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Alterations in juvenile flatfish gill epithelia induced by sediment-bound toxicants: A comparative *in situ* and *ex situ* study

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ABSTRACT

Juvenile *Solea senegalensis* were exposed in the laboratory (*ex situ*) and field (*in situ*) to different sediments of a moderately impacted estuary (the Sado, Portugal) for 28 days. A qualitative histopathological screening yielded scant lesions to gills, albeit alterations such as epithelial hyperplasia being evident and more frequent in fish exposed *ex situ*. Fully quantitative traits, namely chloride and goblet cell count and size revealed differences between the two bioassay approaches, with *ex situ* experiments likely enhancing bioavailability of toxicants. Chloride cells endured autolytic processes that could, at least in part, relate to contamination by mixed metals and polycyclic aromatic hydrocarbons (PAHs). Goblet cells did not reveal changes in the chemistry of mucous. Still, their number and size was reduced in fish exposed *ex situ* to the sediments most contaminated by PAHs, with evidence for adaptation. Also, copper histochemistry revealed the potential role of mucocytes in the regulation of metals.

1. Introduction

The gills of fish are the main apical entry organ of waterborne toxicants, whether dissolved or associated to suspended particulate matter. The high surface-to-volume ratio of gills should render these organs particularly sensitive to waterborne toxicants, hence the interest of gill histopathology in biomonitoring and aquatic toxicology (see Au, 2004, and references therein). It is acknowledged, though, that histopathological biomarkers in gills hold just as scant specificity as any other histological traits in fish exposed to pollution while, simultaneously, posing higher constraints with respect to collection, preservation and sectioning of samples (see Mallatt, 1985; and Au, 2004, for reviews). Also, several works addressing fish histopathology in several organs developed either in the laboratory or in the field, reported important issues such as reduced sensitivity of gills towards toxicological challenge (especially if compared to the liver) or the effects of parasites as confounding factors (e.g. Stentiford et al., 2003; Costa et al., 2009). Still, studies comparing *in situ* and *ex situ* bioassays are scarce.

The gills of fish are delicate, highly vascularized, structures that evolved to maximize gas exchange and ion transport (being critical in the maintenance of osmotic balance), through an arrangement of thin lamellae attached to filaments that branch out of gill arches. In spite of the conservation of the main morphological arrangement of gills among the Osteichthyes, there is considerable inter-specific variation resulting from adaptation to different environments (e.g. varying in salinity and dissolved oxygen), which confers distinct sensitivity to toxic insult as well (refer to Mallatt, 1985 and Au, 2004). Not surprisingly, fish gills, for being one of the most important water-body interfaces, have also been found to hold an important population of leukocytes, indicating highly active immunological activity against external aggressors (e.g. Lin et al., 1998).

The teleost gill epithelium is comprised of three main cell types: pavement cells, mucocytes (goblet cells) and the mitochondria-rich chloride cells. The latter two are highly specialized and are chiefly involved in protection and ion transport, respectively. Even in absence of conspicuous histopathological traits (e.g. epithelial hyperplasia, inflammation or even necrosis), goblet cells may undergo changes related, for instance, to the chemical nature of secreted mucosubstances, as reported for variations in salinity (Solanki and

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Benjamin, 1982). However, toxicological studies on the subject are scarce, although it has been suggested that mucous may have a preventing role in sequestering metals and other ions, therefore acting as a barrier against toxicants (Handy et al., 1989). On their turn, gill chloride cells, for which the role in the maintenance of osmotic balance is long known (Keys and Wilmer, 1932), may endure metabolic and structural alterations as a consequence of multiple factors, from salinity shifts to exposure to pollutants (e.g. Karnaky et al., 1976; King and Hossler et al., 1991; Arellano et al., 1999; Costa et al., 2009, 2010). However, the effects and responses of epithelial cells toward toxicants under realistic circumstances (with respect to concentrations, source and combination of pollutants) are not well understood. Flatfish in particular, which due to their benthic disposition even possess very specific mechanisms of gill ventilation to protect them from sediment particles (Yazdani and Alexander, 1967).

The present work aimed primarily at evaluating the effects of sediment-bound estuarine contamination onto the gills of an important flatfish species in SW Europe, the Senegalese sole (*Solea senegalensis* Kaup, 1858; Pleuronectiformes: Soleidae) through a comparative *in situ*/*ex situ* bioassay approach. Previous research with this species, through a series of fully laboratory bioassays revealed that sediments collected from estuarine sites judged to the low-moderately contaminated could induce significant histopathological alterations in gills and other organs (Costa et al., 2009, 2010). It was hitherto hypothesized that sediment collection, handling and testing could have increased toxicant bioavailability by disrupting the physico-chemical steady-state, hence the significance of performing simultaneous *in situ* and *ex situ* bioassays with the same batch of animals. The research is the continuance of the work described in Costa et al. (2011a), during which the livers of juvenile *S. senegalensis* were surveyed for histopathological lesions and alterations following a semi-quantitative approach based on the weighted condition indices (i.e. biological significance × dissemination) proposed by (Bernet et al., 1999). The animals were then exposed to in the laboratory (*ex situ*) and in the field (*in situ*) to sediments from distinct sites of a moderately-impacted estuary, the Sado (SW Portugal), in the spring of 2007. Although significant differences were found between the livers of animals exposed to the different sediments and subjected to distinct types of bioassays, gill samples remained archived without being analysed. By analysing these archived samples, the current work aims specifically at: i) identifying histopathological lesions and alterations in the gills of *S. senegalensis* occurring as a consequence of exposure to contaminated sediments; ii) determining alterations in gill epithelial cells through quantitative endpoints, and iii) assessing differences between field and laboratory bioassays.

2. Material and methods

2.1. Study area

The Sado Estuary is a large ($\approx$240 km^2) estuarine basin located in SW Portugal. The area is characterized by the conflict between the need to safeguard both environmental quality and its high socio-economical importance. Part of the estuary is a natural reserve, whereas the city of Setúbal and its adjacent heavy-industry belt and harbours are important sources of toxicants, together with the agricultural and mining grounds upstream (Fig. 1). Nonetheless, the estuary is regarded as ecotoxicologically heterogeneous and moderately polluted, holding the characteristics of a recovering ecosystem (see for instance Caeiro et al., 2009; and Carreira et al., 2013, plus references therein). Three sites with distinct characteristics were chosen for the present work. The reference site (R), located farthest from pollution hotspots and contaminated sites C_1 and C_2, near the Setúbal's harbour and heavy-industry area, respectively. The sediments' physico-chemical properties and contamination profiles determined at the time of the experimental procedure are summarized in Table 1. For methodological aspects refer to Costa et al., 2011a, 2011b).

2.2. Experimental procedure

The experimental array consisted of 28-day simultaneous laboratory (*ex situ*) and field (*in situ*) bioassays performed with juvenile hatchery-brood *S. senegalensis* (61.0 ± 8.4 mm standard length; 3.1 ± 1.6 g total wet weight), all from the same cohort. Details on the procedure are described in Costa et al. (2011a). In brief: Sediments were collected during May 2007 from the three sites (Fig. 1) and processed immediately for *ex situ* bioassay preparation and physico-chemical analyses. The *ex situ* bioassay apparatus consisted of a semi-static arrangement of 15-L capacity polyvinyl tanks in which were allocated 2 L of fresh sediments (allowed to settle for 48 h before the assays) and 10 L of clean seawater, with permanent aeration and water recirculation, both set to avoid sediment disturbance. Photoperiod was set at 12:12 h light:dark. Weekly 25% water changes ensured constancy of water parameters: salinity = 32.1 ± 0.3, pH = 8.0 ± 0.1 and toxic unionized ammonia (NH_3) was maintained within 0.04 ± 0.02 mg/L. Fish were fed with commercial pellets daily. The *in situ* experimental array consisted of 90 × 90 × 30 cm PVC cages lined with 5 mm-mesh plastic net. The cages were placed at the sediment collection sites (7–9 m depth), in direct contact with the sediment floor, by scuba diving, assuring that the bottom was overlaid with sediment. Fish were not fed during the field experiment but were allowed to feed on natural prey, which was confirmed by post-sampling analyses that revealed polychaetes and gastropods amidst stomach contents. Both sets of bioassays were performed in duplicate. Twenty animals were randomly distributed per experimental replicate. At days 14 (T_{14}) and 28 (T_{28}) ten animals per replicate were collected, euthanized by cervical sectioning and processed for analyses.

2.3. Histological analyses

The first and second gill arches (eyed side) were dissected, fixed in Bouin's solution (10% v/v formaldehyde and 7% v/v acetic with picric acid to saturation) for 24 h at 4 °C, washed in water and archived in 70% v/v ethanol. The samples were then dehydrated with a progressive series of ethanol, intermediately infiltrated with xylene and embedded in paraffin. Sections (5 μm thick), obtained with a Jung RM2035 model rotary microtome (Leica Microsystems, Wetzlar, Germany), were stained with a tetrachrome (TC) procedure (Costa and Costa, 2012), modified to replace Harris' alum Haematoxylin with the brown/black acid-resistant Weigert's iron Haematoxylin to enhance contrast (Costa et al., 2014). In brief: deparaffinated and rehydrated sections were stained with Alcian Blue (pH 0.5, 1 and 2.5 for the histochemical detection of sulphated, mixed and non-sulphated mucopolysaccharides, respectively) for 30 min. Sections were then briefly washed and stained with Period Acid-Schiff's reagent (5 + 15 min, respectively) for neutral/basic polysaccharides and with Weigert's Iron Haematoxylin for 10 min. Counterstaining was achieved with saturated aqueous Picric Acid (5–10 min at 60 °C). The slides were dehydrated, cleared with xylene and mounted with DPX resinous media (BDH, Poole, UK). Histochemical procedures included the Rubeanic Acid (Dithiooxamide) test for metals (mostly copper): staining was achieved o/n at 37 °C in glassware rinsed with nitric acid and ultra-pure water, followed by counterstaining with Nuclear Fast Red (NFR). Details on the histological and histochemical methods can be found in Martoja and Martoja (1967).