**Induced CYP1A activity and DNA damage in fishes from the middle reach of Paraíba do Sul River basin, southeastern Brazil**

Short Title: CYP1A activity and DNA damage in fishes

**ABSTRACT.** The Paraíba do Sul River (PSR) drainage basin in Southeastern-Brazil spreads over one of the most industrialized and densely populated regions of the country. The impact of chemical contamination on PSR basin seems to be more pronounced in its middle reach where a number of potentially polluting plants are located. In this study we used hepatic EROD activity – a biomarker of exposure to CYP1A-inducing pollutants (e.g. PAHs, PCDD/Fs, PCBs) - and the incidence of micronucleated erythrocytes (Mn) in the peripheral blood - a biomarker of effect of DNA-damaging agents (e.g. PAHs) – to evaluate the effects of pollution on two native fishes, *Geophagus brasiliensis* and *Pimelodus maculatus*. Results showed that the incidence of Mn and EROD in *G. brasiliensis* and *P. maculatus* from the two most downstream sites (Três Rios Town and Piabanha River) were markedly higher than the incidence and EROD recorded in fishes from the most upstream site (Funil Reservoir). The study findings are consistent with the view that induced CYP1A activity and increased DNA-damage are found in fishes caught in sampling sites located downstream the river basin stretch where there are a number of industries that are potential sources of PAHs and CYP1A-inducing contaminants.

**Keywords:** Ethoxyresorufin-O-deethylase **(**EROD) activity, *Geophagus brasiliensis*, micronuclei, *Pimelodus maculatus*, polycyclic aromatic hydrocarbons (PAHs).

**RESUMO.** O Rio Paraíba do Sul (RPS) drena uma das mais industrializadas e densamente povoadas áreas do Sudeste do Brasil. O impacto de contaminação química no RPS parece ser mais pronunciado no segmento médio da bacia onde localizam-se um grande número de indústrias potencialmente poluidoras. Neste estudo, nos utilizamos a atividade hepática EROD – um biomarcador de exposição a poluente indutores da CYP1A (e.g. HAPs, PCDD/Fs, PCBs) – e a incidência de eritrócitos micronucleados (Mn) no sangue periférico – um biomarcador de efeitos de agentes de danificação do DNA- (e.g. HAPs) – para avaliar o efeito de poluição sobre dois peixes nativos, *Geophagus brasiliensis* e *Pimelodus maculatus*. Os resultados apresentaram que a incidência de Mn e EROD em *G. brasiliensis* e *P. maculatus* nos dois locais no trecho mais baixo (Três Rios e Rio Piabanha) foram marcadamente mais elevados do que a incidência e a atividade EROD registradas em peixes dos locais mais à montante (Reservatório do Funil). Estes resultados são consistentes com a visão de que atividade indutora de CYP1A e elevadoras de danos no DNA são encontradas em peixes capturados em locais abaixo do trecho onde um grande número de indústria que são potenciais fontes de poluição de indutores de contaminantes HAPs e CYP1A.

**Palavras-chave:** AtividadeEthoxyresorufin-O-deethylase **(**EROD), *Geophagus brasiliensis*, micronúcleo, *Pimelodus maculatus*, hidrocarbonetos aromátics policíclicos (HAPs)

**Introduction**

The Paraíba do Sul River (PSR) is one of the longest and most important rivers of Southeastern Brazil. It rises in the Sea Mountain range in Sao Paulo state and flows 1.140 km before meeting the Atlantic Ocean on the Northern Coast of Rio de Janeiro State (Fig. 1). Since the PSR drainage basin spreads over one of the most industrialized and densely populated regions of the country, it has suffered a strong impact from human activities. The PSR basin is a major source of potable water supply for Greater Rio de Janeiro City and for a number of other smaller cities, and two hydroelectric power plant reservoirs are located in the middle reach of the river (Funil and Lajes Reservoirs). The pollution of PSR waters by industrial effluents and untreated domestic sewage, however, has been cause for deep concern during the last decades (Nascimento, Araújo, Gomes, Mendes, & Sales, 2012; Santos, Albieri & Araújo 2013).

 The impact of chemical contaminants on river waters and biota seems to be more pronounced in the middle reach of PSR basin where many potentially polluting plants are located, including several chemical and metallurgic industries. Since a huge amount of coal (coke) is burned in furnaces of steelworks, high levels of PAH were found in the PSR sediments in the vicinity of Volta Redonda town where is located one of the oldest and largest Brazilian steelworks (Torres, Malm, Vieira, Japenga & Koopmans 2002). In August 1988, a fire in a plant of Thyssen Foundry Brazil where old transformers and capacitors were stored, resulted in a discharge of an estimated amount of 200 kg of Askarel, a mixture of PCB congeners, in the middle reach of PSR near Barra do Pirai Town, Rio de Janeiro State (Coelho, 1990). Although it has been reported that PSR sediments are contaminated by heavy metals, PAHs and POPs, there have been relatively few studies on the impact of this chemical pollution on the river native biota.

This study was undertaken to investigate the effect of pollution on liver EROD activity and on the incidence of micronucleated erythrocytes in two native fish species (*Geophagus brasiliensis* (Quoy & Gaimard, 1824)*,* Cichlidae, and *Pimelodus maculatus* Lacépède, 1803*,* Pimelodidae) which are both abundant and widely distributed along the PSR Basin. EROD is catalyzed by CYP1A, a CYP enzyme well conserved among vertebrates (Stegeman, Woodin, Singh, Oleksiak & Celander 1997). Since expression and activity of CYP1A are enhanced by AhR ligands, such as PCDD/Fs, PCBs, PAHs and other compounds, induction of EROD activity has been one of the most widely employed biomarkers of exposure to CYP1A-inducing pollutants (Haasch, Prince, Weiksnora, Cooper, Leach, 1993; Goksoyr, 1995; Bainy, Woodin & Stegeman, 1999; Parente, De-Oliveira, Silva, Araújo, & Paumgartten, 2004; Parente, De-Oliveira & Paumgartten 2008, Parente, De-Oliveira, Beghini, Chapeaurouge, Perales, & Paumgartten 2009, Parente, Santos, Oliveira, Torres, Araujo, Delgado & Paumgartten, 2015; Pathiratne, Chandrasekera & Pathiratne, 2009). The incidence of micronuclei in peripheral blood erythrocytes, on the other side, is increased by exposure to xenobiotics that cause DNA damage (genotoxic and clastogenic agents). The foregoing biomarkers were thus used to verify whether fishes examined in this study were affected by organic pollutants, which are CYP1A inducers and or genotoxic agents. As aforementioned, the intensive industrial activity along the PSR basin is a potential source of environmental contaminants with CYP1A-inducing (PCDD/Fs, PCBs, PAHs) and genotoxic properties (PAHs).

**Materials and methods**

**Study area**

The Paraíba do Sul River (PSR; 20º26’-23º38’S, 41º00’-46º30’W) is 1140 km long, with a 57,000 km2 watershed (Fig.1). PSR middle reach flows 400 to 600 m above sea level and drains ancient, predominantly sedimentary, soils formerly covered by a tropical rain forest. This eco-region is characterized by both unconsolidated and semi consolidated sand, gravel, silt and clay, with basalt outcroppings, low mountains, low nutrient soils, fragmented semi-deciduous seasonal rain forest, and poor croplands (Carvalho & Torres, 2002). The climate is mesothermic with high relative humidity, hot and wet summers and dry winters. Annual rainfall ranges from 1000-3000 mm, with the annual averages generally surpassing 2000 mm (DNAEE, 1983). Temperature ranges from a minimum of 20-22 ºC in June-August and a maximum of 28-29 ºC in December-February. The river flow in the studied reach averages 318 m3s-1, ranging from 109 m3s-1 in the dry season to 950 m3s-1 in the wet season (Hydroscience, 1977). The studied river segment was 350 km long, covering a drainage area of approximately 33,663 km2 within a single ecoregion.

Fishes were caught at seven sampling sites along PSR middle reaches (Fig. 1): **Site 1** (Funil Reservoir) is located in the PSR largest eutrophic reservoir. A number of large industrial plants are located downstream the Funil Reservoir Dam (Klapper, 1998). The waters of which are turbid with a retention time ranging from 10 to 50 days; **Site 2** (Lajes reservoir) is located in an oligotrophic reservoir with high water quality and surrounded by well-preserved stretches of Atlantic forest, with minor human activity; the habitat complexity is relatively low, the waters are transparent (2-4 m) and retention time averages 280 days with a flow ranging from 18 to 22 m3sec-1; **Site 3** (Volta Redonda, PSR) – is located in a region that has been considered as the most polluted section of the river (Pfeiffer et al. 1986) owing to the proximity of a number of textile, chemical and food industries, and of one of the largest Brazilian steel plants; 1) **Site 4** - is located on the banks of Preto river, an sparsely populated mountainous area with no intensive agriculture and industries; **Site 5** (Paraibuna River) – this sampling site is located on the banks of a major tributary of PSR approximately 60 km downstream a large urban and industrial area; **Site 6** (Três Rios, PSR) – this site in PSR is approximately 140 km downstream of site 3 and near to the connection of two major tributaries; **Site 7** (Piabanha River) – this site is located on the banks of this tributary river that drains a large industrial area approximately 55 km upstream of the site.

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## Figure 1- Paraiba do Sul River (PSR) basin (upper, middle-upper, middle-lower, and lower reaches) and sampling sites. All sites were located in the middle-lower reach of PSR Basin and are indicated as follows: 1 – Funil Reservoir; 2 – Lajes Reservoir; 3 – PSR nearby Volta Redonda Town; 4 – Preto River, 5 – Paraibuna River; 6 – PSR nearby Três Rios Town; 7 – Piabanha River.

## Sampling

## Two fishes which were abundant and widely distributed all over the PSR watershed were selected for the study: 1) acará or pearl eartheater, *Geophagus brasiliensis* (Perciformes, Cichlidae), and 2) yellow or spotted mandi, *Pimelodus maculatus* (Siluriformes: Pimelodidae). *Pimelodus maculatus* is a detritivorous fish with tendency to carnivory that is close associated to the substrate, while *G. brasiliensis* is an omnivorous fish that uses preferably the shallow river margins. Fishes were collected between May and September 2006 by using gill and casting nets. Immediately after capture, all fishes were anesthetized in ice and killed by decapitation. After killing, blood smears were prepared and livers were removed and frozen in liquid nitrogen as quickly as possible. All fishes were weighed and measured for total length. Voucher specimens were deposited in the ichthyological collection of the Laboratório de Ecologia de Peixes of the Universidade Federal Rural do Rio de Janeiro under number: LEP-UFRRJ #582, 587, 1158, 1420.

**Chemicals**

Substrates (ethoxyresorufin), the reaction product (resorufin), ß-NADP, glicose-6-phosphate, glucose-6-phosphate-dehydrogenase, bovine serum albumin and the Bradford reagent were all purchased from Sigma Chemical Company, St Louis MO, USA. TRIS, MgCl2 and other salts were of analytical grade and supplied by Merck SA Industrias Quimicas, Rio de Janeiro, Brazil.

**EROD assay**

Frozen fish livers were thawed on ice and homogenized in a cold buffer solution (50 mM Tris, 1 mM EDTA, 250 mM sucrose, 20% glycerol, pH 7.4) by using a motor-driven glass Potter-Elvejhem homogenizer equipped with a Teflon pestle. Hepatic homogenates were subsequently centrifuged at 9000g for 30 min at 4°C. Aliquots (1 ml) of the supernatant (liver S9 fraction) were transferred to cryotubes and stored in liquid nitrogen until they were assayed for monooxygenase activity. Protein concentrations in the S9 fractions were measured by a colorimetric method using Coomassie brilliant Blue G dye and bovine serum albumin as the standard (Bradford, 1976).

Ethoxyresorufin-*O*-deethylase (EROD) activity in the hepatic S9 fractions were assayed essentially as described by Burke et al. (1985) except for the use of a NADPH regenerating system. EROD reactions took place in quartz cuvettes at 37 °C and were started by the addition of the regenerating system, which consisted of 0.25 mM b-NADP, 2.5 mM MgCl2, 5 mM glycose-6-phosphate, and 0.5 units of glucose-6-phosphate-dehydrogenase per ml of incubation mixture (De-Oliveira et al., 1999). The rate of resorufin formation was measured by using a spectrofluorimeter (Shimadzu RF-5000) with excitation and emission wavelengths set at 550 nm and 582 nm, respectively and a 5 nm band slit width.

**Micronuclei frequency**

Peripheral blood smears were air-dried, coded, fixed in methanol and stained with 10% Giemsa solution. The micronuclei frequency (Mn) was evaluated under a light microscope (Olympus BX 45 microscope, 1000 x magnification) by a skilled evaluator kept unaware of fish species and sampling site. Frequencies of Mn were estimated by evaluating at least 1000 red blood cells per fish and only erythrocytes with intact cellular and nuclear membrane were examined. Round or ovoid-shaped non-refractory particles with color and structure similar to chromatin, with a diameter 1/3 - 1/20 of the main nucleus and clearly detached from it were interpreted as Mn.

**Statistical analysis**

Group means were compared by one way analysis of variance (ANOVA) followed by Tukey HSD multiple comparison test (Zar, 1999). Proportions were arcsin transformed before using this parametric analysis. In any case, a difference was considered as significant when *p* < 0.05.

**Results**

The cichlid fish (acará, *G. brasiliensis*) was caught in all sampling sites and their average sizes and weight ranged from 158.3 to 248.3 mm Total Length - TL, and 84.0 to 277.0 g, respectively, (Table 1). The yellow mandi fishes (*P. maculatus*),however, were captured only in sites 1, 5, 6 and 7 and their average sizes and body weights ranged from 197.9 to 272.5 mm TL and 63.6 to 249.8 g, respectively (Table 2). Livers from yellow mandi and blood smears from acará fishes captured in site 5 were lost and respective EROD and Mn incidence values are missing in Tables 2 and 3.

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| **Table 1** Ethoxyresorufin–*O*–deethylase (EROD) activity in the liver S9 fraction from *G. brasiliensis* caught at different sampling sites along the middle reach of Paraíba do Sul River Basin. Sampling sites: 1, Funil Reservoir; 2, Lajes Reservoir; 3, PSR nearby Volta Redonda Town; 4, Preto River; 5, Paraibuna River; 6, PSR nearby Três Rios Town; 7, Piabanha River. *N*, number of individuals. Data on EROD, length and weight are shown as means ± SD. EROD activity was compared by ANOVA and Tukey multicomparisons test. |
| Samplingsite | Length (mm) | Weight (g) | *N* | EROD activity  |
| (pmoles/mg ptn/min) | A ≠ B ; *p*<0.05 |
| 1 | 202.9±3.6 | 151.8±75.5 | 7 | 62.1±1.8 | A |
| 2 | 248.3±3.6 | 251.4±83.8 | 6 | 131.8±4.9 | A |
| 3 | 230.0±3.4 | 277.0±114.6 | 10 | 86.7±2.2 | A |
| 4 | 169.8±2.1 | 93.0±41.5 | 9 | 130.2±46.7 | A |
| 5 | 158.3±2.5 | 85.3±30.7 | 3 | 182.5±65.7 | A |
| 6 | 222.1±2.5 | 225.0±75.1 | 7 | 134.4±60.2 | A |
| 7 | 167.5±1.7 | 84.00±33.6 | 5 | 284.1±100.3 | B |

The EROD activity measured in the S9 fraction from livers of *G. brasiliensis* caught in site 7 (Piabanha river) was higher than activities determined in cichlid fishes collected in all other sites (F=22.5; *p*=0.0000). As shown in Table1, there was no other difference between sites regarding EROD activity of *G. brasiliensis*. Hepatic EROD activities of *P. maculatus* caught at sites 6 and 7 were also higher than activities determined in fishes from site 1 (F=4.49; *p*=0.012) (Table 2). Taken together the aforementioned data on EROD activity suggested that exposure of *G. brasiliensis* and *P. maculatus* caught in sites 6 and 7 to CYP1A inducing agents were more pronounced than that of fishes collected at other sampling sites.

The incidence of Mn (‰) in peripheral blood erythrocytes of *G. brasiliensis* caught at sites 6 and 7 was markedly higher than the Mn frequency found in fishes from any other site (F=26.91; *p*=0.001) (Table 3). Along the same vein, frequencies of Mn (‰) in erythrocytes of *P. maculatus* collected at sites 5, 6 and 7 were higher than the frequency found in the blood of fishes from site 1 (F=4.98; *p*=0.010) (Table 3).

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| **Table 2** Ethoxyresorufin–*O*–deethylase (EROD) activity in the liver S9 fraction from *P. maculatus* caught at different sampling sites along the middle reach of Paraíba do Sul River Basin. Sampling sites: 1, Funil Reservoir; 6, PSR nearby Três Rios Town; 7, Piabanha River. *N*, number of individuals. Data on EROD length and weight are shown as means ± SD. EROD activity was evaluated by ANOVA and Tukey multicomparisons test.  |
| Samplingsite | Length (mm) | Weigth (g) | *N* | EROD activity |
| (pmoles/mg ptn/min) | A ≠ B ; *p*<0.05 |
|  |  |  |  |  |  |
| 1 | 272.5±4.3 | 249.8±104.6 | 4 | 42.3±0.99 | A |
| 6 | 246.7±4.3 | 185.1±100.1 | 6 | 72.8±9.57 | B |
| 7 | 197.9±3.4 | 63.6±44.0 | 8 | 92.8±29.68 | B |

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| **Table 3** Occurrence of micronucleated erythrocytes (‰) in the peripheral blood of *G. brasiliensis* and *P. maculatus* caught at different sampling sites along the middle reach of Paraíba do Sul River Basin. 1 – Funil Reservoir; 3 – PSR nearby Volta Redonda Town; 4 – Preto River, 5, Paraibuna River; 6 – PSR nearby Três Rios Town; 7 – Piabanha River -. *N*, number of individuals. Data are shown as means ± SD of individual fish frequencies per sampling site. Comparisons of arcsin transformed ratios were made by ANOVA and Tukey multicomparisons test. |  |
| SamplingSite | *G. brasiliensis* |  | *P. maculatus* |
| *n* | Mn (‰)  |  | A ≠ B; *p*<0.05 | *N* | Mn (‰)  | A ≠ B ; *p*<0.05 |
| 1 | 7 | 0±0 |  | A | 4 | 0.25±0.5 | A |
| 3 | 9 | 0.56±0.73 |  | A | 0 | - |  |
| 4 | 8 | 0.13±0.35 |  | A | 0 | - |  |
| 5 | 0 | - |  | - | 6 | 1.8±1.2 |  |
| 6 | 6 | 3.3±1.4 |  | B | 5 | 3.2±1.6 |  |
| 7 | 5 | 2.2±0.4 |  | B | 9 | 1.6±1.0 | B |

**Discussion**

The high incidence of micronucleated erythrocytes in the peripheral blood of fishes caught in sites 6 and 7, contrasting with the low frequency noted in fishes from site 1, strongly suggested that acaras and yellow mandis collected in the two most downstream sampling sites were exposed to DNA damaging agents. It is of note that acará fishes caught in sites located between sampling sites 1 and 6-7 (i.e. in sites 3 and 4) showed intermediate frequencies of micronucleated erythrocytes in the peripheral blood. The foregoing findings are thus consistent with the view that the lower middle reach of PSR basin suffered the impact of discharges of PAH polluting plants (e.g. steelworks) located along this stretch of the river and that these genotoxic contaminants were to some extent bioavailable to both fish species.

Similarly, EROD activity (a marker for CYP1A) was markedly higher in acaras from site 7 as compared to the activity found in those caught in site 1, while cichlids from sampling sites 2, 4, 5 and 6, showed intermediate values regarding hepatic EROD (Table 1). It should be pointed out that values of EROD activity in the liver of *G. brasiliensis* collected at site 1 found in this study are comparable to those found in fishes of the same species from clean reference sites in a previous study (Parente et al., 2008; Parente et al., 2015). The EROD activity of yellow mandis captured at sites 6 and 7 were also higher than the activities determined in fishes from site 1. As commented in the Introduction of this paper, induced liver EROD activities is one of the most widely used biomarkers of exposure to CYP1A (AhR ligands) inducing agents, including planar halogenated (e.g. PCBs, PCDD/Fs) and polycyclic aromatic hydrocarbons (PAHs). The clearly induced CYP1A-mediated activities in fishes (*G. brasiliensis* and *P. maculatus*) from the two most downstream sampling sites evaluated in this study suggested that the middle reach of PSR basin is contaminated with PAHs and or with other AhR ligands (PCBs, PCDD/Fs). Other similar indications of impacts using EROD activity as biomarker of xenobiotics have been recorded eslsewhere (e.g. Chiang et al., 2012; Karimzadeh & Zahmatkesh, 2013; Kammann et al., 2014; Franco-Bernardes, Maschio, Azeredo-Oliveira & Almeida, 2015).

**Conclusions**

Taken together, results from this study indicated that pollution in the middle reaches of the PSR basin by industrial discharges and accidents and untreated domestic sewage affected two native fish species. Results presented here also suggested that these chemical contaminants, which were detected in river sediments (e.g. PAHs) in some previous studies (Torres et al., 2002), were bioavailable to cichlids and pimelodids. It is of note that the most altered biomarkers of individuals of the two species were those caught in sites 6/7 that were located downstream the place (site 3, Volta Redonda Town vicinity) where there is a huge steelworks that releases a large amount of PAHs into the environment. Surprisingly, fishes captured in the sampling site 3 were apparently less affected than those caught in the two most downstream sites 6 and 7. It should be borne in mind, however, that fish – contrasting to bivalve mollusks - are not static bioindicators and that alterations of biomarkers such as EROD and Mn depend on the way and the extent to which these contaminants that are found mainly in river sediments become bioavailable to fishes.

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