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Gill histopathological and oxidative stress evaluation in native fish captured in Portuguese northwestern rivers

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ABSTRACT

The Northwestern Portuguese region is densely populated and highly industrialized, suffering from high anthropogenic pressure. To assess the biological effect of the several pollutants that are constantly released to the water, a biomarker-based biomonitoring is a promising approach that may provide early-warning signals of pollutants exposure. Fish gill is the first target of pollutants action, thus histopathological and biochemical changes may constitute potential biomarkers. To evaluate this hypothesis, three native fish species (barbel—*Luciobarbus bocagei*, chub—*Squalius carolitertii* and nase—*Pseudochondrostoma* sp.) were sampled in Northwestern Portuguese rivers, the gill histopathological changes were qualitative and quantitatively analyzed and the lipid peroxidation and glutathione-S-transferase activity were determined. A multivariate statistical analysis was performed to establish correlations between these biological responses, environmental variables and ecological status. The quantitative evaluation of the main histopathological changes and oxidative stress responses emphasize the differences, among species, in the responses to the presence of contaminants in water. Discriminant canonical analysis showed that filament epithelium proliferation, necrosis and GST activity were the main contributors to discriminate the ecological status classification. In addition, the results showed that a wide range of environmental factors are influencing fish physiology. In conclusion, the gill biological responses, although not reflecting specific contaminants, can be used as biomarkers of ecosystems perturbation.

1. Introduction

The northwestern region is a densely populated and highly industrialized area of Portugal. For many years, it has been subjected to multiple effluents discharges, without any previous treatment (Araujo et al., 1998; Soares et al., 1999). This fact caused a progressive decline in the water quality, which is traduced in poor ecological status of the water bodies. In this Portuguese region, the main problems are the organic contamination and turbidity, the eutrophication and the pollution, which are due to domestic, agricultural and industrial discharges (Oliveira et al., 2005). Chemical analyses may not be sufficient to properly assess the adverse effects of the complex mixture of water contaminants (He et al., 2011). Thus, to assess the action of these on aquatic populations, a biomarker-based biomonitoring is a promising approach to provide early-warning signs of exposure (Viarengo et al., 2000; Au, 2004; Zorita et al., 2007; Tlili et al., 2010).

According to Water Framework Directive (WFD), fish represent one of the key elements to evaluate the rivers ecological status (Scardi et al., 2008; Hermoso et al., 2010). They are present virtually in all environments, and many species have been found to be susceptible to environmental pollutants (Van der Oost et al., 2003). Fish gills are particularly sensitive to water quality, constituting the first target of pollutants, due to their anatomic location, direct contact with the water and quick absorption (Pandey et al., 2008).

The complexity of the environmental contaminants may induce a variety of biological responses, not necessarily correlated (Viarengo et al., 2000; Lopes et al., 2001; De la Torre et al., 2005; Ozmen et al., 2008; Tlili et al., 2010). Accordingly, Pinto et al. (2010) suggested that the biomonitoring process should include the different levels of biological organization, from sub-cellular

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and cellular analysis of tissues and organs, to the of population and community levels.

Several biomonitoring programs have used the histological changes observed in fish organs as biomarkers of aquatic ecosystems ecological quality (Lang et al., 2006; Blazer et al., 2007; Pinto et al., 2010). The histopathology allowed the identification of several changes induced by environmental factors (Schwaiger et al., 1997). The analysis of histological changes in different fish tissues and target organs, such as gill (Nero et al., 2006; Monteiro et al., 2008), has been an instrument widely used in aquatic toxicology to biomonitoring acute and chronic situations (Stentiford et al., 2003; Van Dyk and Pieterse, 2008). Changes in gill epithelium are a consequence of a range of contaminant exposures, with the severity of changes depending on the pollutant concentration and exposure period (Santos et al., 2011). Thus, fish gill histopathology can provide an assessment method to evaluate of the effects of environmental stressors on fish populations (Au, 2004).

Biochemical and physiological indicators can also be used to identify possible environmental contaminations, before the threatening of aquatic organisms' health (Osman et al., 2010). Indeed, biochemical responses, like lipid peroxidation and antioxidant enzymes activities, were previously tested as potential biomarkers of oxidative stress (Dabas et al., 2012). Oxidative damage occurs when the animal's defense mechanisms, responsible for eliminating reactive oxygen species (ROS) are inadequate or insufficient, promoting oxidative stress (Winterbourn and Hampton, 2008; Simonato et al., 2011). ROS are essential for many physiological processes (Droge, 2002), but at high concentrations, it may be deleterious (Oliveira et al., 2010). The oxidative damages may modify DNA, proteins and lipids, leading to mitochondrial bioenergetics failure and, consequently, to cell apoptosis or necrosis (Chuang, 2010). Lipid peroxidation is frequently used as an indicator of cell membrane damage by ROS (Valavanidis et al., 2006).

Xenobiotics biotransformation is an important process because it changes the biological activity and enhances toxic compounds excretion, preventing cell damage. This process includes different enzymes, which function is to render compounds more water-soluble, facilitating their excretion (Simonato et al., 2011). One of these is the phase II enzyme, glutathione-S-transferase (GST) that plays an important role in the detoxification and excretion of xenobiotics. This biotransformation enzyme conjugates xenobiotics with glutathione (GSH), promoting their elimination from the organism (Richardson et al., 2008).

This study aims to evaluate if the gill histopathological and biochemical responses, as biomarkers of exposure, are able to discriminate the ecological status classification of sampling locations. To achieve this, gills from three native species, representative of the northwestern Portuguese rivers ichthyofauna, were collected in locations with different ecological status, previous classified according to the WFD. Gills histopathological changes (e.g. filament epithelium proliferation, lamellar fusion, necrosis and aneurisms) and oxidative stress responses (GST activity and lipid peroxidation) were determined and used to explore possible associations with environmental descriptors and ecological status classifications.

2. Material and methods

2.1. Study area

Portuguese northwestern sampling locations (Fig. 1) were selected taking into account the distance to urban areas, the presence of industrial and/or agricultural activities and the hydrographical structure of the rivers, especially the presence of physical barriers to fish migrations. Furthermore, sampling locations were representative of different ecological status: good, poor and bad. These ecological status, defined according to EU WFD 2000/60/EC (art. 2), express the quality of the structure and functioning of the aquatic ecosystems associated with surface waters. The assessment of the ecological status of water bodies includes a list of parameters indicative of biological, physico-chemical, chemical and hydromorphological quality elements, (Gottardo et al., 2011), classifying it into five quality classes: high, good, moderate, poor and bad (EC (European Commission), 2000).

In the present work, the sampling locations (SL) with a good ecological status classification were Pinhão (SL 7-41°20′38.26″N; 7°35′25.67″W) and Tejão (SL 8-41°21′52.58″N; 7°55′41.08″W), both in Douro river basin. Locations classified with a poor ecological status were: Prado (SL 1-41°36′2.93″N; 8°28′15.99″W), in Cávado river; Ponte da Junqueira (SL 2-41°23′18.01″N; 8°41′24.66″W), in Este river; Trofa (SL 5-41°20′44.30″N; 8°33′50.13″W), in Ave river and Vizela Santo Adrião (SL 6-41°22′14.65″N; 8°16′29.75″W), in Vizela river. Locations classified with a bad ecological status were Graça (SL 3-41°21′59.57″N; 8°41′49.83″W), in Ave river and Caldas de Vizela (SL 4-41°22′24.62″N; 8°18′38.19″W), in Vizela river.

2.2. Sample collection

Water and fish sampling was carried out simultaneously in July of 2011. At each sampling location, water was collected for organic and inorganic parameters analysis. Water temperature, dissolved oxygen, pH and conductivity were measured *in loco* using multiparametric probes (HQ40d Multi, HACH; YSI Ecosense EC300) (Appendix A). The remaining physico-chemical parameters were determined in the University of Trás-os-Monte and Alto Douro chemical analysis laboratory. The procedures employed and the limits of detection are specified in Appendix B.

The choice of the sampling area was made according to the WFD indications. Briefly, in rivers with less than 30 m wide, the length of the sampling stretch was twenty times the wide; in rivers with more than 30 m, the length was ten times its wide. In all sampling points, five to ten individuals of each species (barbel—*Luciobarbus bocagei*, chub—*Squalius carolitertii* and nase—*Pseudochondrostoma* sp.) were captured using pulsed direct current backpack electrofishing equipment with a DC-500 V generator. Fish were then anaesthetized by immersion in 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222), weighed and measured, and immediately euthanized by decapitation. For each fish, two gill arches were randomly sampled for histopathology and preserved in ten percent formaldehyde. The remaining arches, destined for biochemical assays, were immediately frozen, in liquid nitrogen, and kept at −80 °C until further use. The condition factor (CF) was calculated to each fish, according to the following formula: $CF\ (g/cm^3) = (\text{weight}/\text{length}^3) \times 100$.

The fish samplings were conducted in accordance with Ethical Guidelines of the European Union Council (86/609/EEC) and the Portuguese Agricultural Ministry (Portaria no. 1005/92) for the protection of animals used for experimental and other scientific purposes.

2.3. Gill histopathology

After 24 h of fixation, gills were dehydrated in graded ethanol series and embedded in paraffin. The 5 μm thick sections, made in a rotary microtome (Leica RM 2135), were stained with hematoxylin–eosin (H&E) and observed in a light microscope. One gill arch was chosen randomly and, in average, 30 entire filaments per arch were analyzed. Firstly, a qualitative evaluation was made, where the histopathological changes observed in each individual were recorded and the prevalence of each type of change determined. This was calculated as the percentage of fish showing that particular type of lesion. Secondly, and according to Monteiro et al. (2008), a severity gradation scale (SGS) with six degrees (0–5) was applied. The SGS combines the extent and severity of each lesion. The extent was defined as the percentage of filaments with a kind of lesion in each fish sampled. To quantify the severity of each histopathological change, the different levels of severity were attributed following (Monteiro et al., 2008). The severity of each lesion per average of affected filament, was determined as the number of lamellar and interlamellar spaces affected by a given level of severity, divided by the number of filaments showing that type of histopathological change. The degree zero was given to values found in fish with less histopathological changes, and to define the remaining degrees, the extent and severity levels were combined to show an increasing numbers of lamellae and interlamellar spaces per injured filament. All values obtained, from the extent and severity counting, were then divided into numerical intervals and combined to generate the SGS (Appendix C).

2.4. Oxidative parameters and enzyme measurements

Gill samples were weight and homogenized in phosphate buffer (0.1 M, Na_2EDTA 0.001 M, pH = 7.5). Afterwards, homogenates were centrifuged, at 21651 × g (Sigma 3K30) for 20 min at 4 °C, and supernatants were used for all biochemical assays.