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The Cichlidae fish *Geophagus brasiliensis* (Quoy & Gaimard, 1824) has suitability as a sentinel species for changes caused by xenobiotics?

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ABSTRACT. Industrial wastewater and agricultural practices are among the main activities discharging organic pollutants, such as Polychlorinated biphenyl (PCB) congeners and organochlorine insecticides (e.g. DDT - dichloro diphenyl trichloroethane), to the environment. In this study, we used the native Cichlidae fish species *Geophagus brasiliensis* as a sentinel to evaluate the hepatic 7-ethoxyresorufin-*O*-deethylase EROD activity, a biomarker of exposure to CYP1A-inducing pollutants, to assess the bioavailability of xenobiotics in two reaches of a large lotic system in Southeast Brazil: a less disturbed area (site 1) in the upper stretch, and an area in the middle stretch, which receives various industrial and agricultural effluents from upstream cities (site 2). In addition, *G. brasiliensis* were exposed to a single dose of 50 mg kg^{-1} beta-naphthoflavone (BNF) or of 50 mg kg^{-1} dimethylbenzoanthracene (DMBA) to test the effects on, respectively, the hepatic EROD activity in S9 supernatant fraction, and the frequency of micronucleated erythrocytes three days after the i.p. treatments, and compared to an unexposed group, to test its potential as a sentinel for biomonitoring studies. The EROD activity was approximately two-fold higher in fish from the impacted stretches than in fish from the less disturbed stretches ($p < 0.05$). Micronuclei (MN) frequency was also significantly different ($p < 0.01$) in DMBA-treated fish. The induced EROD activity in the impacted site suggests that organochlorinated pollutants are reaching the biota of the Paraíba do Sul River, confirming the suitability of *Geophagus brasiliensis* as a useful sentinel species to detect changes caused by xenobiotics.

Keywords: EROD; micronuclei; fish; sentinel species; river; biomonitoring.

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Brief history of pollutants and biomarkers

Human activities have contributed to ecosystem degradation, being among the main causes of the current environmental crisis (Monastersky, 2015, Young, McCauley, Galetti, & Dirzo, 2016, Libang, Jie, Xiaoyan, Fang, & Wenjuan, 2019, Nathaniel, Yalçiner, & Bekun, 2021). Environmental contaminants promote intense degradation, resulting in both ecosystem alterations and harmful outcomes to organisms (Hernández et al., 2013, Biswas et al., 2018). Chemical compounds, pesticides, and effluents released by industries and other human activities and settlements are frequently the causes of environmental contamination. Their effects are often threats to the biological diversity of impacted ecosystems, as well as to human health (Rose & Ruppel, 2015, Gavrilescu, Demnerová, Aamand, Agathos, & Fava, 2015, Burri, Weatherl, Mooeck, & Schirmer, 2019).

Aquatic ecosystems are often misused as sinks for many organic chemicals, such as polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCs), polychlorinated biphenyls (PCBs) and polychlrorinated dibenzo-dioxin/furan (PCDDs/PCDFs) (Perelo, 2010, Verhaert et al., 2017). Impacts of such contaminants are often only identified once organism or population level effects are evident (Adams, 2001). Even if these substances reach rivers and lakes in small amounts, they tend to bioaccumulate along the food chain, and eventually can reach harmful concentrations in top predators, including humans (Costa and San'tana, 2008, Verhaert et al., 2017), and may affect sensitive species or larval stages of the biota, impairing the ecosystem healthy functioning and compromising their services (Chagnon et al., 2015, Gilbert, 2016). Biochemical markers (biomarkers) represent the initial, sub-organism, biological responses to contaminants, and have been developed and largely used to aid the detection of early indications of environmental impact

(Van Der Oost, Beyer, & Vermeulen, 2003, Braga et al., 2018). One of the most widely used biomarker of exposure to organic pollution is the induction of hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity in fish (Whyte, Jung, Schmitt, & Tillitt, 2000). Increased levels of EROD are indicative of elevated detoxification activity induced upon exposure to organic pollution (Van Der Oost et al., 2003). The ability to upregulate the EROD activity above basal levels varies among fish species (Whyte et al., 2000) and, the selection of a species with an appropriate potential to induce EROD activity in response to contamination is crucial to the early assessment of environmental impacts (Adams, 2001).

The evaluation of biomarker responses in sentinel fish species is widely applied to biomonitoring programs as early warning tools to indicate the exposure to xenobiotics and damages caused by acute or chronic exposure (De La Torre, Ferrari, & Salibián, 2005). The use of most biomarkers applied to environmental monitoring has been standardized in species from the northern hemisphere (e.g. rainbow trout, *Oncorhynchus mykiss*; Atlantic salmon, *Salmo salar*; and mummichog, *Fundulus heteroclitus*) (Van Veld, Vogelbein, Cochran, Goksøyr, & Stegeman, 1997, Blewett, Weinrauch, Delompré, & Goss, 2017, Stankevičiūtė, 2018).

Here, we analyzed the expression of CYP1A activity and frequency of micronuclei in native cichlid *Geophagus brasiliensis* (Quoy & Gaimard 1824), which is abundant and widely distributed in the coastal drainages along the eastern and southern Brazilian coast, in both lotic and lentic conditions and even in brackish coastal lagoons (Menezes et al., 2007, Fishbase, 2019). This species feeds mainly on invertebrates by stirring up the substrate (Pereira, Smith, & Espíndola, 2004), presents sexual dimorphism, with males reaching larger sizes than females (Reddon, Gutiérrez-Ibáñez, Wylie, & Hurd., 2009).

In the present study, 7-ethoxyresorufin-*O*-deethylase (EROD) activity, a reaction catalyzed mainly by the CYP1A enzyme (Whyte et al., 2000), was used to assess fish exposure to CYP1A-inducing pollutants in two municipalities in the upper and upper middle Paraíba do Sul River, Paraibuna and Canas, respectively. Complementarily, we carried out an initial assessment of the *Geophagus brasiliensis* ability to induce the EROD activity and the frequency of micronucleated erythrocytes (MN). While the induction of EROD activity is a suggestion of exposure to classical CYP1A inducers (e.g. PCBs and PAH), an increased MN frequency indicates damage caused by the exposure and absorption of mutagenic pollutants. The aim of this study was to test the hypothesis that *Geophagus brasiliensis* is suitable as a sentinel species to detect changes caused by xenobiotics.

Study area

The Paraíba do Sul River - PSR (Figure 1) is 1,150 km long and crosses the three most industrialized and densely inhabited states of Brazil, namely São Paulo, Rio de Janeiro and Minas Gerais. Its headwaters are located in Bocaina Mountains, at 1,800 m altitude and the estuary is in the Atlantic Ocean to the north of the state of Rio de Janeiro. The basin is situated between the coordinates 20° 26' and 23° 39'S, and 41° and 46° 30'W, covering an area of 55,500 km^2. Water quality decreases substantially as the river descends from its headwaters towards the river mouth (Carvalho & Torres, 2002). The upper reaches are less changed as they are far from big cities and plantations, and the middle-upper reaches are critically changed.

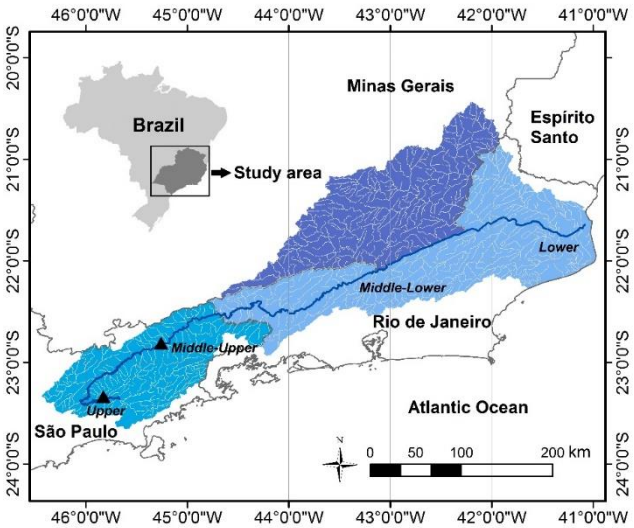

Figure 1. Paraíba do Sul River basin, the two sampled stretches (upper and middle upper reaches) are indicated by black triangles.

Fish collection and handling

Fish were caught at two sampling sites along the PSR upper and middle reaches (Figure 1): 'Site 1' (Paraibuna – less disturbed site, with high water quality and surrounded by well-preserved stretches of Atlantic Forest). This was considered our control or the least disturbed site, because it is the least altered by anthropogenic influence; and 'Site 2' (Canas - further downstream, which receives various industrial and agricultural effluents from upstream cities).

Fish used in bioassays were caught in the Preto River (22º05′02″S; 43º33′35″W), a tributary of the Paraíba do Sul River located at the Mantiqueira mountains, in a relatively well-preserved area, far from large pollution sources. To keep fish alive after capture, individuals were placed in a water tank of 500 L, with continuous water aeration and transported to the fish ecology laboratory of the *Universidade Federal Rural do Rio de Janeiro* (LEP/UFRRJ), where they were acclimated for one week in three 700 L tanks with running water, aerated and naturally dechlorinated. The average water temperature was 24°C (23 - 25°C) and fish were subjected to natural photoperiod. All individuals were fed a pelleted diet twice a day.

This study was authorized by a permanent license for collection of zoological material – SISBIO 10707/Normative Instruction ICMBio 03/2014, and follows the ethical rules applicable to the use of animals in teaching and/or research based on the provisions of the Brazilian law (Federal Law 11794 as of October 08, 2008). All individuals were weighed and measured for total length.

Exposure to xenobiotics

The tested individuals were grouped into three categories: 1 - control, n = 9; 2 - treated with beta-naphthoflavone (BNF), n = 4; and 3 - treated with dimethylbenzoanthracene (DMBA), n = 2. The control group did not receive any treatment and individuals were euthanized for evaluation of basal EROD activity and micronuclei frequency. The other two groups received 50 mg kg^{-1} of the correspondent drug, via intraperitoneal injection (i.p.). Three days after exposure, fish were euthanized by decapitation, the liver was removed quickly and carefully, wrapped in aluminum foil and stored in liquid nitrogen for transportation to the environmental toxicology laboratory of the *Fundação Instituto Oswaldo Cruz* (Fiocruz), when they were transferred to a -80°C freezer.

Microsomal fraction preparation

Livers were thawed in ice bath, homogenized in Potten Elvehjem homogenizer with Teflon pistils in Tris-EDTA buffer (50 mM Tris, 1 mM EDTA, 250 mM sucrose, 20% glycerol, pH 7.4) in a volume corresponding to four times their weight. Homogenate was then centrifuged at 9,000 g, at 4°C, for 30 minutes and the supernatant was collected and stored in -80°C freezer until protein dosage.

Determination of total protein concentration

The quantification of total proteins in S9 fractions was performed using the colorimetric method described by Bradford (1976), adapted for microplate (Molecular Devices SpectraMax Plus 384). Calibration curves were constructed with bovine serum albumin - BSA (Sigma Chemical) 1.4 mg mL^{-1} diluted in phosphate buffer (50 mM KH_2PO_4 and 150 mM NaCl, pH 7.2) to obtain appropriate concentrations. For the determination of protein concentration, S9 fractions were diluted 1:30, 5 μL the diluted sample was poured into microplate wells, followed by the addition of 250 μL Bradford reagent (Merck) in each well. Each value of optical density was converted to protein concentration, using the standard curve and correcting for the dilution factor. Protein concentration in S9 fractions was calculated as the mean value of three replicates for each sample, with a maximum coefficient of variation of 10%, and expressed as mg protein mL $S9^{-1}$.

Determination of EROD activity

EROD activity was measured in a spectrofluorometer microplate (Molecular Devices SpectraMax Gemini). Initially, dibasic potassium phosphate buffer was added to each well. Then, the appropriate volume of S9 fraction to sum 25 micrograms of total protein per well and the substrate 5μM ethoxyresorufin (Sigma) were added. The microplate was incubated at 30° C for 2 minutes and the reaction initiated by adding NADPH regeneration system in each well (0.25 mM ß-NADP, 2.5 mM $MgCl_2$, 5 mM glucose-6-phosphate, and 0.5 units glucose-6-phosphate dehydrogenase per milliliter incubation mixture). The reaction occurred for 10 minutes, and quenched by the addition of acetonitrile. The final product, resorufin, was quantified in a spectrophotometer (550 nm excitation, 582 nm emission).

Procedures for micronuclei (MN)

The MN frequency was evaluated in erythrocytes of *G. brasiliensis*. The final result of each group was expressed as the mean value of the frequency found in each fish of the same group (‰). Only nucleated red blood cells with intact nuclear and cytoplasmic membranes were counted. Micronuclei were considered as corpuscles when presented 1/3 - 1/20 nucleus size, being clearly separated, with distinguishable edges, same color and refringence.

A drop from peripheral blood was directly smeared on slides and air-dried. Smears were subsequently fixed in methanol for 10 min., and stained with 10% Giemsa solution for 6 min. The MN frequency evaluated, by scoring at 1,000× magnification using an Olympus BX 45 microscope, a minimum of 1,000 cells, and the final results were expressed as the number of micronuclei per 1,000 cells. For standardizing and reducing abstraction errors, all cells were examined by the same person.

Statistical analysis

The mean EROD activity and micronuclei frequency (MN) of the three groups (control; treated with BNF; treated with DMBA) were compared by analysis of variance (ANOVA) followed by *a posteriori* Tukey test ($p < 0.05$) to determine which means were significantly different. Data were arcsine transformed before these parametric analyses.

All individuals used in this study were adults, weighing from 45 to 205 g and with total length ranging from 133 to 228 mm (Table 1).

Results and discussion

The mean EROD activity measured in the liver S9 fractions of *G. brasiliensis* collected at site 2, in the upper-middle reaches of the Paraíba do Sul River, was approximately two-fold higher ($p < 0.05$) than the values found in fish sampled in the least disturbed area, site 1, in the upper stretch (Table 1).

Although significant ($p < 0.05$), the induction factor (i.f.) of EROD activity three days after exposure to BNF (i.p. 50 mg kg^{-1}) (Table 2; Figure 2) in the liver of *G. brasiliensis* (i.f. = 1.3) was below the expected, considering the results for other species of this fish family (Parente, Oliveira, & Paumgartten, 2008). *Oreochromis niloticus*, another Cichlidae species, showed a 7-fold higher in EROD activity after exposure to the same agent using the same protocol (Parente et al., 2008). Nonetheless, EROD induction in *G. brasiliensis* has been used for monitoring purposes, with i.f. varying from 4 to 16-fold higher (Wilhelm Filho, Torres, Tribess, Pedrosa, & Andsoares, 2001, Parente et al., 2008, Clemente et al., 2010, Morado, Parente, Araújo, Paumgartten, & Gomes, 2018). At least two other Cichlidae species have been tested for EROD response after exposure to potential CYP1A inducers. *Pseudetroplus maculatus* exposed to one-fifth and one-tenth of chlordecone' LC50 had, respectively, a 10 and 5-fold increase in liver EROD, while the exposure of *Australoheros facetus* to endolsufan had no effect on EROD activity (Crupkin et al., 2013, Asifa & Chitra, 2017).

Table 1. EROD activity measured in liver S9 fractions of *Geophagus brasiliensis* in two stretches of the Paraíba do Sul River: site 1 – upper section (least-disturbed), and site 2 – middle-upper section (disturbed).

Sites	Weight (g)	Total Length (cm)	Liver weight (g)	EROD	*n*
1	82.50 ± 40.78	15.93 ± 1.73	0.95 ± 0.54	97.40 ± 4.51	10
2	152.90 ± 42.41	20.07 ± 1.92	1.30 ± 0.54	170.30 ± 8.11 *	6

Table 2. Summary for biological data of *Geophagus brasiliensis* used in this study. Control = untreated; BNF = treated with beta-naphthoflavone; DMBA = treated with dimethylbenzoanthracene.

Group	Weight (g)	Total Length (cm)	Liver weight (g)	*n*
CONTROL	87.6 ± 36.0	17.6 ± 2.1	1.6 ± 0.2	9
BNF	94.4 ± 51.8	17.9 ± 3.0	1.6 ± 1.2	4
DMBA	148.6 ± 83.6	20.8 ± 4.7	2.0 ± 1.7	2

The induction factor observed in fish sampled in Site 2 had the same magnitude than detected in response to the exposure to 50 mg kg^{-1} BNF, suggesting that fish in site 2 of the Paraíba do Sul River are exposed to relevant concentrations of organic pollutants.

The exposure to DMBA caused a 4.5-fold increase in the frequency of micronucleated erythrocytes in *Geophagus brasiliensis* (Figure 2). This induction factor is higher to the one reported for the same species in response to 10 and 43 μM of copper sulphate (i.f. 2) (Benincá et al., 2012), but lower to the one reported after

exposure to 0.04 mg g^{-1} body mass of cyclophosphamide (i.f. 19) (Campos Júnior et al., 2016, Morais et al., 2016) and in specimens caught in polluted waterbodies (Benincá et al., 2012, Campos Júnior et al., 2016, Morais et al., 2016). Other Cichlidae species, *Australoheros facetus*, showed a similar increase when exposed to endosulfan (i.f. 4) and methyl methanesulfonate (i.f. 5) (Crupkin et al., 2013).

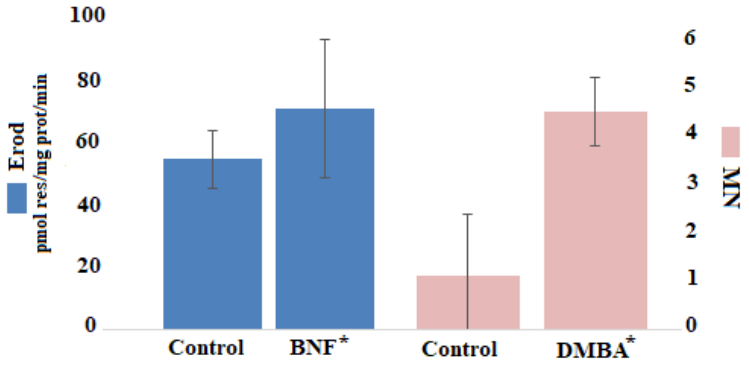

Figure 2. Induction of EROD activity three days after intraperitoneal exposure to 50 mg kg^{-1} β-naphthoflavone (BNF). EROD values (mean ± standard error) are shown for the control (cont) and in the BNF - treated groups. * indicate statistical significance ($p < 0.05$). Micronuclei frequency (MN, ‰) (mean ± standard error) in different groups: control (cont.) and treated with dimethylbenzoanthracene (DMBA).

Here, a native Cichlidae species with a wide-range distribution, *Geophagus brasiliensis*, showed only mild responses to classical toxins, which can cause strong responses in EROD activity and the frequency of micronucleated erythrocytes in species from temperate climate. Nonetheless, EROD activity was efficient to detect differences between the control and the impacted site, indicating the presence of xenobiotics, probably due to the chemical substances used in industries and plantations of upstream municipalities. This indicates that *G. brasiliensis* is suitable as a sentinel species for changes caused by xenobiotics. In this sense, the higher CYP1A activity in *G. brasiliensis* in site 2 – upper middle stretch (disturbed) comparing to both site 1 – upper stretch (less disturbed) and to the bioassay result, suggests warning levels of xenobiotics in the Paraíba do Sul River stretch, site 2. Therefore, further studies are required to better understand the chemical contaminants in this Paraíba do Sul River stretch, as well as their dynamics and effects on environment and living organisms.

Conclusion

The Cichlidae fish *Geophahus brasiliensis* is a suitable sentinel species for changes caused by xenobiotics in Neotropical rivers. These pollutants are chemical substances used in industries and plantations that are discharged into rivers that drain cities and other populated areas. It is suggested that organochlorinated pollutants are reaching the biota of the Paraíba do Sul River. However further studies are required to better understand the effects of these contaminants on living organisms from freshwater systems.

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