Acta Scientiarum

http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v40i1.36666

GENETICS

Induced CYP1A activity and DNA damage in fish from the middle Paraíba do Sul River basin, southeastern Brazil

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ABSTRACT. The Paraíba do Sul River (PSR) drainage basin in Southeastern Brazil covers one of the most industrialized and densely populated regions of the country. The impact of chemical contamination on the 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 effects of DNA-damaging agents (e.g. PAHs) - to evaluate the effects of pollution on two native fish species, *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 of Mn and EROD recorded in fish from the most upstream site (Funil Reservoir). Our findings are consistent with the view that CYP1A-inducing activity and increased DNA-damage are found in fish caught in sampling sites located downstream of the stretch where there are several 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).

Atividade induzida de CYP1A e danos de DNA em peixes do trecho médio da bacia do rio Paraíba do Sul, sudeste do Brasil

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 se localiza grande número de indústrias potencialmente poluidoras. Neste estudo, foi avaliada 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) foi marcadamente mais elevada 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 elevadora de danos no DNA são encontradas em peixes capturados em locais abaixo do trecho onde grande número de indústrias são potenciais fontes de poluição de indutores de contaminantes HAPs e CYP1A.

Palavras-chave: Atividade Ethoxyresorufin-O-deethylase (EROD); *Geophagus brasiliensis*; micronúcleo; *Pimelodus maculatus*; hidrocarbonetos aromáticos 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 the State of São Paulo and flows 1,140 km before meeting the Atlantic Ocean on the Northern Coast of the State of Rio de Janeiro (Figure 1). Since the PSR drainage basin covers 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 hydropower 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 the PSR basin, where many potentially polluting plants are located, including several chemical and metallurgical industries (Molisani, Salomão, & Ovalle, 2005; Linde-Arias, Inácio, Novo, Albuquerque, & Moreira, 2008; Nascimento et al., 2012; Parente et al., 2015). Since a huge amount of coal (coke) is burned in furnaces of steelworks, high levels of Polycyclic Aromatic Hydrocarbons (PAH) were found in the PSR sediments in the vicinity of Volta Redonda town where it 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 Askarel, a mixture of PolyChlorinated Biphenyl (PCB) congeners, in the middle reach of the PSR near Barra do Piraí Town, State of Rio de Janeiro (Coelho, 1990). In 2003, 600,000 m^3 of wastewater from wood industry were discharged into an important tributary of Paraíba do Sul (Pomba River) and in 2008, about 8,000 L of the pesticide Endosulfan was also discharged into this river, with both events resulting in large fish mortalities. Although it has been reported that PSR sediments are contaminated by heavy metals, PAHs and Persistent Organic Pollutants (POPs), there have been relatively few studies on the impact of this chemical pollution on the river native biota (e.g., Pfeiffer, Fiszman, Malm, & Azcue, 1986; Linde-Arias et al., 2008; Terra, Araújo, Calza, Lopes, & Teixeira, 2008; Parente et al., 2015).

This study was undertaken to investigate the effect of pollution on liver 7- ethoxyresorufin O-deethylation (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 Cytochrome P450, family 1, subfamily A (CYP1A), a Cytochrome P450 (CYP) enzyme well conserved among vertebrates (Stegeman, Woodin, Singh, Oleksiak, & Celander, 1997). Since expression and activity of CYP1A are enhanced by Aryl Hydrocarbon Receptor (AhR) ligands, such as Polychlorinated Dibenzo-p-Dioxins and Polychlorinated Dibenzofurans (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, Oliveira, Silva, Araújo, & Paumgartten, 2004; Parente, Oliveira, & Paumgartten, 2008; Parente et al., 2009; Pathiratne, Chandrasekera, & Pathiratne, 2009; Parente et al., 2015). 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 fish 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).

Material and methods

Study area

The Paraíba do Sul River (PSR; 20° 26' - 23° 38' S, 41° 00' - 46° 30' W) is 1,140 km long, with a 57,000 km^2 watershed (Figure 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 of the Paraíba do Sul river basin is characterized as subtropical hot, with annual average temperature oscillating between 18°C and 24°C. The maxims precipitations occur in the headwaters of the basin and at the highest points of the mountains of the Mar and Mantiqueira, reaching values of 2,250 mm $year^{-1}$. The summer period is characterized as rainfall with cumulative precipitation between 200 and 250 mm $month^{-1}$, in months with maximum precipitation (December and January), while in winter we have the interval between the months of May to July the driest period, with precipitation less than 50 mm $month^{-1}$ (Marengo & Lincoln, 2005). The studied river segment was 350 km long, covering a drainage area of approximately 33,663 km^2 within a single ecoregion.

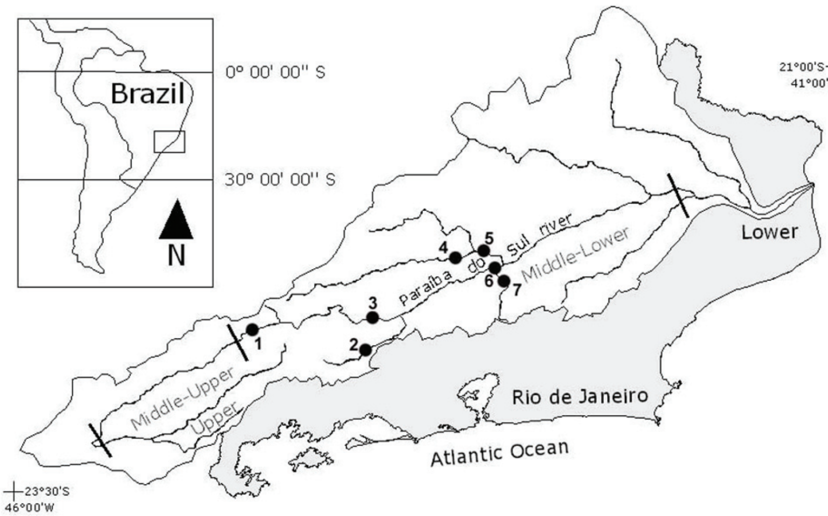

Figure 1. Paraiba do Sul River (PSR) basin (upper, middle-upper, middle-lower, and lower reaches) and sampling sites. All sites were located in the middle-lower reach of the PSR Basin and are indicated as follows: 1) Funil Reservoir; 2) Lajes Reservoir; 3) PSR nearby Volta Redonda Town; 4) Preto River,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

Fish were caught at seven sampling sites along the PSR middle reaches (Figure 1): 'Site 1' (Funil Reservoir) is located in the PSR largest eutrophic reservoir. A number of large industrial plants are located downstream of the Funil Reservoir Dam (Klapper, 1998). The waters 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 interference; the habitat complexity is relatively low, the waters are transparent (2-4 m) and the retention time averages 280 days with a flow ranging from 18 to 22 m^3 s^{-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; 'Site 4' is located on the banks of Preto River, an sparsely populated mountainous area with no intensive agriculture and industries; it is considered our control or reference site, because it is the least altered by anthropogenic influences. 'Site 5' (Paraibuna River) is located on the banks of a major tributary of the PSR approximately 60 km downstream of a large urban and industrial area; 'Site 6' (Três Rios, PSR), this site in the PSR is approximately 140 km downstream of site 3 and near the confluence of two major tributaries; 'Site 7' (Piabanha River), this site is located on the banks of this tributary that drains a large industrial area approximately 55 km upstream of the site.

Sampling

Two fish species, which are 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 detritivorous tending to carnivory and is close associated to the substrate, while *G. brasiliensis* is an omnivorous fish that uses preferably the shallow river margins. Fish specimens were collected between May and September 2006 by using gill and casting nets. Immediately after caught, fish were anesthetized in ice and killed by decapitation. After, blood smears were prepared and livers were removed and frozen in liquid nitrogen as quickly as possible. This project received Permanent license for the collection of zoological material - SISBIO 10707/Normative Instruction ICMBio 03/2014, and follows the ethics rules applicable to the use of animals in teaching and/or research based on the provisions of the Brazilian law (Federal Law 11794 of October 08, 2008). All individuals were weighed and measured for total length. Voucher specimens were deposited in the fish 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 (Nicotinamide Adenine Dinucleotide Phosphate), 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 (2-Amino-2-(hydroxymethyl) propane-1,3-diol), $MgCl_2$ and other salts were of analytical grade and supplied by Merck SA Indústrias Químicas, Rio de Janeiro State, Brazil.

EROD assay

Frozen fish livers were thawed in ice and homogenized in a cold buffer solution (50 mM Tris, 1 mM EDTA (Ethylenediaminetetraacetic acid), 250 mM sucrose, 20% glycerol, pH 7.4) by using a motor-driven glass Potter- Elvehjem homogenizer equipped with a Teflon pestle. Liver homogenates were subsequently centrifuged at 9,000g at 4°C for 30 min. Aliquots (1 mL) of the supernatant (liver S9 fraction) were transferred to cryotubes and stored in liquid nitrogen until 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 (Nicotinamide Adenine Dinucleotide Phosphate) 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 $MgCl_2$, 5 mM glycose-6-phosphate, and 0.5 units of glucose-6-phosphate-dehydrogenase per mL of incubation mixture (Oliveira, Fidalgo-Neto, & Paumgartten, 1999). The rate of resorufin formation was measured by using a spectrofluorimeter (Shimadzu RF-5000) with excitation and emission wavelengths set at 550 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, 1,000 x magnification) by a skilled evaluator kept unaware of fish species and sampling site. Frequencies of Mn were estimated by evaluating at least 1,000 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's HSD (Honest Significant Difference) multiple comparison test (Zar, 1999). Proportions were arcsine transformed before using this parametric analysis. In any case, a difference was considered as significant when $p < 0.05$.

Results and discussion

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

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 the 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; and 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's test.

Sampling site	Length (mm)	Weight (g)	*N*	EROD activity	
				(pmoles mg^{-1} ptn min^{-1})	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 those of cichlid fish collected in all other sites ($F = 22.5$; $p = 0.00001$). As shown in Table 1, 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 fish from site 1 ($F = 4.49$; $p = 0.012$) (Table 2). Taken together, the aforementioned data on EROD activity suggested that exposure of *P. maculatus* caught in sites 6 and 7 and *G. brasiliensis* in site 7 to CYP1A-inducing agents were more pronounced than that of fish collected at other sampling sites.

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 the Paraíba do Sul River Basin. Sampling sites: 1) Funil Reservoir; 6) PSR nearby Três Rios Town; and 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's test.

Sampling site	Length (mm)	Weight (g)	*N*	EROD activity (pmoles mg^{-1} ptn min^{-1})	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

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 the 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; and 7) Piabanha River. *N*: number of individuals. Data are expressed as means ± SD of individual fish frequencies per sampling site. Comparisons of arcsine transformed ratios were tested by ANOVA and Tukey's test.

Sampling Site	*G. brasiliensis* *N*	Mn (‰)	A ≠ B; p < 0.05	*P. maculatus* *N*	Mn (‰)	A ≠ B; p < 0.05
1	7	0±0	A	4	0.25±0.5	A
2	5	0.45±0.31	A	0		
3	9	0.56±0.73	A	0	-	
4	8	0.13±0.35	A	0	-	
5	0	-	-	6	1.8±1.2	B
6	6	3.3±1.4	B	5	3.2±1.6	B
7	5	2.2±0.4	B	9	1.6±1.0	B

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 fish from any other site (F = 26.91; p = 0.001) (Table 3). Likewise, 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 fish from site 1 (F = 4.98; p = 0.010) (Table 3).

EROD activity (a marker for CYP1A) was markedly higher in acarás 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 fish of the same species from reference sites in previous studies (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 fish from site 1. As previously commented, 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 fish (*G. brasiliensis* and *P. maculatus*) from the two most downstream sampling sites evaluated in this study suggested that the middle reach of the 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 elsewhere (e.g. Chiang et al., 2012; Karimzadeh & Zahmatkesh, 2013; Kammann et al., 2014; Franco-Bernardes, Maschio, Azeredo-Oliveira, & Almeida, 2015).

The high incidence of micronucleated erythrocytes in the peripheral blood of fish caught in sites 6 and 7, contrasting with the low frequency in fish from site 1, strongly suggested that acarás and yellow mandis collected in the two most downstream sampling sites were exposed to DNA-damaging agents. It is worth noting that acará fish caught in sites located between sampling sites 1 and 6-7 (i.e. at 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. Parente et al. (2015) reported that the contamination of PSR fish meat by a diverse range of industrial pollutants (genotoxic and AhR ligand agents, and toxic metals) are relevant to human health at the present time and that it should be borne in mind that the biomarkers respond to the bioavailability of contaminants to target organisms. Although direct evaluation of water quality has not been done in this study, EROD activity and the high incidence of micronucleated erythrocytes in the peripheral blood of fish are robust evidence that agent pollutants occur along the PSR river basin, mainly in the most downstream sites. A continuous biomonitoring program should be implemented in this region to obtain a more comprehensive investigation to identify the origins of any sources of pollution.

Conclusion

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. Importantly, 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 are huge steelworks that release a large amount of PAHs into the environment. Surprisingly, fish 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, found mainly in river sediments, become bioavailable to fish.

Acknowledgements

This research was funded by CNPq-CTHidro (*Conselho Nacional de Desenvolvimento Científico e Tecnológico*), Process and 555274/2006-3 and by Faperj (*Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro*), Process 102.704/2008 and 101.966/2009. We also thank Ruan Manágma Vasconcellos for field and laboratory assistance. This research was conducted under Sisbio Collection of Species Permit 10707 issued by ICMBio, Brazilian Environmental Agency.

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Received on April 11, 2017.
Accepted on October 17, 2017.