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Determination of 54 pesticides in waters of the Iberian Douro River estuary and risk assessment of environmentally relevant mixtures using theoretical approaches and *Artemia salina* and *Daphnia magna* bioassays

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ABSTRACT

As a case study, the estuary of the international Douro River (Iberian Peninsula) was sampled over a year (2010) at six sampling sites to determine the presence of 56 pesticides of different categories (insecticides, herbicides, and fungicides). 96% of measured pesticides were detected in 79% of the quantified samples. Individual average pesticide concentrations ranged from 39 to 1 265 ng/L, indicating a ubiquitous presence of the selected compounds; moreover, twelve pesticides were above the 2013/39/EU Directive limits. Due to its highly impacted profile, a theoretical hazard assessment was done considering the average and maximum environmental mixtures of all measured pesticides to identify the most sensitive trophic level. For both environmental mixtures, the theoretical approach suggested that invertebrates were the most sensitive group. Therefore, short-time exposure assays using both invertebrates *Artemia salina* and *Daphnia magna,* were done using the referred mixtures. Data demonstrated significant toxic effects — high mortality rate and abnormal swimming behaviour — of the exposed animals. Both approaches (theoretical and experimental) support the analytical results, alerting for an intervention on this estuarine environment and of other comparable.

1. Introduction

Estuaries are very rich and dynamic environments that are vital to local and migratory fauna and flora, being also important for the wide range of services and goods they provide to humankind. However, due to anthropogenic activities, these ecosystems are continuously being loaded with pollutants, attaining concentrations of individual and/or mixtures of compounds capable of inflicting negative consequences. Among several contaminants, pesticides are transferred to these aquatic systems, where many times their final concentrations attain values above the legal limits (EU, 2013). Portuguese brackish water systems, like Ria Formosa Lagoon, Tagus and Mondego Rivers, are examples of that status (Cruzeiro et al., 2015, 2016b, 2016c). According to the Directive 2013/39/EU (EU, 2013), pesticides like pentachlorobenzene (PeCB), terbutryn, cypermethrin (alpha), endosulfan, heptachlor and others, were above the average annual environmental quality standards (EQS) fixed for superficial waters.

As one of the most important aquatic systems of Iberian Peninsula, the Douro River crosses Spain and Portugal, ending up in one of the biggest estuaries of Portugal, located between two largely populated cities (Porto and Gaia). According to Ribeiro et al. (2016), for decades the surface and groundwater of this river are being used for local rural activities, electric power generation and industry, and as drinking water supply to half of the residing population of the metropolitan area of Porto city. The river margins are also characterized by a predominant agricultural activity (grape production), to which several pesticides are known to be applied (such as simazine, terbutryn, azinphos-methyl, phosmet, and endosulfan alpha) and that ultimately reach (at least many of them) the Douro River estuary (Rocha et al., 2012).

In this context, one purpose of this work was to analyse estuarine surface water samples of Douro estuary by gas chromatography-mass spectrometry (GC–MS) to assess the presence of pesticides along a year and check differences between seasons. A total of 56 pesticides were selected, according to national and European databases (DRAP, 2014; European Union, 2008) as well as other published scientific works that include approved, not approved and banned pesticides (European

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Union, 2008).

Most of the environmental monitoring studies are addressing the exposure and consequences of single-toxicants, leaving out the heterogeneity of pesticide mixtures that are found at different locations (Rojo-Nieto et al., 2012). Therefore, a theoretical approach, using the average and maximal concentrations of all measured pesticides, was used herein to evaluate the potential risk of these mixtures in different trophic levels (algae, crustacean, and fish). Afterwards, acute toxicity tests using the reference models *Artemia salina* (brine shrimp) and *Daphnia magna* (water fleas) were performed. Characteristics, such as short life cycle, small body, simple hatching procedure, promote both models as appropriate organisms for short toxicology tests with low costs in routine and research practices (Lu et al., 2012; Janssen and Persoone, 1993; Vanhaecke et al., 1980).

Considering the above, the specific aims of this work were to: (a) quantify 56 selected pesticides from surface waters collected in the Douro River estuary over a year; (b) analyse data taking in consideration the European legislation; (c) run a theoretical hazard assessment; and (d) use artemia and daphnia as biologic models for acute-toxicity tests that assess the potential hazardous effects of the unveiled environmental mixtures.

2. Material and methods

2.1. Study area and sample collection

The watershed of Douro River cover an area of 98 000 km^2, shared between Spain and Portugal that drains into the Atlantic Ocean. It has an average depth of 8 m and tides with a semidiurnal range of 2–4 m. The residence time changes between 0.3 and 16.5 days with the increase or decrease of freshwater flow that is controlled by the Crestuma–Lever dam, situated 21.6 km from the mouth (Bordalo and Vieira, 2005).

For this work, six intertidal sampling sites were selected along the estuary (Fig. 1). At the north margin, there were three sampling stations in the estuary, *i.e.*, S1 (41° 08′ 52.2″ N, 8° 39′ 04.2″ W), S2 (41° 08′ 36.5″ N, 8° 35′ 06.5″ W), and S3 (41° 04′ 23.3″ N, 8° 29′ 29.1″ W). Three other sites were located at the south margin of the estuary, S4 (41° 04′ 9.9″ N, 8° 29′ 59.2″ W), S5 (41° 08′ 18.5″ N, 8° 35′ 19.4″ W), and S6 (41° 08′ 25.6″ N, 8° 39′ 23.4″ W).

Surface water samples (2.5 L) were collected during ebb tide, at the shore, in four different months (March, May, July, and September 2010, covering all four seasons) into pre-rinsed amber glass bottles. In the laboratory, the samples (1 L) were immediately filtrated (0.45 µm glass fibre filter; Munktell, Germany), adjusted with H_2SO_4 for a pH = 7 (to prevent degradability of the target pesticides), and stored at 4 °C for no more than 24 h.

2.2. Analytical work

2.2.1. Sample preparation and pesticides extraction

The target 56 pesticides were extracted by solid phase extraction (SPE, OASIS HLB), according to Cruzeiro et al. (2015). Briefly, cartridges were conditioned sequentially with 5 mL of ethyl acetate, followed by 5 mL of methanol, and 2.5 mL of ultrapure water at a flow rate of 1–2 mL/min. The surface water samples (500 mL), added with the IS (atrazine-$_{d5}$ and 4,4′-DDT-$_{d8}$), were loaded into SPE cartridges at a constant flow-rate of 5 mL/min. The cartridges were dried and then eluted with 6 mL of ethyl acetate, at 1 mL/min. The extracts were concentrated into 200 µL of hexane and kept in vials at −80 °C. The final range of concentrations was 10 – 400 ng/L for all pesticides and 160 ng/L for the IS. Blanks at an intermediate concentration (160 ng/L) were used to ensure both the absence of contamination and the existence of a quality control.

Reagents and pesticides information are described in the supplementary material SM1.

2.2.2. Instrumental methods, quality assurance and quality control procedures

The extracts were analysed in a gas chromatograph (Trace GC ultra, Thermo Finnigan Electron ©), (coupled with an ion trap mass spectrometer detector Thermo Scientific ITQ™ 1100 GC-MS^n), an auto sampler (Thermo Scientific TriPlus™), and equipped with a Trace GOLD column (TG-5SILMS, 30 m × 0.25 mm × 0.25 µm). Column oven temperatures were programmed according to a previously published method (Cruzeiro et al., 2015).

The performance of the methods was checked daily, using method blanks (solvent controls), quality controls (two-fold higher than the limit of quantification), fortified samples spiked with both surrogates, and using, weekly, new calibration curves. The limits of detection (LODs) and quantification (LOQs; see Table 1) for each pesticide were defined as LOD = $3.3(\alpha/S)$ and $LOQ = 10(\alpha/S)$; here, α is the standard

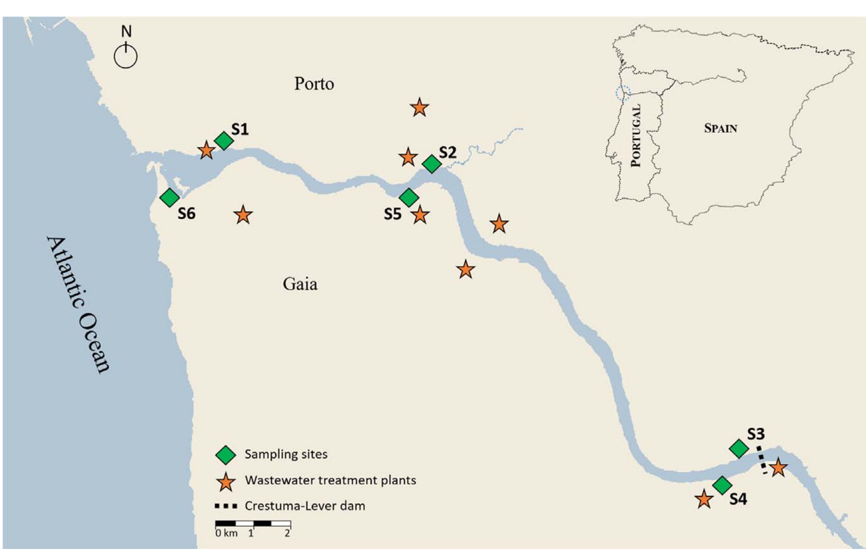

Fig. 1. Location of sampling sites within the Douro River estuary (S1 to S6) along with the waste water treatment plants (WWTPs); map adapted from Microsoft MapPoint, 2010.