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Identification of compounds bound to suspended solids causing sub-lethal toxic effects in *Daphnia magna*. A field study on re-suspended particles during river floods in Ebro River

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Identifying chemicals causing adverse effects in organisms present in water remains a challenge in environmental risk assessment. This study aimed to assess and identify toxic compounds bound to suspended solids re-suspended during a prolonged period of flushing flows in the lower part of Ebro River (NE, Spain). This area is contaminated with high amounts of organochlorine and mercury sediment wastes. Chemical characterization of suspended material was performed by solid phase extraction using a battery of non-polar and polar solvents and analyzed by GC–MS/MS and LC–MS/MS. Mercury content was also determined for all sites. Post-exposure feeding rates of *Daphnia magna* were used to assess toxic effects of whole and filtered water samples and of re-constituted laboratory water with re-suspended solid fractions. Organochlorine and mercury residues in the water samples increased from upstream to downstream locations. Conversely, toxic effects were greater at the upstream site than downstream of the superfund Flix reservoir. A further analysis of the suspended solid fraction identified a toxic component eluted within the 80:20 methanol:water fraction. Characterization of that toxic component fraction by LC–MS/MS identified the phytotoxin anatoxin-a, whose residue levels were correlated with observed feeding inhibition responses. Further feeding inhibition assays conducted in the lab using anatoxin-a produced from *Planktothrix agardhii*, a filamentous cyanobacteria, confirmed field results. This study provides evidence that in real field situation measured contaminant residues do not always agree with toxic effects.

1. Introduction

The adsorption of chemicals onto sediment particles is an important process through which many contaminants are removed from the water column. Bottom sediments may act both as a sink and as a long-term source of toxicants (Burton, 2002). This dual role is particularly relevant during floods, in which pollutants stored in sediments may be easily remobilized by sudden increases of river flow and the consequent increase of toxicant concentrations. Nowadays, in fact, due to climate change, we are suffering an increase of severe weather conditions for certain regions, often characterized by an alternation of extreme events such as drought and flash floods, thus growing the awareness to the impacts caused by floods (Ikeda et al., 2005; Kleinen and Petschel-Held, 2007). Therefore, there is an increasing challenge among environmental toxicologists to identify substances within suspended particular matter having the potential to harm the biological communities (Stachel et al., 2005; Wölz et al., 2010).

Traditionally, identifying environmental contaminants has been performed using targeted chemical analysis following a prioritisation/ranking process. This approach is an effective way of analysing environmental samples, but has the drawback that certain unknown contaminants may be missed (e.g., components with low concentrations and high target toxicity, metabolites, transformation products or natural products). In recent years the development of effect-directed analysis (EDA) or toxicity identification procedures (TIE) have allowed to address the problem of unknowns using bioassays as diagnostic tools. Using these meth-

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ods a complex environmental sample is extracted, fractionated and then analyzed using both biological assays and chemical analysis in order to link the presence of one or more compounds to their biological effects. Finally, the identified compounds need to be confirmed by testing uncontaminated samples spiked at concentrations measured in the obtained fractions. In these procedures, the selection of bioassays to be used in whole samples and their fractions are crucial. Therefore, there is a need to develop more ecologically relevant in vivo bioassays that can be used both for the whole samples and fractions. This is most problematic when trying to assess toxicity of particle bound contaminants in suspended material in water, since the very few already existing bioassays have been only tested in sediments (Bosch et al., 2009; Phillips et al., 2009; Schmitt et al., 2011; Wölz et al., 2010). In this regard, particle-feeding organisms are of special interest since contaminated particles might end up in their gastrointestinal tract and exert toxic effects (Bosch et al., 2009). Recently, the use of cost effective sub-lethal *Daphnia magna* feeding tests combined with Toxicity Identification Evaluation (TIE) procedures have allowed the identification of water soluble and particle-bound compounds in sediments causing toxic effects (Bosch et al., 2009).

Likewise many other rivers located in arid or semi-arid climate regions, dams in the Ebro River (NE, Spain) are used to regulate surface-water cycles, especially when water demand and its availability are imbalanced (Petrovic et al., 2011). Flushing flows have been used typically to mitigate dam-induced impacts such as deterioration of riparian habitats (Gibbins et al., 2007) or reduction of sediment transport (Batalla and Vericat, 2009). As a counter effect, contaminants that are accumulated in sediments have been shown to be mobilized during these flood events (Kirchner et al., 2000; Quesada et al., 2014). This is especially problematic in Flix reservoir, where an organochlorine industry operates since the beginning of the 20th century. In fact, this long operational period, along with the construction of a dam next to the factory around 1960, resulted in the accumulation of high amounts of heavily polluted sediments in the adjacent riverbed (Bosch et al., 2009; Fernández et al., 1999; Grimalt, 2006). Major pollutants reported in these wastes include hexachlorobenzene (1900 ng/g), polychlorobiphenyls (39,000 ng/g), DDEs-DDTs (1300 ng/g), polychlorostyrenes (360 ng/g), polychloronaphthalenes (1100 ng/g), mercury (49 μg/g), cadmium (2.3 μg/g), chromium (210 μg/g) and nickel (67 μg/g), mean values (Grimalt, 2006). Bosch et al. (2009) reported that mercury bound to waste sediment particles severely impaired grazing rates of filter feeders like *D. magna*. Furthermore, pollutants originated at Flix site are carried downstream by the Ebro River to its delta located 90 km away, where they can bioaccumulate and affect biota (Faria et al., 2010; Navarro et al., 2009; Pastor et al., 2004). Nevertheless, there is no information of toxic effects of re-suspended industrial waste sediment material during flood events.

Another environmental problem associated to dams is the proliferation of noxious substances produced by cyanobacterial blooms (Agha et al., 2012; Herry-Allani and Bouaïcha, 2013). Several studies have reported the occurrence of toxic cyanobacteria species (e.g., *Anabaena*, *Planktothrix*), known to produce toxins such as microcystins and anatoxins in Ebro reservoirs at and downstream of Flix (de Hoyos et al., 2004; Quesada et al., 2004). The cyanobacteria mentioned above or/and their phytotoxins exert toxic effects to grazers like *Daphnia*, which are known to graze on them (Claska and Gilbert, 1998; Demott et al., 1991; Freitas et al., 2014).

This study aimed to use a TIE protocol implemented with in vivo *D. magna* feeding inhibition responses to evaluate and identify toxic compounds present in suspended solids during an unusual period of prolonged flushing flows along a contaminated area located at the low Ebro River Basin.

2. Material and methods

2.1. Water sampling

Thirty superficial water samples were collected from January 18th to June 22nd 2013 along the lower course of Ebro River (NE Spain) between its tributary, the Cinca river (CI) that ends at Riba-Roja reservoir, just before its junction with the reservoir and the village of Mora d'Ebre, which is located 50 Km downstream (M; Fig. 1A). Samples were collected at five different periods during a prolonged flash flow of 155 days (Fig. 1C). Sampling sites included locations situated upstream the industrial sediment wastes (e.g., Cinca river, CI and Riba-Roja pier at Flix reservoir, RR), in front of the wildlife reserve located on the riverbank opposite to the factory (FR) and at the meander located immediately downstream from the dam (MU, MD) (Fig. 1A and B). On June 22nd it was possible to take samples on the river bank opposite of MU at MF (Fig. 1B). According to the river flow, MF site should have a greater contribution of mobilized industrial wastes than sites FR, MU and MD. Three locations further downstream were also sampled and included both river margins at Ascó village (Ascó pier, A; and Ascó village, AV) and at Mora village (M) (Fig. 1A and B). Part of the water from Flix reservoir is diverted through a bypass below the village of Flix to generate electrical power and it is released just downstream of MD. This means that a portion of the re-suspended sediment particles coming from the industrial sediment wastes, which are located at the left margin of Flix reservoir, may not reach MU, MF and MD. Conversely, water samples from Ascó and Mora should be fully enriched from re-suspended particles from contaminated Flix sediments.

In addition to the above water samples, three additional samples were included as negative controls. Two were obtained from elutriates of sediments obtained between the Meander and Ascó in a previous study (sediment samples S5, S6; (Bosch et al., 2009)). Elutriates were obtained after mixing 1 g of freeze dried and sieved (60 μm size) sediment in 1 L of ASTM hard water to obtain the fine particulate fraction. Another water sample was obtained outside the Ebro basin from the lagoons of Las Tablas de Daimiel National Park, south-central Spain.

2.2. Physico-chemical characterization of water samples

A set of environmental variables were measured on each sampling. Water physico-chemical parameters including temperature (*T*; °C), pH, conductivity (μS/cm), dissolved oxygen (O2, mg/L) and suspended solids (SS, mg/L) were obtained following (Damásio et al., 2008) procedures. Briefly, *T*, pH, conductivity and O_2 were measured in situ by using a WTW Multi 340i handheld meter. Suspended solids were obtained after filtering 1 L of water sample through a Whatman grade GF/F glass fiber filter paper (0.7 μm pore size) previously washed with acetone, pre-combusted at 400 °C and pre-weighted. Following filtering, the filter was freeze dried, weighed to assess suspended solids and then used to determine total mercury or organochlorine residues. Further characterizations of the suspended matter were conducted in water samples from January and May. Particle size analyses were performed using a Laser Diffraction particle size analyser (LS 13,320 MW, Beckam Coulter, Inc., USA), whereas carbon, nitrogen and hydrogen content was determined by Elemental Microanalyzer (A5) model Flash 1112 (Thermo Scientific, UK), performed by the modified Pregl-Dumas technique (dynamic flash combustion), using helium as carrier gas.

Analysis of total Hg in filters was performed following Carrasco et al. (2008) with minor modifications by means of the Advanced Mercury Analyzer AMA-254 (Altec, Prague, Czech Republic). In each run appropriate blanks (freeze dried filters previously loaded with 1 L of nanopure water) were used. The AMA instrument is based on catalytic combustion of sample, its pre-concentration