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Applying quantitative and semi-quantitative histopathology to address the interaction between sediment-bound polycyclic aromatic hydrocarbons in fish gills

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ABSTRACT

Even though PAHs are considered priority marine pollutants, information on the interaction effects between these compounds is scarce, furthermore under ecologically-relevant circumstances. Semi-quantitative and quantitative histological analyses were enforced on the gills of the seabass (*Dicentrarchus labrax*), exposed to two model PAHs, single or combined, through a series of 28-day laboratory bioassays. Fish exposed to sediments contaminated with either PAH (250–800 ng g^{-1}), isolated or combined, exhibited most significant gill histopathological alterations after 28 days of exposure, as determined through weighted condition indices, especially in animals exposed to the potential carcinogen benzo[b]fluoranthene (B[b]F) and to mixtures of this compound with its lower, non-carcinogenic counterpart Phenanthrene (Phe). Negative correlations between interlamellar hyperplasia (the most remarkable alteration) and goblet cell counts suggest that fish exposed to sediments contaminated with B[b]F or mixed PAHs increased the thickness of epithelial cells as a response to insult, albeit compromising cell differentiation, to which is likely added impaired gas exchange and osmotic balance. In contrast, animals exposed to Phe increased the number of chloride and goblet cells relatively to control fish at early stages of exposure, suggesting then a more efficient protective mechanism. The results also showed that histopathological alterations in mixture-exposed animals do not match the expected additive effects. Overall, the findings indicate that chronic exposures to sediment-bound PAHs, under realistic scenarios, may induce lesions in gills that may imply significant hindering of basal metabolic/homeostatic pathways in marine fish whose interpretation may be hindered by complicated interaction effects and unknown factor involving, more that dose-response, time-dependent effects.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are long listed as priority pollutants, especially due to the known link between these toxicants and neoplastic disease, inclusively in marine fish (e.g., Baumann et al., 1996; Vethaak et al., 2009; Myers et al., 2003). Due to their high risk, environmental quality guidelines have been draw as threshold levels, such as the Sediment Quality Guidelines originally developed by (Chapman, 1990). However, guidelines for PAHs, as many other toxicants tend to dismiss the mixture factor. On their turn, aquatic sediments are regarded as reservoirs of complex mixtures of xenobiotics, primarily, those that have hydrophobic properties, like PAHs, which are invariably present as mixtures. These compounds, characterised by having two or more fused benzene rings are usually originated by incomplete combustion of fuel and other organic matter and tend to be transported to the aquatic milieu through continental runoffs, air born particles and direct anthropogenic inputs (see for instance Douben (2003), for a review). Natural and human-drives processes, such as the remobilization of sediments associated to storm and dredging activities, respectively, may favour the release of PAHs from adsorbed particles back to the water column, potentially rendering them more bioavailable (Martins et al., 2012). The intrinsic physico-chemical properties of PAHs, such as their octanol-water coefficient (K_{ow}), molecular weight and structure,

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interferes with desorption and therefore, bioavailability.

At the same time, PAH uptake, accumulation and detoxification differs between organisms and is also modulated by the chemical properties of the compounds (Porte and Albaigés, 1993; Meador et al., 1995). Thus, distinct mechanisms and thresholds of toxicity are expected, among PAHs, regardless of chemical similarities among the class (Mayer and Reichenberg, 2006), which further complicates estimating the effects of these compounds onto ecosystems. Also, the effects and underlying mechanisms triggered by PAHs have been mostly drawn from experimental research where ecological relevance was often omitted, with respect to concentrations, model organisms, and, most importantly mixtures.

The toxicity of PAHs is derived from detoxification processes, during which these hydrophobic substances are metabolised into more electrophilic, thus more soluble but also more reactive, metabolites through the action of phase I microsomal CYP mixed-function oxygenases (see for instance Stegeman and Hahn (1994)). These mechanisms, termed PAH bioactivation, produce metabolites (e.g. epoxides and dihydrodiols) that may bind to proteins and to DNA (Stegeman and Lech, 1991; Xue and Warshawsky, 2005). Also, the bioactivation generates reactive oxygen species (ROS) (e.g., Cavalieri and Rogan, 1995; Penning et al., 1996; Ohnishi and Kawanishi, 2002, for details on PAH activation and radical formation).

As for other vertebrates, fish have high PAH bioactivation ability through CYP mixed-function monooxigenases (Livingstone, 1998; Uno et al., 2012, for a review), which may render highly reactive and mutagenic PAH metabolites. In fact, it has been suggested that PAHs in aquatic environments are an important risk factor for various health aspects of fish, such as adverse histopathological lesions (Costa et al., 2009; Martins et al., 2015a; Martins et al., 2015c) and genotoxicity (e.g. Martins et al., 2015a), among others.

Although not usually regarded as the primary organ involved in xenobiotic biotransformation (unlike the liver), gills are in direct contact with contaminated water and may endure significant lesions, compromising critical physiological functions, from gas exchange and osmoregulation to excretion. For such reason, fish gill histopathology has long been regarded as an important method in marine environmental toxicology (see for instance, Mallatt (1985), Pandey et al. (2008) and Au (2004)). However, more mechanistic data on the formation of gill histopathological lesions and alterations in fish exposed to mixed PAHs is scarce, furthermore under ecologically-relevant scenarios, such as PAHs bound to sediments in realistic concentrations.

As such, the aim of the present work was to evaluate the histopathological effects of the interaction between PAHs, with distinct chemical and toxicological properties, in the gills of marine fish. For the propose, the European sea bass *Dicentrarchus labrax* (Perciformes: Moronidae), was taken as model due to its high ecological and economical importance, availability through laboratory-assisted brooding and preceding information on exposure to toxicants, since it a species of growing interest among ecotoxicologists (Hallare et al., 2011). Two PAHs were selected as models, both included in the List of Priority Substances attached to the European Water Framework Directive (WFD, updated through the Directive 2008/105/EC), later followed by the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC) and both usually present on contaminated coastal sediments (e.g. Martins et al. (2012)): the non-carcinogen phenanthrene (Phe) and the carcinogen benzo[b]fluoranthene (B[b]F). Phenanthrene, the simplest three-ringed PAH with a bay region, although generally considered to have no carcinogenic activity (being classified by IARC as group 3), has been shown to cause genotoxicity (Oliveira et al., 2007; Martins et al., 2013, 2015a) and to generate reactive oxygen species (Sun et al., 2006; Yin et al., 2007; Oliveira et al., 2007; Martins et al., 2015b) in aquatic organisms. On its turn, benzo[b]fluoranthene consists of five fused aromatic rings and is classified as possible carcinogenic to humans (IARC, group 2B) and has been shown to cause genotoxicity (Martins et al., 2013, 2015a) to clams and fish and to cause carcinogenicity in rats and mice skin (Weyand et al., 1993).

2. Material and methods

2.1. Experimental design

Twenty eight-day laboratorial bioassays were conducted with artificial sediments contaminated with two model PAHs, phenanthrene (Phe) and benzo[b]fluoranthene (B[b]F), singly or combined, at equitoxic and realistic concentrations. Ecological relevance and approximate equitoxicity was endeavoured by selecting two target concentrations (termed C1 and C2), accordingly to the sediment toxicity thresholds guideline retrieved from MacDonald et al. (1996), namely the Threshold Effects Level (TEL) and the Probable Effects Level (PEL): 86.7 and 544 ng g^{-1} (Phe), 88.8 and 763 ng g^{-1} (B[b]F), respectively. Artificial sediments (6% total organic matter, 42.2% fine fraction) were spiked, as described by Martins et al. (2015a,b), with stock solutions of Phe (2500 μg mL^{-1}) and B[b]F (1020 μg mL^{-1}) and with DMSO only (control tests) resulting in nine experimental groups: control, Phe-C1, Phe-C2, B[b]F-C1, B[b]F-C2, co-exposure to Phe-C1 and B[b]F-C1 (M1), to Phe-C2 and B[b]F-C2 (M2), to Phe-C2 and B[b]F-C1 (M3), Phe-C1 and B[b]F-C2 (M4).

The bioassays were prepared according to Martins et al. (2013, 2015b,c). Briefly: 2 L of sediments per replicate was placed in 15 L-capacity tanks to which 12 L of filtered seawater was added. The bioassays were performed in duplicate, with 10 randomly-selected juvenile *Dicentrarchus labrax*, belonging to the same cohort (85.2 ± 8.5 mm standard length; 9.90 ± 2.31 total wet weight) and obtained commercially (MARESA, Spain), being placed in each tank. Fish were fed once a day, aeration was constant and the photoperiod was set at 12 h light: 12 h dark. The physico-chemical parameters of water were monitored daily. To ensure their constancy (temperature = 19.0 ± 0.2 °C, salinity = 31 ± 1, pH = 7.8 ± 0.2, dissolved oxygen between 90% and 94%, and total ammonia = 1.5–2 mg L^{-1}), 25% of the total water volume was changed weekly. Five fish per tank were collected at days 14 and 28 (hereafter referred to as T_{14} and T_{28}), euthanized by cervical sectioning and dissected immediately. All animals were measured for total weight (ww_t) and standard length (L_s). Water and sediment samples were also collected at T_0, T_{14} and T_{28} and stored at −20 °C for PAH analyses.

2.2. Phe and B[b]F analysis in water

Water samples were percolated through speedisks (J.T. Baker) previously conditioned with ethylacetate and methanol, followed by the elution of both PAHs with a mixture of ethylacetate/dichloromethane (v/v) under vacuum, as described by Martins et al. (2013). PAHs were quantified by gas chromatography-mass spectrometry (using Thermo DSQ) in selected-ion monitoring (SIM) mode (Martins et al., 2008).

2.3. Histology

Gill samples (first arches of each animal) were prepared for histological analyses following Martoja and Martoja (1967). In brief: fresh samples were immersed in Bouin-Hollande's fixative (10% v/v formaldehyde and 7% v/v acetic acid to which picric acid was added to saturation) for 48 h. Afterwards, samples were