Vol.58, n.4: pp. 605-612, July-August 2015
http://dx.doi.org/10.1590/S1516-8913201500130
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Enzymatic Activity in the Gastrointestinal Tract of *Pimelodus maculatus* (Teleostei, Siluriformes) in Two Neotropical Reservoirs with Different Trophic Conditions

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ABSTRACT

Enzymatic activities for digestion of proteins and carbohydrates were compared among three organs of the digestive system of Pimelodus maculatus *in two reservoirs with different trophic conditions during the winter of 2006. The aim was to test the hypothesis that enzymatic activity for the digestion of proteins and carbohydrates differed among organs and that such activities differ between the trophic state of the environment. Enzymatic activities were determined through the assays of specificity for trypsin, chymotrypsin and β-glucosidase enzymes. The intestine had higher trypsin-like enzymatic activities compared to the stomach and liver. The highest β-glucosidase activity was found in the liver compared to the stomach and intestine in the oligotrophic reservoir only. Overall, enzymatic activity did not differ between the eutrophic and oligotrophic reservoirs, although the intestinal chymotrypsin was comparatively higher in the eutrophic reservoir and the hepatic β-glucosidase was higher in the oligotrophic reservoir. These findings indicated that most digestive activity occurred in the intestine for* P. maculatus*, which was probably related to its omnivorous/carnivorous feeding habits. The highest proteolytic activity in the intestine was expected for most fishes, but the high hepatic β-glucosidase in the oligotrophic reservoir was unexpected. The hepatic β-glucosidase as well as the intestinal chymotrypsin-like activity could be considered as the candidates for biomarkers of environmental quality.*

Key words: enzymes, catfish, physiology, freshwater fishes, digestive tract

INTRODUCTION

Fishes are among the preferred organisms to monitor the environmental conditions in freshwater systems, such as lotic and lentic systems (Van der OOST et al. 2003; Lionetto et al. 2012). Different environmental conditions may affect the activity of digestive enzymes in fishes and comparison of biochemical parameters between the individuals from different environments can be useful to furnish the biomarkers of pollution and other ecological parameters. Measurements of variation in the enzymatic activity and specificity are widely used to assess the environmental quality and some enzymes are particularly important such as carbonic anhydrase and hydrolases (Kuz'mina et al. 1996, 2003, 2013; Lionetto et al. 2012; Filippov et al. 2013), apart from cytochrome P450 that is directly involved in the transformation of xenobiotics (Uno et al. 2012).

A Siluriformes fish species widely distributed in the rivers and reservoirs of Southeastern Brazil was chosen for this study. The long-whiskered catfish *Pimelodus maculatus* Lacepède, 1803 is a benthic species, which has comparatively less dependency on the substratum compared to other Siluriformes. It is able of perform limited

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reproductive seasonal migration to spawn (Dei Tos et al. 2002) and has omnivorous feeding habit with tendency for carnivory, presenting intestine size of almost 1.5-folds the total length (Barbosa et al. 1998). The intestinal enzymatic activity is probably adapted to digest a wide source of foods, mainly those of animal origin; therefore, feeding habits for this species is probably highly dependent on the presence of peptidase enzymes (Lundstedt et al. 2004; Xiong et al. 2011).

Studies conducted in artificially polluted environments lead to more conclusive results, such as those reported by Kuz'mina et al. (2013) who showed that *Cyprinus carpio* (Linnaeus, 1758) individuals submitted to mercury-enriched food showed an increase in the activity of intestinal peptidases and a corresponding decrease in the activity of intestinal glycosidase. Nevertheless, studies performed with fishes collected from their natural environment are more realistic, even if the chosen enzymatic activity is not a clear biomarker, data showing spatial variations and comparison of enzymatic profile among species are relevant to the freshwater ecology and important for further investigations.

Brito et al. (2012) assayed the activities of hepatic glutathione-S-transferase and catalase, branchial and renal carbonic anhydrase, and muscle acetylcholinesterase from *P. maculatus* collected from three different reservoirs of the Paraíba do Sul River. Despite some seasonal variation found between the individuals collected in winter and summer, significant enzymatic activity differences between the fishes of the three reservoirs were not found. A study was conducted in which the activities of digestive enzymes of *P. maculatus* were compared with that of another Siluriformes species, *Hypostomus auroguttatus* Kner, 1854 (Duarte et al. 2013). Significant differences in peptidase and β-glucosidase activities were found between the two species that were attributed to feeding habits and to the relative size of the gastrointestinal tract. Nevertheless, the activities of these enzymes did not clearly varied to reflect the changes between a lotic and a lentic environments located in the middle reaches of Paraíba do Sul river basin (Duarte et al. 2013).

The aim of this study was to assay the glucosidase and peptidase activities of *P. maculatus* from the reservoirs with different trophic state and to compare such activities from distinct organs of the digestive system. In particular, enzymatic activities of *P. maculatus* were compared in (1) three organs of the digestive system (stomach, intestine and liver), and (2) eutrophic and oligotrophic reservoirs. The hypothesis tested was the activities of enzymes for proteins (peptidases) and carbohydrates (β-glucosidase) hydrolysis in the gastrointestinal tract (including the epithelium) or in the liver change according to the organs and environmental conditions.

MATERIAL AND METHODS

Study Area

Funil Reservoir

The Funil Reservoir (22°30′–22°38′S, 44°32′–44°42′W, 440 m above sea level) is located in the middle reaches of the Paraíba do Sul River within the Atlantic Forest biome of Southeastern Brazil (Fig. 1). This reservoir is the largest artificial impoundment on the river, with an area of 40 km^2, maximum depth of 70 m, and water retention time of 10–50 days. The climate is subtropical with monthly mean water temperatures of 18–24°C. Rainfall is highest in the summer months (December–January; 200–250 mm per month) and lowest in the winter months (June–August), with less than 50 mm per month (Marengo and Alves 2005). Approximately, 1.8 million people lived in the municipalities upriver from the reservoir. It meant that the river received large pollutant loads, mainly domestic and industrial effluents (Pinto et al. 2006). The high nutrient loads brought into the reservoir gives rise to eutrophication processes such as algal blooms and high productivity (Soares et al. 2008). There is little soil cover around the reservoir because of agricultural practices and fluctuating water levels that erodes the shoreline increasing suspended sediment in the reservoir (Branco et al. 2002).

Santa Branca

Santa Branca Reservoir (23°18'-23°29'S; 45°45'45°53'W) is located in the upper stretches of the Paraiba do Sul River in Southeastern Brazil and has a typical branched reservoir area of ca. 31 km^2 (Fig. 1). This reservoir is a typical oligotrophic system (Cetesb 2013), located approximately 30 km below the Paraibuna Reservoir, the upper most reservoir of the Paraiba do Sul River. This was constructed for flow control but the hydroelectric plant only became operational in 1997. The dam blocks the entire river course, completely restraining fish movements from up to downriver and vice-versa.

Seasonal rainfall peaks dictate the dynamics of reservoir water level. The reservoir has a maximum volume of 438.500 hm³ (average of 307.300 hm³), a retention time of 62.65 days, and a wide water-level oscillation, which contributes to marginal erosion and sedimentation.

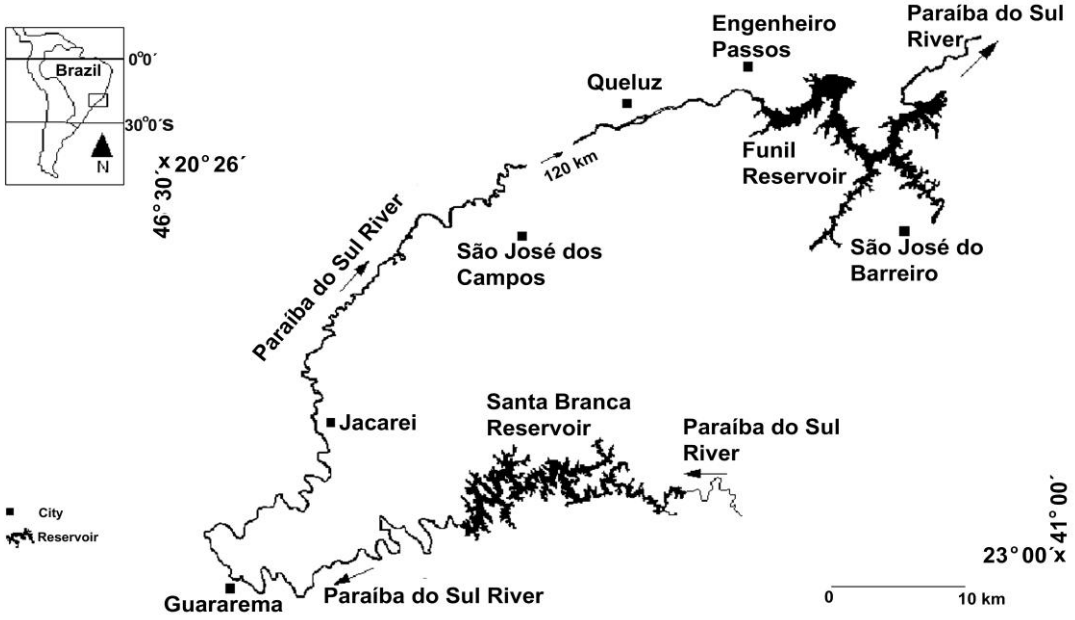

Figure 1 - Map of the study area with indication of the two reservoirs in the Paraiba do Sul river basin.

Fish Collection and Handling

Twenty-four individuals of *P. maculatus* averaging 252 ± 5.4 mm (TL- total length ± SD) and weighing 184 ± 13.1 g (TW- total weight ± SD) were collected in the winter (July/September) 2006 in Funil (twelve individuals) and Santa Branca (twelve individuals) reservoirs. Gill nets (30 m long, 2.5 m height) with different mesh sizes (2.5, 4.5 and 6.5 cm stretched knots) were employed for catching the fishes. The nets were deployed next to the shoreline during the afternoon and retrieved in the following morning, fishing for approximately 15 h. Voucher specimens were deposited in the fish collection at the Laboratory of Fish Ecology of the Universidade Federal Rural do Rio de Janeiro (UFRRJ-LEP 0996-0999).

Tissue Preparation for Digestive Enzyme Assays

Fishes were transported alive to the laboratory, where they were killed by immersion in water at 4ºC and dissected. Stomachs were removed by cutting at the esophagus end and at the pyloric sphincter. For the intestinal tissue, only a distal part was selected for this study. This corresponded to a cut at 60-150 mm from the anus (c.a. 1/3 intestine length). The liver was also removed and these three organs were washed with Milli-Q™ water and processed in an appropriate manner for the enzymatic analyses.

The internal contents of the intestine and stomach were removed by washing with 0.9 % (w/v) saline solution. Then, the stomach, intestine and liver were weighed and homogenized into saline solution. Five grams of the tissue were extracted with 1.0 mL of saline solution. Thus, both epithelial tissue and lumen enzymes were extracted. The extracts were centrifuged at 5,200 *g* at 4ºC for 60 min (Skea et al. 2005) in a Sigma 4K15 desktop centrifuge (11156 rotor). Following centrifugation, the supernatant of each tube was gently pipetted into four separate polypropylene vials and frozen at -80°C with aliquots corresponding to each one enzymatic analysis, that is, trypsin-like, chymotrypsin-like and β-D-glucosidase while the fourth aliquot was reserved for total protein assay.

Chemicals and equipment

Chromogenic substrates used in the enzymatic assays (benzoyl-DL-arginine-4-nitroanilide, BApNA; benzoyl-L-tyrosine-4-nitroanilide,

BTpNA and 4-nitrophenyl-β-D-glucopyranoside, PNPG) were products from Sigma-Aldrich (St Louis, M.O., USA). The Folin-Ciocalteu reagent used for the protein quantification was a Vetec (Rio de Janeiro, Brazil) product. All other reagents used were of analytical grade and were used without further purification. Water was always type 1, ultra-pure (Milli-Q™). The absorbance for the enzymatic activities were determined using a Shimadzu UV 160A spectrophotometer (Shimadzu, Kyoto, Japan) with temperature control.

Peptidase Activity with Trypsin-like or Chymotrypsin-like Specificity

This assay was conducted accordingly to Duarte et al. (2013). The enzymatic assay was initiated by the addition of 0.9 mL of a 0.1 mol/L Tris-HCl buffer, pH 8.0, containing 20 mmol/L $CaCl_2$, to a 1.5 mL polypropylene tube. Then, 50 µL of BapNA solution (at 5 mmol/L in dioxane) or BTpNA solution (at 1.0 mmol/L in dimethyl sulfoxide) was added and the mixture was incubated at 37ºC for 10 min. The tissue samples were then defrosted and the microtubes were centrifuged at 14,500 *g* for 15 min for particulate matter removal. Fifty microliters of the tissue extract were added to the reaction mixture and the enzymatic action occurred at 37ºC for 10 min. The reaction was interrupted by the addition of 250 µL of aqueous acetic acid solution at 60% (by volume). Then, samples in the microtubes were centrifuged at 14,500 *g* for 15 min and the absorbance values of the supernatants were recorded at 410 nm.

$$V_t = \frac{Abs}{\varepsilon t.[prot]}$$

where V_t is the specific peptidase activity, *Abs* is the absorbance, ε is the p-nitroaniline molar absorptivity (7,680 $M^{-1}{\cdot}cm^{-1}$), *t* is the reaction time and *[prot]* is the protein content of the corresponding tissue extract volume. A blank was prepared with water instead of the tissue extract and incubated in the same experimental conditions as the samples containing enzymes.

β-D-glucosidase Activity

This assay was conducted accordingly to Duarte et al. (2013). The enzymatic assay was done by the addition of 900 µL of 0.2 mol/L citrate buffer, pH 6.0, to a 1.5 mL polypropylene tube. Then, fifty microliters of a 4-nitrophenyl-β-D-glucopyranoside solution (30 mmol/L in dioxane) were added and the mixture was incubated at 37ºC for 10 min. The tissue samples were then defrosted and the microtubes were centrifuged at 14,500 *g* for 15 min for particulate matter removal. Fifty microliters of the tissue extract were added to the reaction mixture and the enzymatic action occurred at 37ºC for 10 min. The reaction was stopped by the addition of 500 µL of 0.5 mol/L glycine buffer, pH 10.0. Then, the samples in the microtubes were centrifuged at 14,500 *g* for 15 min and the absorbance values were recorded at 400 nm. After dilution corrections, specific β-D-glucosidase activity was calculated according to the following equation:

$$V_g = \frac{Abs}{\varepsilon t.[prot]}$$

where V_g is the specific β-D-glucosidade activity, *Abs* is the absorbance, ε is the p-nitrophenol molar absorptivity at pH 10 (16,640 $M^{-1}{\cdot}cm^{-1}$), *t* is the reaction time and *[prot]* is the protein content of the corresponding tissue extract volume. A blank was prepared with water instead of the tissue extract and incubated in the same experimental conditions as the samples containing enzymes.

Total Protein Quantity of Tissue Extracts.

Total protein quantity (in micrograms) of the stomach, intestine, and liver extract were quantified following Lowry (1953) against bovine albumin solution as standard for building a standard curve.

Statistical Treatment.

Sampling design was planned to test the variation in enzymatic activity between the three organs and the two systems (eutrophic and oligotrophic). One way Analysis of Variance followed by a Tukey test was used to compare the mean values ($p < 0.05$), since data met the requirements of normality and homoscedasticity according to the Shapiro Wilk´s and Levene tests, respectively (Zar 1999).

RESULTS

The fishes from Santa Branca oligotrophic reservoir showed significantly higher intestinal trypsin-like activity than either stomachal or hepatic activities as expected for a typical digestive enzyme. Nevertheless, for peptidases with chymotrypsin specificity, the hepatic activity

was significantly higher than the stomachal activity and it was similar to the intestinal activity. Concerning the carbohydrate digestion, hepatic β-glucosidase activity was significantly higher than either intestinal or stomachal activities in the oligotrophic reservoir only (Fig. 2; Table 1).
No significant difference of β-glucosidase activity was found among the organs of the fishes from Funil eutrophic reservoir (Funil). β-glucosidase activities were similar and generally of low magnitude in the liver and in the gastrointestinal tract of individuals collected in the eutrophic reservoir. However, differences between the intestinal trypsin and chymotrypsin activities were significantly higher than the stomachal activity (Fig. 2; Table 1).

Table 1 - Comparison (means ± standard deviation) of enzymatic activities (trypsin-like, chymotrypsin-like and β-D-glucosidase) among digestive organs (stomach, intestine, and liver) from *Pimelodus maculatus* collected in the Funil eutrophic reservoir and the Santa Branca oligotrophic reservoir. Letters indicate significant difference levels from ANOVA at $p < 0.05$.

Oligotrophic	**F (p)**	**Stomach**	**Intestine**	**Liver**
Trypsin	6.1 (0.005)	309.7 ± 77.8^{b}	577.8 ± 134.8^{a}	244.3 ± 37.4^{b}
Chymotrypsin	4.3 (0.03)	85.32 ± 10.2^{b}	147.9 ± 21.8	252.5 ± 43.9^{a}
β-Glucosidase	16.8 (0.00)	66.3 ± 15.9^{b}	123.9 ± 33.7^{b}	350.3 ± 45.2^{a}
Eutrophic				
Trypsin	4.8 (0.015)	123.42 ± 31.0^{b}	328.9 ± 106.1^{a}	228.06 ± 35.8
Chymotrypsin	6.4 (0.005)	118.4 ± 28.5^{b}	305.6 ± 79.4^{a}	191.8 ± 31.7
β-Glucosidase	2.06 (0.14)	84.9 ± 16.9	96.9 ± 34.0	44.8 ± 7.9

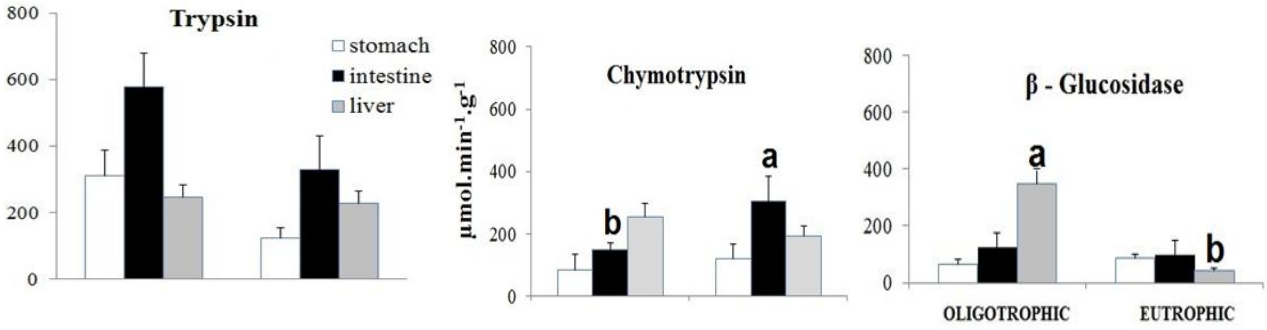

Figure 2 - Enzymatic activity of hydrolytic enzymes in stomach, intestine and liver of *Pimelodus maculatus* collected in the oligotrophic Santa Branca Reservoir and in the eutrophic Funil Reservoir. Letters indicate significant differences in enzymatic activities in fish from the two reservoirs according to ANOVA at $p < 0.05$.

Results showed only minor differences in the enzymatic activities in the reservoirs (Fig. 2; Table 1). Hepatic β-glucosidase activity was significantly higher in the fishes from the oligotrophic reservoir than those from the eutrophic one ($F=91.2$; $p = 0.001$), and the intestinal chymotrypsin-like activity was significantly higher in the eutrophic reservoir compared with the oligotrophic reservoir ($F=5.2$; $p=0.03$). The intestinal β-glucosidase activity was slightly higher in the individuals from the Santa Branca oligotrophic reservoir than in the individuals from the Funil eutrophic reservoir and there was also a tendency of higher activities of tryptic hepatic enzymes of the fishes from the oligotrophic than the eutrophic reservoir.

DISCUSSION

Pimelodus maculatus is one of the freshwater fish species most widely employed in the studies of biomarkers of pollution in tropical freshwater systems (Coppes de Achaval et al. 1982; Rodriguez et al. 1989). Brito et al. (2012) measured the activities of hepatic, renal and branchial enzymatic activities in the specimens collected in three different reservoirs of the Paraíba do Sul system, but no significant

differences were found among the reservoirs. In the present study, experiments were conducted in two reservoirs (oligotrophic versus eutrophic). The activities of digestive peptidases and β-glucosidase were assayed, since these enzymes could be affected by the feeding habits and by ecological conditions (Chakrabarti 1995; Kuz'mina et al. 1996, 2003; 2013).

P. maculatus is an omnivorous species with tendency to carnivory (Duarte el al. 2013). For this species, protein digestion is expected to begin in the stomach. This was confirmed in this study because trypsin-like and chymotrypsin-like activities were detected in the fish stomachs. Since the experiments were conducted at pH 8.0, the stomachal peptidase activities were probably derived from the intracellular enzymes extracted from the mucosa. Digestion in the stomachal lumen occurs in acidic milieu and probably the activities detected in this study are reactions occurring in the mucosa cells after the absorption of small peptides. The peptidase activities were generally higher in the intestine than in the stomach of fishes, which was an expected result since both extracellular and intracellular activities could contribute to protein digestion in alkaline milieu. Chong et al. (2002) showed that either chymotrypsin-like or trypsin-like activities were higher in the intestine than in the stomach of the juvenile carnivorous fish, *Symphysodon aequifasciata.* Lundstedt et al. (2004) found high peptidase activity in the stomachs of spotted sorubim *Pseudoplatystoma corruscans*. Nevertheless, the aim of the present study was to investigate the enzymatic activities as biomarkers of pollution, and thus a thorough profile of enzymatic digestion was not conducted.

Activities of the digestive enzymes in the fishes may vary among the organs, which can be affected by the environmental conditions. Thus, monitoring of enzymatic activities in different organs is important to furnish data for the intraspecific variations to allow further comparisons among different environments. In this study, gastrointestinal and hepatic activities for trypsin-like and chymotrypsin-like peptidases and for β-glucosidase were relatively with low variation among the specimens. This was relevant, since the biochemical parameters such as enzymatic activities were usually affected by the pollutants (Van der Oost et al. 2003; Pathiratne et al. 2009; Kuz'mina et al. 2013). Thus, before specific comparisons between the two reservoirs, one must point out that these hydrolases are promising biomarker for future studies given the acceptable low intraspecific variations and the easy assaying of their activities with chromogenic or natural substrates. Chakrabarti et al. (1995) investigated the enzymatic activities (alpha-amylase, cellulase, invertase, esterase, acidic and alkaline peptidases) of eleven teleostean fishes and found that the activities were higher in the intestine than in the stomach or in the liver, independently of the feeding habit. Difficulty in finding clear biochemical indicators of feeding habits made it even more difficult to compare among the individuals from different environments, such as oligotrophic and eutrophic ones. Thus, every piece of biochemical information is relevant.

Duarte et al. (2013) showed that the enzymatic activities of the peptidases and β-glucosidase were generally higher in the illiophagous/detritivorous *H. auroguttatus* than in the omnivorous *P. maculatus*. This was in agreement with the reports on enzymatic digestive activities depending upon the feeding habits (Hidalgo et al. 1991; Chan et al. 2004; Corrêa et al. 2007). Another important issue, specifically concerning the digestion of carbohydrates, was the source of the glucosidases (Nelson et al. 1999). Some authors have suggested that the fish itself could produce some cellulolytic enzymes but it seemed more probable that those enzymes were produced by the microorganisms associated with the liver and gastrointestinal tract (Bairagi et al. 2002). Izvekova (2005) investigated the microbiota contribution to peptidases and amylases secreted by the bacteria tightly bound to the fish intestine. Thus, since the hepatic β-glucosidase activity was significantly higher in the oligotrophic reservoir than in the eutrophic, and the intestinal activity showed a tendency to be higher in the individuals from the oligotrophic reservoirs than in those from the eutrophic system, it would be important to compare the hepatic and intestinal microbiota from the fishes from the two reservoirs in future studies. The increased activity in the hepatic β-glucosidase observed in the oligotrophic reservoir deserves additional investigation and should be associated with the microbiota studies to furnish a combined biochemical and microbiological biomarker of the freshwater systems. Thus, hepatic β-glucosidase activity as well as intestinal chymotrypsin activity could be considered as the candidates for biomarkers of environmental quality.

The effect of organic pollutants upon the activity of digestive enzymes has been studied. Filippov et al. (2013) reported that maltase activity was lower in the fishes collected in most polluted regions of the Rybinsk reservoir in the Russian Federation, whereas peptidase activities were higher when compared to the fishes collected in a more oligotrophic area of the reservoir. Lentic environments have more variation in the water quality among areas of different trophic conditions than lotic systems in which the water flow contributes to diminish such differences. Benthic fishes must adapt to those variations (Agostinho et al. 2004). Thus, it is important to find the biochemical alterations that occur in those species in different parts of a river course. It is important to point out the relevance of using fish species in the ecological studies to assess environmental quality. This is particularly important in the Paraíba do Sul river basin that have many different lentic environments in a highly populated area under increasing pressure from anthropogenic activities.

CONCLUSION

The peptidase activities were generally higher in the intestine than in the stomach of *P. maculatus*, which was an expected result, associated with its omnivorous feeding habit. Decreased activity in hepatic β-glucosidase and increased intestinal chymotrypsin-like activity observed in the oligotrophic reservoir deserved additional investigation and could be related to the microbiota activity. Studies on both enzymatic and microbiota activity are necessary to furnish a combined biochemical and microbiological biomarker of environmental quality in the freshwater systems. However, it was concluded that hepatic β-glucosidase activity as well as intestinal chymotrypsin activity could be considered as the candidates for biomarkers of environmental quality in tropical reservoirs.

ACKNOWLEDGMENTS

We thank Ademar P. Santos, Bernardo H. S. Bambirra, Daniel Jr. Leite, Camila C. Lara, Fabrícia Fonseca, Jacqueline Barbosa, Leandro H. S. Bambirra, Marco Antonio R. Paiva, Rodrigo S. Reston, Vinício T. S. Coelho and Jamil Silvano de Oliveira for helping in field and laboratorial analyses. We also thank Dr. Dirceu Marzulo Ribeiro, Paulo Sérgio Formagio, Marcos Antonio Evangelista and Maria das Neves for infrastructure support. Furnas Centrais Elétricas S/A financially supported this research by the Program of Research and Development (P & D), process number 016206. The Carlos Chagas Filho Research Support Foundation of the Rio de Janeiro State (FAPERJ) gave a sponsorship to SD. MPB and FGA are recipients of CNPq fellowships.

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Received: September 04, 2014;
Accepted: May 08, 2015.