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Diffuse sources of contamination in freshwater fish: Detecting effects through active biomonitoring and multi-biomarker approaches

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ABSTRACT

Aquatic organisms are usually exposed to a mixture of xenobiotics that may exert a large effect even in low concentrations, and when information is obtained exclusively from chemical analyses the prediction of the deleterious effects is potentially hindered. Therefore, the application of complementary monitoring methods is a priority. Here, in addition to chemical analyses, an active biomonitoring study using multiple biomarker responses in Nile tilapia *Oreochromis niloticus* was conducted to assess the effects of a contamination gradient along four reservoirs in Iguaçu River. Chemical analysis in the muscle showed high levels of metals in fish from the reservoir closest to an industrialized and environmentally degraded area, however fish exposed to all studied reservoirs showed hepatic alterations (necrosis and inflammatory processes). Also, significant variations of biochemical biomarkers were observed with no clear indication of contamination gradient, since an indicative of higher impact was found in an intermediary reservoir, including high concentrations of biliary polycyclic aromatic hydrocarbons (PAHs). However, nuclear morphological alterations (NMA) were less frequent at the same reservoir. Thus, the multi-biomarker approach allied to active biomonitoring is a practical and important tool to assess deleterious effects of contamination in freshwater, providing data for monitoring and conservation protocols.

1. Introduction

Aquatic ecosystems are under constant chemical stress which may compromise water quality and consequently the health of resident organisms. Complex mixtures of pollutants enter freshwater environment from diffuse sources due to increased urbanization process, and industrial and agricultural activities (He et al., 2011; Liu et al., 2014). These diffuse sources are particularly problematic in large rivers such as Iguaçu River (Paraná State, Southern Brazil).

According to recent data the Iguaçu River is the second most polluted urban river in Brazil (IBGE, 2010) due to several impacting activities occurring at different intensities from the river upper reaches to its outfall. In addition to high rates of domestic and industrial waste disposal in its upper reaches, there is a continuous runoff of contaminants from agricultural activities at its intermediate and lower reaches (Freire et al., 2015). Therefore, the Iguaçu River may present distinct sets of degradation along its shores, which demands a more comprehensive approach to encompass the complexity of biological responses to these environments.

Biomarker methodologies have previously been combined with active biomonitoring (ABM) (He et al., 2011; Wepener et al., 2005), which involves the transplantation of organisms from one place to another to compare the biological responses. In general, organisms are moved from clean to polluted sites (He et al., 2011; Smolders et al., 2002), although the reciprocal procedure can be applied to demonstrate the recovery of organisms (Sutton et al., 2004). ABM studies have been conducted using several taxa, including bivalves, polychaetes, algae and fish (Rotter et al., 2011; Díaz-Jaramillo et al., 2013; Tsangaris et al., 2011; Oikari, 2006), usually placed in assay chambers or cages. This approach allows: (i) reducing the sources of variability related to biotic factors by using standard organisms (surrogate species), presenting similar physiological characteristics; (ii) providing an environmental realist exposure and thus the integration of the effect of many factors (temperature, pH, conductivity, contamination changes over the time) that may influence the toxicity of aquatic systems; and (iii) using life history traits related to population dynamics (survival, growth, reproduction and feeding rates) as toxicity markers, what is not possible with harvested native organisms (Liber et al., 2007)

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Biomarkers are widely used in monitoring programs due to their sensitivity to environmental stressors. By measuring biological responses at different levels of organization in the same organism, i.e. a multi-biomarker approach, it is possible to obtain an integrative overview of how exposure to pollutants impacts the biota (Popovic et al., 2015, Babić et al., 2016). This approach is much more reliable and it has become crucial to evaluate effects of chronic exposure to complex and dynamic mixture of pollutants (Wepener et al., 2005; Linde-Arias et al., 2008; Osório et al., 2013; Freire et al., 2015; Yamamoto et al., 2016).

In a multi-biomarker approach, changes in the antioxidant system of cells are one aspect that can be investigated and may reflect an environmental impact on organisms at a subcellular level (van der Oost et al., 2003; Hellou et al., 2012). An impaired antioxidant system may lead to damage to biomolecules, such as lipids, proteins and DNA. Since vertebrates metabolize the polycyclic aromatic hydrocarbons (PAHs) the detection and quantification of these metabolites in the bile fluid is another important marker of exposure. The effects of pollutants may also be observed in organisms' organs since the structure and organization of tissues are tightly coupled to cellular and molecular processes. Morphological alterations of target organs can indicate disorders resulting from both acute and persistent exposure to toxic metals, organic pollutants and complex mixtures (Oliveira Ribeiro and Narciso, 2014).

Oreochromis niloticus (Nile tilapia) is a widely distributed species in tropical ecosystems and is often used as a model organism in experimental studies, due to its high tolerance to variations in salinity, temperature and adaptability to rearing systems (Linde-Arias et al., 2008; Turra et al., 2010).

The effectiveness of monitoring programs relies on the selection of relevant biomarkers allied to a comprehensive experimental design. For this reason, an active biomonitoring was conducted to assess the effects of a putative gradient of pollution along four cascading reservoirs in Iguaçu River (Segredo - SE, Salto Santiago - SS, Salto Osório - SO and Salto Caxias - SC), using *Oreochromis niloticus* as the model species. Segredo (SE) is the closest reservoir to the metropolitan region of Curitiba (MRC), which receives substantial discharges of pollutants due to intense urbanization and industrial activities (IAP, 2009). Owing to its distance from MRC, the Salto Caxias reservoir is characterized by a low degree of contamination, expected to have a better water quality, and so considered in the current study as a reference site.

Thus, we assume there is a pollution gradient from Segredo to Salto Caxias reservoirs, following a significant input of complex mixtures of pollutants from MRC, and we hypothesize that if the water quality is indeed compromised, then biomarkers will show altered patterns of response along this gradient. Due to the proximity to MRC, fish would show pronounced changes at all biological levels at Segredo reservoir compared to the reference site, i.e. increased morphological alterations in liver and gill tissues and overall significant differences in biochemical and genetic biomarkers. This pattern of response is expected to consistently subside towards Salto Caxias, following a decrease in contamination, which will be measured by metal concentration in the water and fish tissue and PAHs metabolites in fish bile.

2. Materials and methods

2.1. Study area

The Iguaçu River (1.060 km of extension) crossing all Parana State, Southern Brazil, is the most important river in a 72.000 km^2 basin. This river is marked by the presence of cascading reservoirs jointly operating to generate electricity at its lower reaches: Segredo, Salto Santiago, Salto Osório and Salto Caxias (Fig. 1).

2.2. Fish sampling and transplantation experiments

Two hundred and forty adult specimens of *O. niloticus* (21.2 ± 3.0 cm length; 187.2 ± 74.8 g weight; mean ± standard deviation) were obtained from an aquaculture facility located in the city of Toledo, Paraná - Brazil. The fish were transported under constant oxygenation and allocated to 8 m^3 net-cages (60 fish per cage) randomly installed across the four reservoirs on October 2015 and kept for 60 days until biological sampling for biomarkers analyses. Considering that mortality could occur at the beginning of the study, we started the experiment with a large number of animals (60 fish) so that we could have an adequate number of individuals at the end of the experiment in each tank 20–30).

After 60 days of exposure in reservoirs water, 20–30 individuals were retrieved per studied site and anesthetized with MS222 (tricaine methane sulfonate, 0.02 g l^{-1}). The blood was sampled through caudal vein puncture with heparinized syringes and smeared on glass slides for counting the micronucleus and other nuclear morphological alterations. Then, the individuals were killed by cervical section and dissected in the field to minimize stress. Biological samples of liver (half of the organ) were fixed in ALFAC solution for light microscopy, and gills were fixed in Karnovisk solution for scanning electron microscopy.

For biochemical analysis, a portion of muscle and liver (half of the organ) samples were preserved at −80 °C. All procedures using these fish were performed according to the NIH guidelines, and were approved by the Committee on Ethics in Animal Experimentation of the Federal University of Paraná (http://www.bio.ufpr.br/portal/ceua/).

2.3. Metal detection in water and fish

Metals (Co, Cd, Cr, Cu, Mn, Ni, Pb, Zn) were quantified in the water, and fish's liver and muscle. The water was sampled at 30 cm below the surface, preserved with 0.5% of HNO_3, and kept at 4 °C protected from light (Yamamoto et al., 2016). The liver and muscle of fish were stored at −75 °C and toxic metals bioaccumulation was analyzed according to Cotta et al. (2006). After acid digestion (Method US EPA 3005A for water; Method US EPA 3050B for tissue samples), the detection of metals was performed using a Flame Atomic Absorption Spectrometer (FAAS, Varian, AA 240FS).

2.4. Polycyclic Aromatic Hydrocarbons (PAHs) in Bile

PAHs (2–6 rings) were detected by fixed-wavelength fluorescence according to Aas et al. (2000) with minor modifications. The bile extracted from 3 individuals were pooled and stored in amber glass vials at −80 °C until analysis. Samples were diluted in 48% ethanol (1:1000) and added to black 96-well microplates for fixed wavelength fluorescence (FF) measurement. Excitation-emission wavelength pairs 288:330, 334:376, 364:406 and 380:422 nm were employed to detect naphthalene-derived metabolites (2-ring), pyrene-derived metabolites (4-ring), benzo[a]pyrene-derived metabolites (5-ring) and benzo(ghi) perylene (6-ring), respectively (Aas et al., 2000).

2.5. Histopathological biomarkers

Liver samples were preserve by immersion in ALFAC solution (70% ethanol, 4% formaldehyde, 5% glacial acetic acid) for 16 h, dehydrated in a graded series of ethanol, diaphanized in xylene, and embedded in Paraplast Plus® (Sigma, St Louis, USA) in a Micron® Tissue Processor. Sections of 5 μm thick were obtained, stained with hematoxylin/eosin and observed under a light microscope for histopathological analyses, according to Bernet et al. (1999) with minor modifications.

The second right gill arch was washed in phosphate buffer saline and preserved by immersion in a modified Karnovisk's solution (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for at least 24 h. Gills were dehydrated in ethanol, submitted to the critical point drying using liquid CO_2, metalized with gold and examined using a JEOL JSM-6360LV scanning electron microscope.