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Human hepatoma cells exposed to estuarine sediment contaminant extracts permitted the differentiation between cytotoxic and pro-mutagenic fractions

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ABSTRACT

Complex toxicant mixtures present in estuarine sediments often render contaminant screening unfeasible and compromise determining causation. HepG2 cells were subjected to bioassays with sediment extracts obtained with a series of progressively polar solvents plus a crude extract. The sediments were collected from an impacted area of an estuary otherwise regarded as pristine, whose stressors result mostly from aquaculture effluents and hydrodynamic shifts that enhance particle deposition. Compared to a reference scenario, the most polar extracts yielded highest cytotoxicity while higher genotoxicity (including oxidative damage) was elicited by non-polar solvents. While the former caused effects similar to those expected from biocides, the latter triggered effects compatible with known pro-mutagens like PAHs, even though the overall levels of toxicants were considered of low risk. The results indicate that the approach may constitute an effective line-of-evidence to infer on the predominant set of hazardous contaminants present in complex environmental mixtures.

1. Introduction

Ecosystems such as estuaries are particularly sensitive to anthropogenic pressures, in large part owing to limited self-renewal. Aquatic sediments, in particular, can become significant reservoirs of persistent contaminants that, adsorbed to the typically complex combination of fine particles and organic matter, may be released back to the water column through events disrupting the sediments' physico-chemical balance (see Chapman and Wang, 2001; Chen and White, 2004). Mixtures of environmental contaminants may induce adverse effects to biota and humans at distinct levels of biological organization, from population down to molecular-level (Ohe et al., 2004; for a review). Surveying the toxicological hazards of sediment contaminant mixtures remains, however, a challenge to environmental toxicologists, mostly due to difficulties in establishing cause- and dose-effect relationships in living organisms. These difficulties largely come from i) factors affecting bioavailability; ii) inter-species differences regarding the effects and responses to toxicants and iii) the effects of contaminant interactions. Furthermore, identifying and quantifying hazardous substances extracted from sediment samples, especially organic compounds, is expensive and time-consuming. For such reasons, researchers worldwide have been relying on bioassays to determine the risk inherent to full toxicant sets as an alternative to the identification of a specific substance, or few substances of concern. Among those substances, the ones classified by the International Agency for Research on Cancer (IARC) as genotoxic, mutagenic or carcinogenic to humans are long regarded as a priority. The latter include some polycyclic aromatic hydrocarbons (PAHs), pesticides and other anthropogenic toxicants long known to contaminate sediments of impacted coastal areas (Kennish, 2002).

Bioassays became important tools to determine the potential genotoxic or mutagenic effects of aquatic sediment contaminants,

Abbreviations: BC, Binucleated cell; CBPI, Cytokinesis-block proliferation index; DCM, Dichloromethane; DDT, Dichlorodiphenyltrichloroethane; FPG, Formamidopyrimidine-DNA glycosylase; HCB, Hexachlorobenzene; hex, *n*-Hexane; met, Methanol; MNBC, Micronucleated binucleated cell; MNi, Micronuclei; NR, Neutral red; PAH, Polycyclic aromatic hydrocarbon; PCB, Polychlorinated biphenyl; RI, Replication index; ROS, Reactive oxygen species; SEQ/mL, Sediment equivalent/mL.

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using different approaches and different experimental models, among which fish have been regarded as adequate target subjects due to their ecological importance and ability to act as surrogates for high-order vertebrates (see Bolis et al., 2001). However, under the *Three R* principle (Replacement, Reduction and Refinement), some researchers in the field of environmental toxicology began to perform studies on fish cell lines to determine risk of sediment contamination (e.g. Woo et al., 2006; Yang et al., 2010; Šrut et al., 2011). Even though these approaches may render an estimate of ecological risk, predicting effects to humans from these data remains speculative. While studies with murine cell lines (e.g. Aouadene et al., 2008) may permit a link between sediment contamination and risk to mammals, research on human cell lines remains scarce. Nevertheless, Shea et al. (2008) and Higley et al. (2012) investigated metallothionein induction and endocrine disrupting effects of sediment-bound toxicants, respectively. The human hepatoma HepG2 cell line has been widely used in pharmacological and toxicological research, because it retains the enzymatic apparatus typically involved in hepatic detoxification processes as, for instance, CYP monooxygenases (Knasmüller et al., 2004). However, there is little exploration of this particular cell line in studies focused on crude environmental mixtures of toxicants, in spite of the cell line's potential to contribute to an effective line-of-evidence in risk assessment procedures.

The present case study, the Mira Estuary (SW Portugal, Fig. 1), is considered one of the least impacted estuaries in the country by anthropogenic stressors (Vasconcelos et al., 2007; Cardoso et al., 2011). Consisting of a small 30 km-long area, the estuary retains its hydrodynamics mainly influenced by tidal conditions. Deprived of significant urban and industrial activities along its margins, its pressures arise mainly from a small town located at the mouth of the estuary, which receives seasonal tourism activities. Other anthropogenic pressures are related to non-intensive agriculture, livestock production and aquaculture (Vasconcelos et al., 2007; Chainho et al., 2008). Recent studies revealed a contamination hotspot inside the estuary, located at an artificial canal built to serve an aquaculture facility. This is the only relevant industrial infrastructure in the estuary and riverine basin that is potentially able to induce effects in living organisms consistent with exposure to hazardous substances (Carreira et al., 2013). This canal is located in a steep corner where the reduction of hydrodynamics and current speed, together with marine and freshwater mixing, likely contributes to increased deposition of toxicants carried by the river. Given the especial conservation status of the estuary, further investigation on the sediments from this site appeared mandatory.

The present work aims essentially at: i) characterizing in the HepG2 cell line the cytotoxic and genotoxic effects of contaminated sediments from an impacted estuarine area and inferring its potential as an indicator of risk and ii) relating the observed effects to a particular set of environmental compounds using several contaminant extraction procedures performed with a progressive series of polar/non polar solvents.

2. Material and methods

2.1. Sediment sampling

Subsamples of the sediment batches that had been previously characterized for contamination and standard physico-chemical parameters (Carreira et al., 2013) were taken for the HepG2 assays. The sediment sample here termed ME (Mira East) was collected from the contaminated channel, at the lowest hydrodynamics site of the estuary (Fig. 1); the reference sediment sample, termed MW (Mira West), consisted of a mostly sandy sediment and was collected at the mouth of the Estuary, in an area with high oceanic influence and a low level of contamination. Table 1 summarizes the main characteristics and contamination patterns of the tested sediments.

2.2. Preparation of sediment extracts

Contaminant extraction was adapted from the protocol of Šrut et al. (2011), with modifications. In brief: sediments were dried at 40 °C in the dark, pulverized and homogenized with a mortar. Contaminants were then extracted with four different sets of solvents. The crude extract was obtained with a mixture of DCM:methanol (2:1). Other sediment samples were extracted using *n*-hexane, DCM or methanol. The solvents were chosen taking into account the increase in polarity (*n*-hexane < DCM < methanol), as proposed by Šrut et al. (2011). The extraction procedure consisted of adding 50 mL of solvent to 30 g of dry pulverized sediment samples. Extraction was done mechanically, by shaking during 15min. Samples were allowed to settle and 25 mL of the solvent supernatant was taken and evaporated at 45 °C. Extracts were then recovered in 3 mL of dimethylsulfoxide (DMSO) and subdivided for cytoassay replication. The final sediment:extract proportion was 10 g of sediment (dry weight) per mL of DMSO. Extract concentrations used in the cell assays were therefore expressed as dry weight sediment-equivalent per volume unit of culture medium (mg SEQ/mL). Extract samples were named as follows: $ME_{DCM/met}$, $MW_{DCM/met}$ (crude extract); ME_{hex}, MW_{hex} (*n*-hexane fraction); ME_{DCM}, MW_{DCM} (dichloromethane fraction); ME_{met}, MW_{met} (methanol fraction).

2.3. Cell culture

The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC No. HB-8065). All culture medium and supplements were obtained from Invitrogen (Carlsbad, CA, USA). Cells were subcultured weekly in DMEM-F12 containing L-Glutamax and HEPES buffer (25 mM), supplemented with 15% heat-inactivated fetal bovine serum (FBSi), 1% penicillin/streptomycin and 1.5% amphotericin B (0.25 mg/mL), in 5% CO_2, humidified

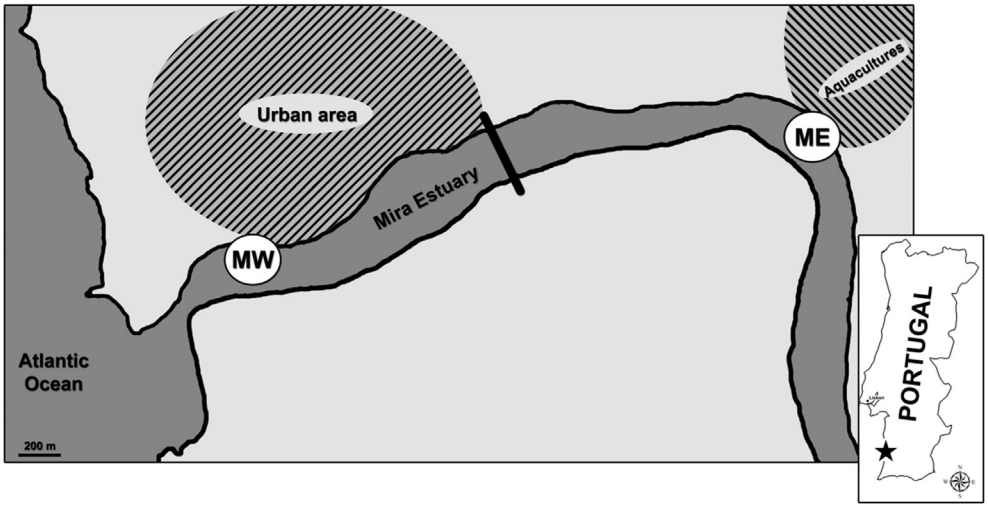

Fig. 1. Map of the Study area, the river Mira Estuary, with the indication of the two sampling sites ME and MW considered in the present work.