On the other hand, toxicological data (e.g., EC50) of the cladoceran are widely available for compounds that belong to different chemical groups, with different modes of action, but toxicity data for insects such as *C. riparius* are still too scarce to perform a TU approach with this species.

The toxicity risk showed by the pesticides in our basins could somewhat be detected in the CCA. The variability in density of the invertebrate species was explained by a 3% for changes in pesticides' levels, mainly OPs. Other relevant compounds that explained the community changes were POPs, mostly PAHs, which presented toxicity, although to a certain extent, in L4 and E1. It is well known that the chemical properties of industrial POPs make them persistent in the sediments of aquatic ecosystems, and they are able to bioaccumulate easily in the tissues of biota and eventually cause toxicological effects (Covaci et al., 2005; Naso et al., 2005; Van Ael et al., 2012).

The sediments of the Guadalquivir River had high toxicity, although it was only detected with the *V. fischeri* short-term bioassay. This toxicity may be related to the toxicity of metals (high TUs for *P. subcapitata*), particularly Cu, Ni and Hg and also to some PhACs (antibiotics). In G2, the SEM/AVS ratio indicated high toxicity due to bioavailability of metals and long-term effects were also detected with the *C. riparius* test. High levels of Ni may be an indicator of recent anthropogenic pollution (Krachler et al., 2003), because it is a metal widely used in industry and it can be found at elevated levels in freshwater areas surrounding heavily developed urban areas, but it may also be related with previous metal-mining activities. The Guadalquivir River basin was particularly affected by this element. An excess of Ni have been found to affect survival of algae (Eisler, 1998; Muyssen et al., 2004) and also of some freshwater gastropod species (Peters et al., 2014; Niyogi et al., 2014). The toxicity of Cu and Zn, can be explained by their bactericidal and antimicrobial nature affecting the enzymatic systems. Additionally, Cu is a strong inhibitor of photosystem II electron transport activity and can alter the energy storage capacity in algae

during photosynthesis and decrease chlorophyll a content (Mallick and Mohn, 2003; Bossuyt and Janssen, 2004 and Sabatini et al., 2009). Cu and Zn have both been found to be more toxic for algae than for daphnids (Ardestani et al., 2014), which could explain why toxic effects were detected with the *P. subcapitata* and *V. fischeri* tests but not with the *D. magna* test. Toxicity of Hg was important in the Júcar River, especially upstream. This metal can bioaccumulate and have severe implications in vertebrate and invertebrate organisms (Cardoso et al., 2012; Cabecinhas et al., 2015). In algae it may cause alterations in enzymatic activities and photosynthetic inhibition (Jonsson and Aoyama 2009) at concentrations in the range than those detected in interstitial water of the sediments of the Júcar River.

De Castro-Català et al. (2015) have found that PhACs and EDCs from water in the same rivers are the most likely chemical families related to benthic invertebrate responses. Regarding water toxicity, Kuzmanovic et al. (2015) have also identified OPs insecticides and alkylphenolic compounds (EDCs) as the main contributors of toxicity for *D. magna* in these four basins. In the present study, metals and some OPs were the main contributors to sediment toxicity, what emphasizes the different fate of pollutants (water or sediment) depending on their chemical characteristics. The properties of compounds (e.g., partitioning coefficient) coupled with the sediment characteristics (e.g., organic carbon content, grain size) are determining the chemical composition of sediments. Mediterranean rivers are characterized by important hydrological fluctuations (Bonada and Resh, 2013). Floods can periodically remove river bed sediment, mainly the fine fraction, and the interaction and persistence of pollutants in sediment are influenced by this hydrological dynamism. In fact, the fine sediment percentage was a significant variable for the community composition (see CCA analysis), which highlights the importance of sediment dynamism in these basins. In addition to the effects of toxic pollutants, excessive fine sediment loads may result in the elimination of certain species, particularly those with sensitive traits (Buendia et al., 2013) and their habitats (Robson et al., 2013).

5. Conclusions

We identified hotspots of sediment toxicological risk in the studied rivers using a combined approach of TUs, a battery of ecotoxicity bioassays and local invertebrate community description. Short-term effects in *V. fischeri* and *P. subcapitata*, and long-term sub-lethal and even lethal effects in *C. riparius* indicate that sediment toxicological risk is highly severe in some sites of the studied rivers, and that it can affect the invertebrate communities, particularly if this exposure is continuous and accumulative over time and along the river. The TU approach helped to identify OPs insecticides (chlorpyrifos) and metals (mainly Cu, but also Hg and Ni) as the main contributors responsible for the toxicological effects in the sediments. These results suggest a strong necessity to keep developing toxicity studies with organisms from different organizational levels, covering the range of different sensitivities to compounds with different modes of action. The development of studies at higher levels of organization (e.g., community) is also crucial to provide ecologically relevant predictions of toxic effects in the environment. Finally, this study also evidences the importance of integrating ecological, toxicological and chemical techniques for an appropriate risk assessment.

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