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Biomarker responses as indication of contaminant effects in *Oreochromis niloticus*

Cleoni dos Santos Carvalho [a,*], Vanessa Aline Bernusso [b], Heloísa Sobreiro Selistre de Araújo [b], Evaldo Luiz Gaeta Espíndola [c], Marisa Narciso Fernandes [b]

[a] Universidade Federal de São Carlos, Campus Sorocaba, Rodovia João Leme dos Santos, km 110, SP-264, CEP 18052-780, Sorocaba, SP, Brazil
[b] Departamento de Ciencias Fisiológicas, Universidade Federal de São Carlos, Caixa Postal 676, Rodovia Washington Luis, km 235, CEP 13565-905, São Carlos, SP, Brazil
[c] Departamento de Hidráulica e Saneamento, Escola de Engenharia de São Carlos, Universidade de São Paulo, Av. do trabalhador São Carlense 400, CEP 13566-590, São Carlos, SP, Brazil

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ABSTRACT

The current study investigated oxidative stress parameters (enzymes activities, metallothionein content and lipid peroxidation) in freshwater fish, *Oreochromis niloticus*, tilapia exposure to Monjolinho River (in 4 months of year: January, April, July and November). One critical site in Monjolinho River (site B) was assessed in comparison to a reference site (site A). Water pH and oxygen concentration was lower than that recommended by CONAMA (Brazilian National Environmental Committee), resolution 357/2005 for protection of aquatic communities, and ammonium and the metals Cu, Zn, Mn and Fe (on all months) concentrations were higher than the maximum concentration recommended. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were significantly decreased in liver and muscle in tilapia from Monjolinho River, throughout the year, in relation to reference except in gills that SOD activity increased. Glutathione S-transferase (GST) activity was significantly increased in liver of the tilapia from Monjolinho River in all sites, in relation to reference except in gills that GST activity increased in July and decreased in November, suggesting that GST activity could be induced to neutralize the pollutants toxicity. On the other hand, GST activity was significantly decreased in white muscle indicating a toxic effect of pollutants, resulting in a decreased ability of tilapia to perform defense reactions associated to GSTs. The decrease of catalase (CAT) activity in gills of the *O. niloticus* together with the increase of SOD activity, could explain the increased lipid peroxidation (LPO) level in this organ. Metallothionein levels in liver and gills were significantly high in all sites. Results indicate that the exposure to metals caused severe damage to tissues; despite the consensually assumed antioxidant induction as a sign of exposure to contaminants the effects seem in part to be mediated by suppression of antioxidant system with SOD, CAT and GPx as potential candidates for tissues toxicity biomarkers of pollutants.

1. Introduction

Aquatic systems are the main recipients of almost all anthropogenic discharges. Metals are major pollutants of aquatic ecosystems due to disposal of industrial effluents, or via direct dumping in the river of waste material, such as sewage sludge and dredge spoil (CETESB, 1994–2002). They are usually toxic at high levels and may accumulate in the aquatic organisms as metals are not biodegradable or eliminated from ecosystems (Linde et al., 1996; Tagliari et al., 2004; Sanchez et al., 2005). Fish in the aquatic environment can be subjected to a multipollution state and the occurrence of sequential exposures is an important aspect of ecotoxicological research. Moreover, chemical analyses are expensive and it is not feasible to measure all classes of chemicals likely to be found in an aquatic environment given the complex mixture.

On the other hand, biomarkers, representing toxicant-induced changes in biological systems, can serve as links between an environmental contamination (cause) and its effects, providing therefore unique information on the ecosystem health (Maria et al., 2009), and provides relevant data about possible pathological process in fish. Metals may interfere in several metabolic pathways of cells and thereby induce different cellular responses depending on concentration and metal proprieties (Almeida et al., 2002; Tagliari et al., 2004; Abdel-Tawwab et al., 2007; Bouraoui et al., 2008; Carvalho and Fernandes, 2008; Casado-Martinez et al., 2009; Monteiro et al., 2009, 2010). These responses may promote oxidative stress by catalyzing the formation of reactive oxygen species (ROSs) such as the superoxide anion (O_2^-), hydroxyl radical (OH^-), singlet oxygen (O_2^1) and hydrogen peroxide (H_2O_2), which may generate DNA alterations and peroxidation of membrane lipids initiating cellular degenerative process (Storey, 1996; Liebler and Reed, 1997; Schlenk et al., 1999; Ahmad et al., 2000; Oakes and Kraak, 2003; Tagliari et al., 2004; Sanchez et al., 2005).

* Corresponding author. Fax: +55 15 32295967.
E-mail address: san-cleo@ufscar.br (C.S. Carvalho).

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ROS can be detoxified by enzymatic and non-enzymatic cell defense systems can be measured as biomarkers of xenobiotic mediated oxidative stress. Defenses against ROS include scavenger compounds like glutathione (GSH) and metallothionein (MT), and such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST) and glutathione reductase (GR) among others (Storey, 1996).

As the changes in the cell antioxidant defenses reflect the exposure to contaminants and/or their toxicity, all of them may be useful biomarkers in monitoring aquatic ecosystems (Almeida et al., 2007; Bouraoui et al., 2008; Ruas et al., 2008; Monteiro et al., 2010). Metals which are discharged daily into water bodies can induce ROS production in fish, consequently, a response of antioxidative defenses (Liebler and Reed, 1997; Schlenk et al., 1999; Ahmad et al., 2000; Ruas et al., 2008; Monteiro et al., 2010). When the ROS production exceeds the basal cell levels and surpassed the defense capacity of the cell the oxidative stress occurs (Sies, 1986; Storey, 1996; Tagliari et al., 2004).

The Monjolinho River, runs through São Carlos country, is one of the main responsible for the water supply in this city. However, this water resource receives great quantity of effluents deriving from industrial and urban wastes. This River receives industrial effluents of approximately 400 industries (food, paper, leather, paint, etc.) (Espíndola et al., 2000; Campagna, 2005; Dornfeld, 2006; Ruas et al., 2008; Printes et al., 2011).

Nile tilapia, *Oreochromis niloticus*, is widely distributed freshwater fish that can persist in a highly polluted habitat and have the potential for development as a biological monitor of an environmental pollution. In this perspective, the assessment of hepatic defense and damage responses in a resident fish species becomes highly relevant in biomonitoring studies, considering the important role of the liver on numerous vital functions such as accumulation, biotransformation, and excretion of contaminants and, subsequently, the proneness to face high levels of contaminants/metabolites and ROS. Gills area critical organ to fish as they represent the primary site for gas exchange, ion regulation, and excretion of metabolic waste products (Cerqueira and Fernandes, 2002; Monteiro et al., 2009). Fish muscle is an important source of animal protein and has higher biological value compared with other muscle proteins.

Thus, this study aimed to investigate the effects of aquatic contaminants, throughout a year, on the antioxidant defenses responses of a resident fish (*O. niloticus*) of an urban area of a known domestic and industrial polluted river. The activity of the enzymatic antioxidant defenses (SOD, CAT, GPx and GST) and the MT concentration were analysed in the liver, gills, and white muscle tissues. LPO was analysed in order to evaluate the effectiveness of antioxidant defenses.

2. Materials and methods

Adult specimens of *O. niloticus* (Body mass, BM = 90–200 g; Body length, BL = 15–20 cm) ($n = 8$, each month) were captured in different months of year: January, April, July and November, at the Monjolinho River (São Carlos, SP, Brazil) using a fishing-net. The sampling sites selection was based along the river entrance, taking into account the various types and sources of contamination as well as the selection of a site, unpolluted reference relatively well-preserved, which is known to be free from the influence of domestic or industrial effluents (site A). The site B (polluted site) was chosen because it was close the reference site and provide pollution inputs (Fig. 1). The water samples and the animals were collected in January, April, July and November 2006.

Immediately after capture, fish were killed by transecting the spinal cord and tissues were then removed, washed with cold saline (0.9% NaCl), divided into three sets, frozen in liquid nitrogen and later stored at −80 °C. The first set was used for further process in oxidative stress studies, the second set for MT measurement and the third and last set for LPO measurement.

2.1. Water analyses

The physical and chemical variables of the water were determined following the procedures described in APHA (1998). Dissolved oxygen (DO), conductivity, temperature, and pH were measured in the field using a Water Quality Checker (Horiba). Ammoniacal nitrogen, nitrate and nitrite were determined, after the water has been filtered, according to the method described by Mackereth et al. (1978). The concentration of metals in the water was determined by atomic absorption spectrophotometry (AAS), according to the quality assurance and quality control (QA/QC) requirements and the SRM 3114 NIST (USA) reference standards. Briefly, the water samples were fixed with nitric acid, filtered in glass filter (0.45 μm pore diameter) and the metal determination were done using a flamer spectrophotometer (SpectrAA 220, Varian).

2.2. Biochemical parameters

All the biochemical assays were measured spectrophotometrically (Biochrom Libra S32) at 25 °C. Samples of each frozen tissue were homogenized in 0.1 M sodium phosphate buffer pH 7.0 at a ratio of 1:10 w/v for LPO levels and SOD, CAT and GPx activity assays; 0.1 M potassium phosphate buffer pH 7.0 keeping the proportion 1:5 w/v for GST activity assay. Samples tissues were centrifuged at 12,000 xg at 4 °C and the supernatant was used for biochemical assays. Total proteins were determined according to the Bradford method (1976), adapted in microplate (Dynex Technologies Ltd., MRXTC, UK) according Kruger (1994) using bovine serum albumin as a standard. Absorbance of samples was measured at 595 nm.

Lipid peroxidation (LPO) in tissues were assessed by Fe^{2+} oxidation in the presence of xylenol orange (FOX-ferrous oxidation-xylenol orange), according to Jiang et al. (1992). The homogenized samples was treated with 10% trichloroacetic acid (TCA) and then centrifuged. The supernatant was applied to a solution-contained 900 μL of Fox reagent (0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange and 4 mM butylated hydroxytoluene) in 90% (v/v) methanol, and incubated at 37 °C for 15 min for color development prior to colorimetric measurement at 560 nm. The molar extinction coefficient of $4.3 \times 10^4\ M^{-1}\ cm^{-1}$ for cumene hydroperoxide was used (Jiang et al., 1991). Cumene hydroperoxide (CHP) was used as a standard. LPO levels were expressed as nmoles of CHP per mg protein.

SOD activity was measured by indirect inhibition assay of reduction of nitro blue tetrazolium (NBT) (Crouch et al., 1981), which was generated by 37.5 mM hydroxylamine in alkaline solution. The assay was performed in 0.5 M sodium carbonate buffer (pH 10.2) with 2 mM ethylenediaminetetraacetic acid (EDTA). The reduction of NBT to blue formazan by superoxide anion was measured at 560 nm. The rate of NBT reduction in the absence of hemolysate was used as a reference rate. One unit of SOD was defined as the amount of protein needed to decrease the reference rate to 50% of maximum inhibition. All data were expressed in units of SOD activity per mg protein.

CAT activity was determined by decreasing the H_2O_2 concentration at 240 nm (Aebi, 1974). Decays in absorbance were recorded during 17 s in 50 mM sodium phosphate buffer (pH 7.0) containing 15 mM H_2O_2 and the enzyme extract. One Bergmeyer units (B.U.) of CAT is the amount of enzyme that liberates half the peroxide oxygen from the H_2O_2 solution in 100 s at 25 °C. CAT activity was expressed as B.U. per gram of protein.

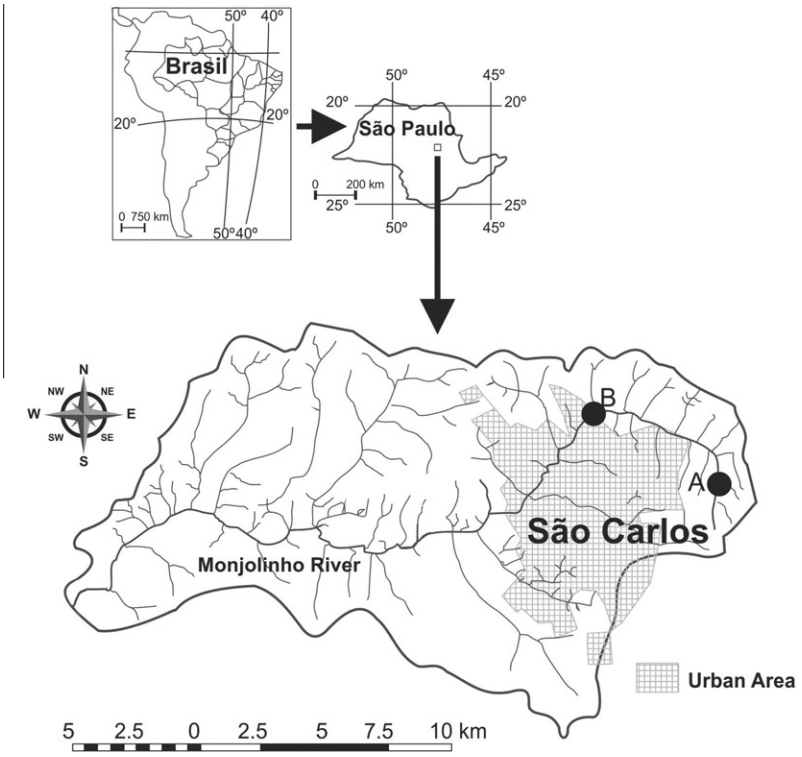

Fig. 1. Map of Monjolinho River, (São Carlos, SP, Brazil) with locations of fish-capture sites. The respective coordinates are as follows: Reference site: Site A (22°00′35.1″S; 47°50′06.1″W); and polluted site: Site B (21°59′26.5″S; 47°53′30.8″W). Sampling Stations (adapted from Printes et al., 2011).

GPx activity was measured using 2,5, dithiobis-tetranitrobenzoic acid (DTNB) reagent, according to the modified Mills' procedure 2 (Mills, 1959) described by Hafeman et al. (1974). Briefly, the samples were incubated at 37 °C for 5 min with a solution containing 80 mM sodium phosphate buffer (pH 7.0), 80 mM EDTA, 1 mM sodium azide (NaN_3), 0.4 mM GSH, and 0.25 mM hydrogen peroxide (reagent solution). The reaction was stopped by adding metaphosphoric acid solution for protein precipitation. After centrifugation, the remaining GSH in the supernatant was determined using 0.4 M sodium phosphate buffer (pH 7.0) and 1 mM DTNB in 1% trisodium citrate solution, and then it was measured at 412 nm. A blank incubation was carried out simultaneously with the samples, since non-enzymatic GSH oxidation by H_2O_2 occurs during incubation. One unit of GPx enzyme activity was defined as μg of GSH consumed per minute (Latha and Pari, 2004). GPx activity was expressed in units per mg protein.

The GST activity was determined according the method described by Habig et al. (1974). The activity of GST was assayed as the increase of absorbance at 340 nm due to the conjugation of 1 mM glutathione (Sigma) to 1 mM of 1-chloro-2,4-dinitro-benzene (CDNB) as a substrate. The activity was measured as the amount of enzyme catalyzing the formation of adduct S-2,4-dinitrophenyl against blank at 25 °C. The molar extinction coefficient used for CDNB was 9.6 mM^{-1} cm^{-1}. The activity was expressed in GST units, where one unit is the amount of enzyme necessary to conjugate 1 nM of CDNB per min and per milligram protein at 340 nm.

Metallothionein (MT) levels were evaluated in the liver, gills and white muscle according to the spectrophotometric method described in Viarengo et al. (1997) based on cysteine residues titration of a partially purified MT extract. The amount of metallothionein was quantified using Ellman's reagent containing DTNB measured spectrophotometrically at 412 nm. The MT levels concentrations were estimated using GSH is reference standard and expressed as μM-SH groups per mg of protein.

2.3. Statistical analysis

Results are presented as means ± standard error (SE). Data differences were determined using one-way ANOVA. Post-test Tukey with at 95% confidence limit was applied to compare mean values whenever the data difference was significant (GraphPad Instat version 3.00, GraphPad Software, USA).

3. Results

3.1. Water analysis

Table 1 show the values of water variables from reference (site A) and polluted sites (site B) during the year. No significant differences were detected in reference sites on water quality. In contrast, the physical and chemical characteristics of water of the polluted site changed during the year. All water variables were higher in the polluted site compared with the reference one, excepting dissolved O_2 (DO) and pH which were lower in the January and November (DO) and April and July (pH = 5.93 and 5.76, respectively). The concentration of ammonium and metals such as Cd, Cu, Zn, Mn and Fe, depending on season, were higher than the values recommended by the Brazilian Environment National Council for biota preservation and human safety (CONAMA 357/2005). In general, the river water in April (autumn) and July (winter) were better than in January (summer) and November (spring).

The activities of antioxidant enzymes and LPO levels in the liver, gills and white muscle are shown in Figs. 2–4. LPO was higher in liver in all months and, in January, it was exceptionally higher. In the liver, the activity of the SOD and GPx were significantly lower (86% and 80%, respectively) in April, July and November while, in January, GPx was higher (100%) than the reference site. CAT activity increased from January to July and decreased significantly in

Table 1
Physical and chemical data of water collected in the reference site (A) and in the polluted site of Monjolinho River (B).

Water	CONAMA (values)	Values							
Variables		January		April		July		November	
		Site A	Site B	Site A	Site B	Site A	Site B	Site A	Site B
DO (mg L^{-1})	5.0	7.45 ± 0.05	3.29 ± 0.5[#]	7.35 ± 0.08	7.52 ± 0.4	7.0 ± 1.0	6.31 ± 0.45	7.7 ± 0.9	2.7 ± 0.04[#]
Temperature (°C)		24.0 ± 1.0	22 ± 1.0	22.0 ± 0.8	23 ± 1.5	20.0 ± 1.2	21.3 ± 1.0	25.0 ± 1.5	22 ± 0.6
pH	6–9	6.9 ± 0.08	6.63 ± 0.6	6.5 ± 0.05	5.93 ± 0.03[#]	7.0 ± 0.08	5.76 ± 0.05[#]	7.20 ± 0.98	6.63 ± 0.07
Conductivity (μS cm^{-1})		28 ± 4	58 ± 4.5	28 ± 4	34 ± 0.9	24 ± 3.4	47 ± 3.2	27 ± 6.1	58 ± 1.9
Silicate (mg L^{-1})		1.64 ± 0.2	10.05 ±	1.58 ± 0.4	3.69 ± 0.25	1.15 ± 0.67	3.00 ± 0.32	1.83 ± 0.9	7.51 ± 1.2
Ammonium (μg L^{-1})	20	56.14 ± 10	432.98 ± 23.0[*]	36.14 ± 0.9	153.77 ± 11.0[*]	42.09 ± 12.9	151.29 ± 6.5[*]	22.17 ± 2.9	313.15 ± 9.3[*]
Nitrite (μg L^{-1})	1000	4.31 ± 0.1	20.47 ± 2.8	8.31 ± 0.15	9.68 ± 0.50	12.98 ± 0.58	2.55 ± 0.025	6.33 ± 0.26	4.52 ± 0.4
Nitrate (μg L^{-1})	10000	115.13 ± 15.6	282.57 ± 18.9	121.09 ± 10.9	110.43 ± 9.4	90.04 ± 12.7	166.93 ± 10	109.10 ± 0.7	163.72 ± 8.2
Cr (μg L^{-1})	50.0	ND	1.50 ± 0.2	ND	1.27 ± 0.5	ND	4.40 ± 1.0	ND	4.50 ± 0.7
Cd (μg L^{-1})	1.0	ND	0.27 ± 0.03	ND	0.06 ± 0.02	ND	1.20 ± 0.13[*]	ND	0.03 ± 0.005
Cu (μg L^{-1})	9.0	ND	ND	ND	5.49 ± 0.07	ND	13.60 ± 0.65	ND	13.88 ± 0.08[*]
Zn (μg L^{-1})	180.0	120 ± 20.0	ND	157 ± 13.0	270.0 ± 6.5[*]	133 ± 19.0	17.0 ± 0.9	182 ± 9.0	490.0 ± 11.8[*]
Mn (μg L^{-1})	100.0	ND	80±	ND	790.0 ±[*]	ND	70.0±	ND	570.0±[*]
Fe (μg L^{-1})	300.0	ND	1060.0 ±[*]	ND	11480.0 ±[*]	ND	1270.0 ±[*]	ND	109360.0 ±[*]

ND = not detected.
[#] Values lower.
[*] Values higher than those established by the Brazilian National Environmental Committee (CONAMA), resolution 357/2005.

November (120%). The activity of the GST was significantly higher in all months studied (Fig. 2).

In the gills the LPO level was higher in all months exceptionally in April was not. SOD activity was higher than the reference site (70%) and, particularly elevated in January (300%); GPx and CAT activities were significant lower (about 20–60%), excepting the GPx, in January. GST activity was higher ($P < 0.05$) in all months (about 30–50%) (Fig. 3).

LPO was higher in all months in white muscle. SOD, GPx, and GST activities were lower (50–95%) than the reference site while CAT activity was higher throughout the year (about 3–30×) (Fig. 4).

The MT levels were quantified by –SH residue content. The MT levels were higher (60–170%) in the liver and gills of the *O. niloticus* from polluted site throughout the year (Fig. 5).

4. Discussion

Studies performed in the Monjolinho River showed metal and pesticide contamination in this area (Espíndola et al., 2000; Campagna, 2005; Dornfeld, 2006; Ruas et al., 2008; Printes et al., 2011). Fish inhabiting the urban region of Monjolinho River are exposed to a complex of different mixtures of contaminants, particularly in April, July and November. Our data clearly showed the ecotoxicological impact of multicontaminantes on fish.

Abiotic factors may influence biomarker responses to contaminants. The levels of dissolved oxygen (DO) were found to be lower in January and November. Low oxygen concentration in water interferes with the fish population, causing death and abnormalities in the offspring. Disturb the balance oxygen supply/demand influencing oxygen levels in tissues, which interfere with antioxidant defenses (Storey, 1996; Oliveira et al., 2010a). Temperature and pH affect the catalytic efficiency and binding capacity of enzymes (Hochachka and Somero, 1984; Carvalho and Fernandes, 2008). Moreover, the bioavailability of several pollutants with special reference to metals is affected by pH changes (Cusimano et al., 1986; Takasusuki et al., 2004; Carvalho, 2003; Carvalho et al., 2004; Carvalho and Fernandes, 2008).

Ammonia (NH_3) plays a role in the regulation of a number of metabolic pathways and the high concentration of ammonia in Monjolinho River could contribute to the altered metabolic status of fish. Tissues may change with the high acute toxicity of NH_3 and can also lead to metabolic changes. According Costa et al. (2008) the excessive presence of NH_3 alters cellular metabolism, resulting in decreased cellular concentrations of ATP. In addition, ammonia inhibits the active transport of sodium ions, which can affect the transport of chloride ions, bicarbonate and water reabsorption in epithelia carriers. Ammonia is a toxic metabolite and excess ammonia is known to trigger the operation of detoxification or utilization systems, chiefly by way for formation of less toxic nitrogenous substances (Begum, 2004).

Metals cannot be degraded by biological or chemical processes, and thus tend to accumulate in soils and aquatic sediments. As a consequence, they can enter the food chain, thereby representing a health risk to humans and animals (Castiglione et al., 2009). The metal content found in the water samples collected during the months January, April, July and November show the presence of chromium, cadmium, copper, zinc, manganese and iron. Besides, some essential elements, such as copper (Cu) and zinc (Zn), are present at elevated, and hence potentially toxic, levels mainly as a result of agricultural practices, and industrial and municipal waste disposal on land.

Several studies have shown enhanced LPO in aquatic organisms exposed to high concentrations of metals (Ahmad et al., 2000; Oakes and Kraak, 2003; Pandey et al., 2008; Ruas et al., 2008; Monteiro et al., 2010). Our study revealed higher LPO levels in liver, gills and white muscle, a clear oxidative stress indication. LPO, a

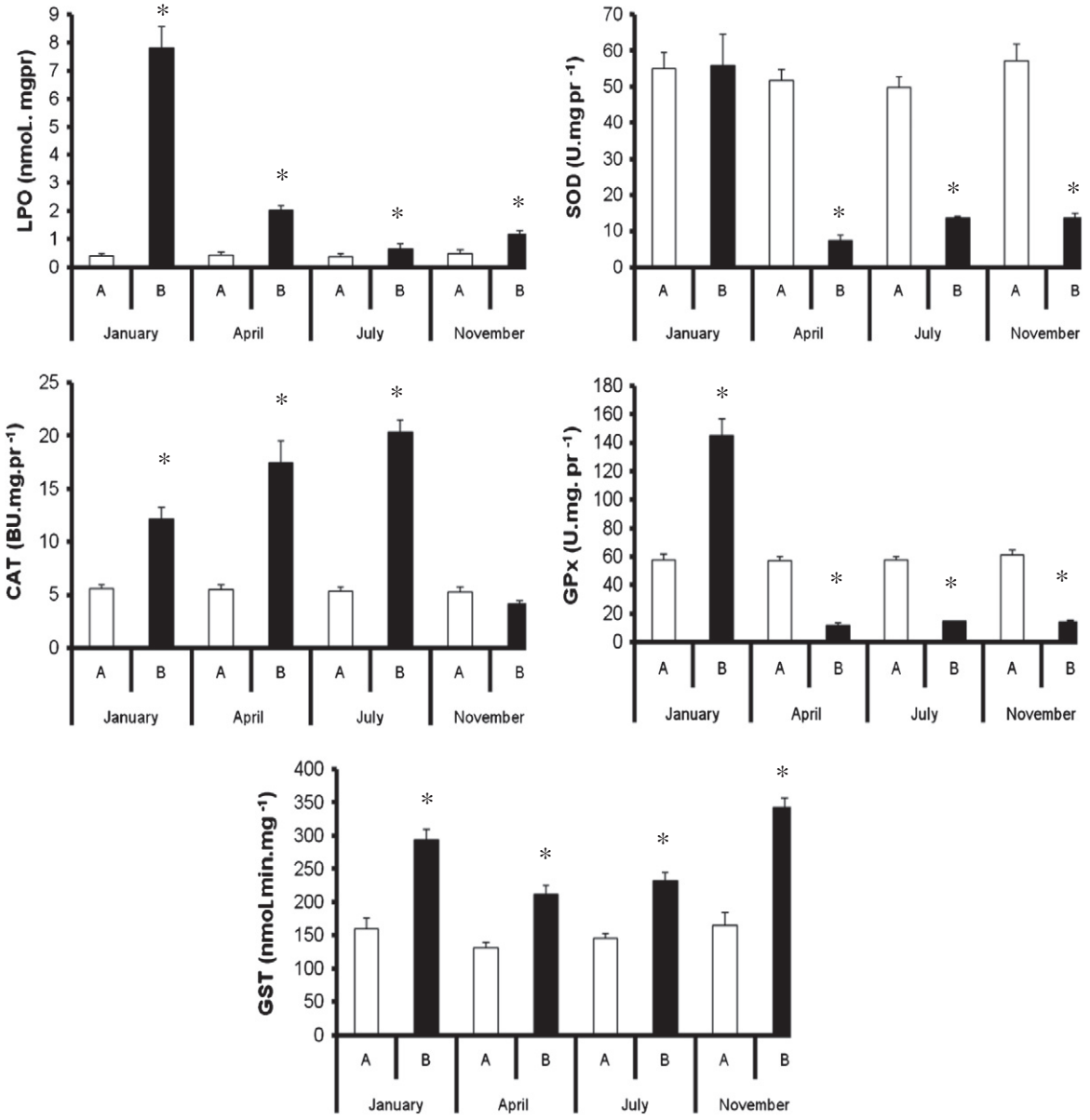

Fig. 2. Antioxidant enzyme activities and levels of lipid peroxidation (LPO) in the liver of *O. niloticus*. Values are mean ± S.E.M. (*) indicates significant difference in relation to control group ($P < 0.05$); $n = 6–8$. A = reference and B = Monjolinho River.

complex process resulting from free radical reactions in biological membranes forms lipid hydroperoxides which decompose double bonds of unsaturated fatty acids and destructs membrane lipids. Once absorbed, contaminants may interact with endogenous substances, causing biological effects that may impair the life quality not only of the exposed organisms but also of the whole ecosystem.

Polluting substances such as metals could act on the organisms directly and/or by forming free oxygen radicals, initiating degenerative processes and causing genotoxic effects (Halliwell and Gutteridge, 1985). In the presence of transition metals, like iron and copper, O_2^- and H_2O_2 can generate OH^- through Fenton reaction (Storey, 1996). Other non-transition metal ions can also be implicated in ROS generation in mitochondria. Cadmium, for example, is known to generate ROS due to an inhibitory effect on mitochondria electron transport (Stohs et al., 2000).

SOD and CAT are the primary defense against oxygen toxicity induced by metals. SOD is a metalloenzymes that play a key role in the defense against ROS by transforming superoxide anions into hydrogen peroxide, which is detoxified by both GPx and CAT activities. SOD and GPx activities decreased in liver and white muscle of the tilapia in relation to reference, except in gills that SOD activity increased. Enzyme activity can be decreased by negative feedback from excess of substrate or damage by oxidative modification. The loss of antioxidant defenses (CAT and GPx) in gills was paralleled by higher LPO levels. However, the decreased activities of CAT and GPx enzymes favor the accumulation of H_2O_2 and this radical accelerate the conversion of Fe^{3+} to Fe^{2+} and the latter serves as a substrate for hydroxyl radical generating reaction leading to enhanced lipid peroxidation (Halliwell and Gutteridge, 1985). A reduced enzyme activity could indicate that is antioxidant capacity was surpassed by the amount of hydroperoxide products lipid peroxidation which can be observed in LPO levels these tissues.

In addition, the marked decreasing in SOD activity (liver and white muscle) may result from direct binding of the metal to the enzyme and leads to oxidative stress and stimulates lipid peroxidation (Bainy, 1993; Liebler and Reed, 1997; Hamed et al., 2003).

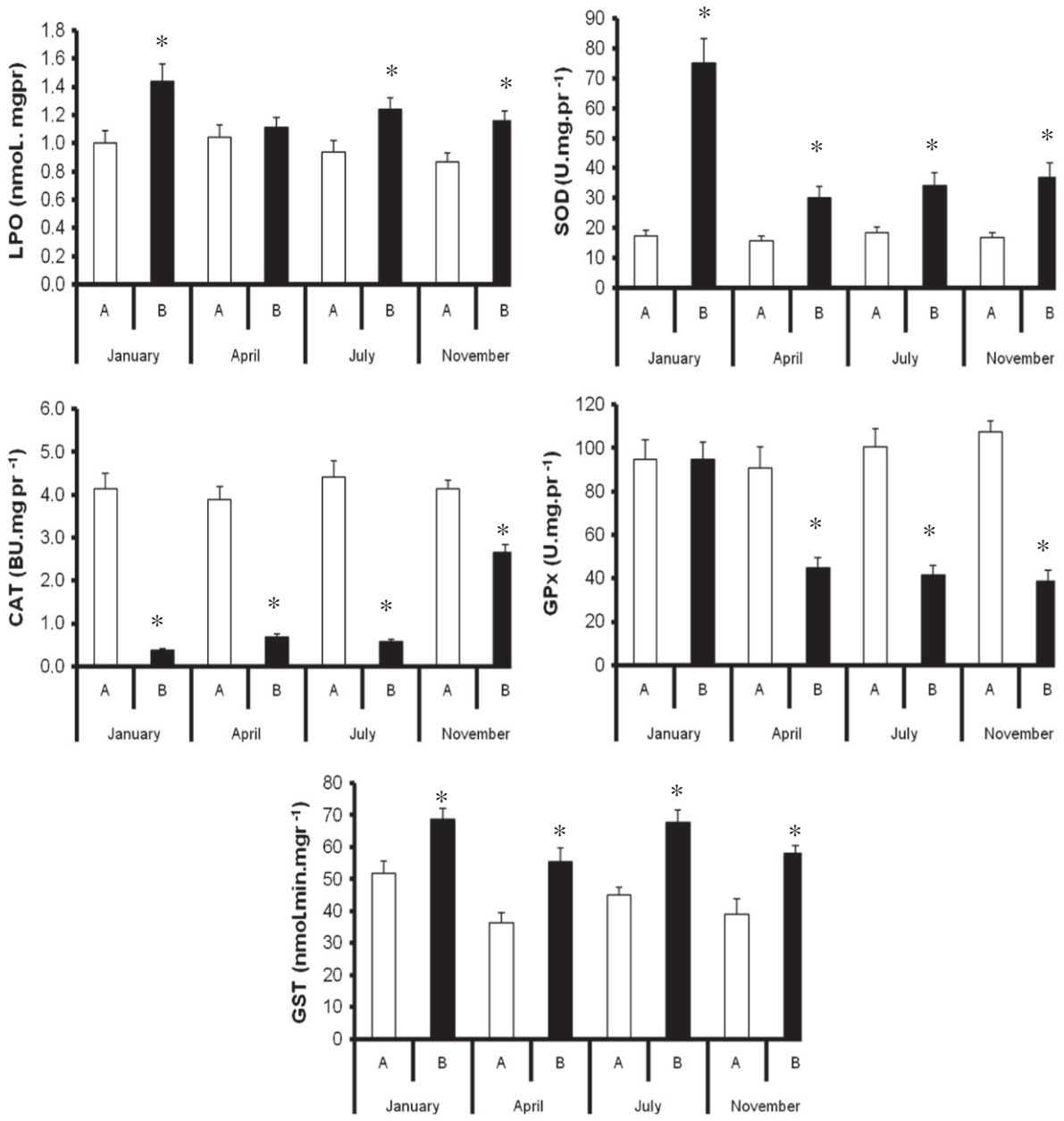

Fig. 3. Antioxidant enzyme activities and levels of lipid peroxidation (LPO) in the gills of *O. niloticus*. Values are mean ± S.E.M. (*) indicates significant difference in relation to control group ($P < 0.05$); $n = 6–8$. A = reference and B = Monjolinho River.

In gills the increase of the SOD activity leads to a rise in H_2O_2 production that was not compensated by the increased CAT and GPx thereby inducing the oxidative stress and stimulates lipid peroxidation. In fact, it was observed in gills of the fish collected in January, July and November. According Padmini and Usha Rani (2009), the antioxidant enzymes activities may be increased or inhibited under metals exposure depending on the intensity and the duration of the stress applied. The elevated levels of SOD in gills shows a possible shift toward a detoxification mechanism under long term exposure to the metals which indicated that the tilapia could protect itself against the toxic effect of superoxide anion radical by increasing its activity. The higher SOD activity in gill than in liver could be an indicator of compensatory tissue response to metal exposure (Fernandes et al., 2008). Liu et al. (2006) related that Cu^{2+} induced the generation of SOD activity when fish were subjected to lower Cu^{2+} and was inhibited with higher Cu^{2+} (>0.01 mg L^{-1}).

CAT activity increase suggests the presence of higher peroxide concentrations. The increase CAT activity and decrease GPx and SOD activities in liver and muscles clearly indicated that oxidant defenses were not enough to prevent in the LPO formation. Hence, H_2O_2 removal by CAT is an important strategy of organisms against oxidative stress. Atli et al. (2006) reported that metals (Cd^{2+}, Cu^{2+}, Cr^{2+} and Zn^{2+}) stimulated, *in vivo*, CAT activity in liver of the *O. niloticus* and was inhibited by Ag^{+} and Cd^{2+}. According to these authors the inhibition of CAT activity has been related to the binding of metal ions to –SH groups of the enzyme, increased hydrogen peroxide and/or superoxide radical.

CAT is the primary enzyme in scavenging H_2O_2, so when CAT activity is inhibited, more H_2O_2 is available for production of hydroxide free radical. In this study, the decrease of CAT activity in gills of the *O. niloticus* could explain the increased LPO level in this organ. The gills, liver and kidney are commonly the primary target organs for many pollutants. Liver exhibits a high metabolism

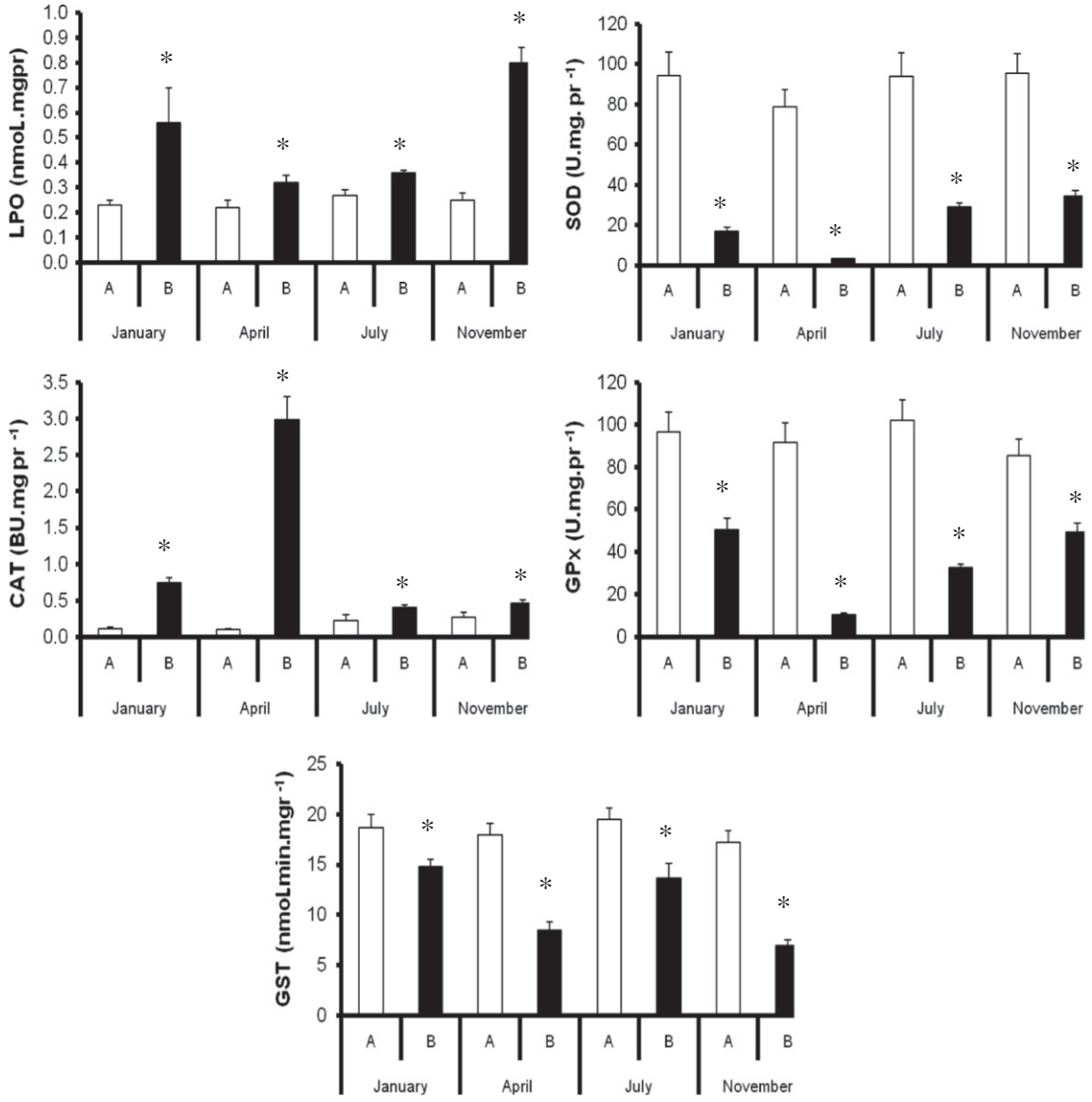

Fig. 4. Antioxidant enzyme activities and levels of lipid peroxidation (LPO) in the white muscle of *O. niloticus*. Values are mean ± S.E.M. (*) indicates significant difference in relation to control group ($P < 0.05$); $n = 6–8$. A = reference and B = Monjolinho River.

and oxygen consumption and it is the main organ of xenobiotic detoxification and its ability to eliminate the oxidative by-products. However, the gills have a wide surface area open to the external milieu and, the metal absorption in fish takes place primarily through gills also the first target to waterbone pollutants metal absorption in fish (Cerqueira and Fernandes, 2002; Monteiro et al., 2009). In addition, exposure to metals induced histopathological changes in the gill such as, hemorrhage at filaments and hypertrophy of epithelial cells (Cerqueira and Fernandes, 2002; Fernandes et al., 2008; Monteiro et al., 2009;). These changes although not leading to lethal outcome, might compromise gill's ability to handle the xenobiotics and infectious agents.

CAT is responsible for the reduction of hydrogen peroxide, while GPx catalyzes the reduction of both hydrogen peroxide and lipid peroxides. However, our results indicated that the decreased of the GPx and SOD activities in tissues of the *O. niloticus*, from Monjolinho River, could be indicated that the abilities to protect against hydrogen peroxide were reduced and are not scavenged by these antioxidant enzymes.

GPx depletion promotes generation of ROS and oxidative stress with the subsequent cascade of effects affecting the functional and structural integrity of cell and organelle membranes (Latha and Pari, 2004; Padmini and Usha Rani, 2009). Inversely, Sanchez et al. (2005) verified in liver of *Gasterosteus aculeatus* exposed to copper (LC 50) increase of enzyme activities of the SOD, CAT and GPx and GSH levels characterizing metal contamination. GPx and SOD activities and LPO levels in erythrocytes of *O. niloticus* showed increased in autumn (corresponding to April) and spring (corresponding to November) from polluted river (Ruas et al., 2008). Monteiro et al. (2010) observed increases in SOD, CAT and GPx in liver, gills, white muscle and heart in *Brycon amazonicus* exposed to 0.15 mg L^{-1} of mercury chloride ($HgCl_2$). Fish liver can be regarded as the body's detoxification organ and hence a target organ of various xenobiotic substances (Padmini and Usha Rani, 2009).

Low levels of GPx in fish may result in a significant accumulation of the high levels of H_2O_2. This could be associated to the O_2^- production or to the action of metals in enzyme synthesis (Bainy, 1993; Padmini and Usha Rani, 2009) and, causing a number of damage cellular for the reason that the impairment in the radical formation.

Ahmad et al. (2000) verified a time dependent increase in activities of GPx in liver of freshwater catfish (*Channa punctatus*) in

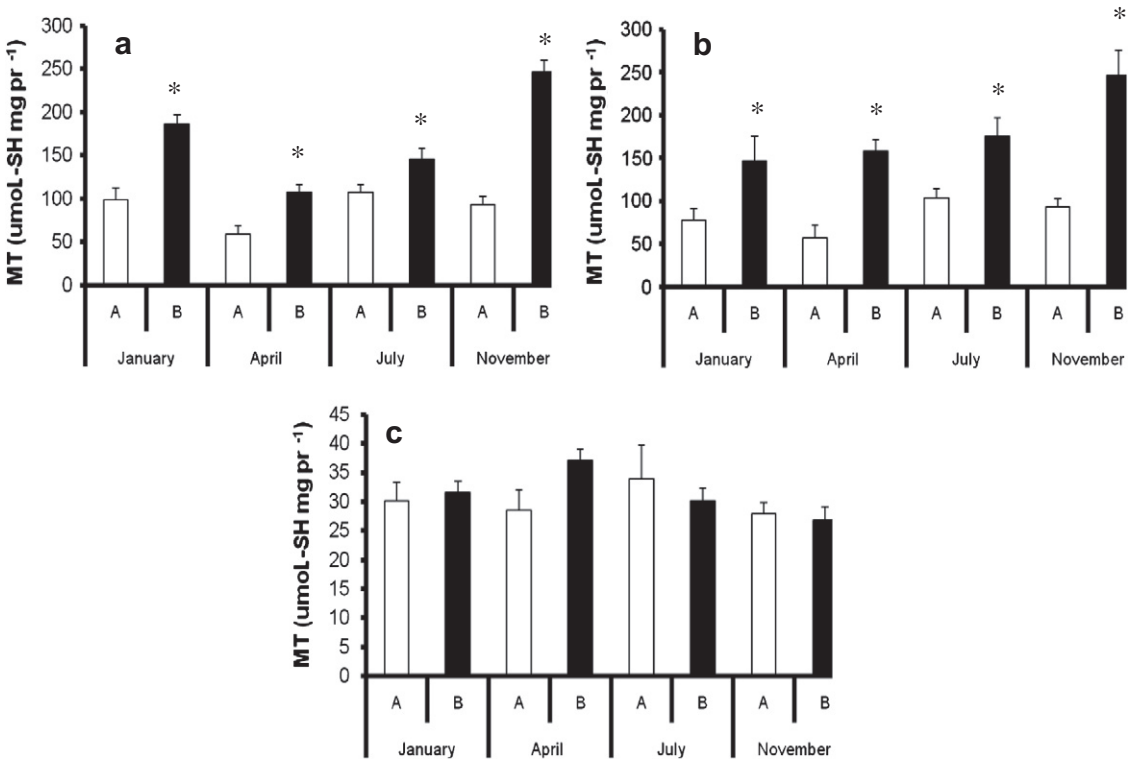

Fig. 5. Metallothionein content in liver (a), gills (b) and white muscle (c) of the *O. niloticus* (μM SH/mg protein). Values are mean ± S.E.M. (*) indicates significant difference in relation to control group ($P < 0.05$); $n = 4–6$. A = reference and B = Monjolinho River.

response to paper mill effluent with different concentrations. *O. niloticus* and *Clarias lazera* industrial polluted sites collected from the Nile River showed an increase of GPx activity in liver and kidney when compared to control (Hamed et al., 2003). Kutlu and Susuz (2004) observed slightly inhibited activity of GPx after exposure to lead acetate in invertebrate, *Gammarus pulex*. Results denoted different patterns of antioxidant enzyme response, suggesting that different toxicants may induce different antioxidant/prooxidant responses depending on their ability to produce ROS and antioxidant enzymes to detoxify them.

GST is a group of widely distributed enzymes that catalyzes the conjugation of reduced glutathione (GSH) with compounds having reactive electrophilic groups (especially xenobiotics, like as metal and pesticide). These enzymes generate less toxic and more hydrophilic molecules (Olsen et al., 2001) and play a role preventing oxidative damage by conjugating breakdown products of lipid peroxides to GSH (Barata et al., 2005; Fernandes et al., 2008). This metabolic pathway allows the protection of nucleophilic groups in macromolecules such as proteins and nucleic acids. Other functions, not associated with detoxification, include repair of macromolecules oxidized by ROS, regeneration of S-thiolated proteins, and biosynthesis of physiologically important metabolites (Freitas et al., 2007).

The increased GST activity in liver and gills of the tilapia from Monjolinho River in all months, in relation to reference, suggests that GST activity could be induced to resist the pollutants toxicity (mainly metals). Induction of GSTs is known to indicate the presence of various xenobiotics like polycyclic aromatic hydrocarbons (PAHs) (Ahmad et al., 2005), mercury chloride (Monteiro et al., 2010) and pesticides (Printes et al., 2011). *Sparus aurata* exposed to Cd chloride (200 μg kg^{-1}) showed GST activation in liver starting from 12 h exposure and maintained until 48 h of exposure (Bouraoui et al., 2008). Our results indicate that this enzyme could be crucial and also shows peroxidase activity towards ROS in the cells under oxidative stress in the *O. niloticus* related to a great input of pollutants into the Monjolinho River. These results show the vulnerability of these organs and, the *O. niloticus* were undergoing oxidative stress pollutant-induced industrial. In addition, show that in liver and gill detoxification processes were not depressed.

GST may be actively involved detoxifying redox cycling chemicals. However, GST activity was significantly decreased in white muscle. The relatively decreasing of the GST activity in white muscle may be related to diminished levels of GSH susceptible of being conjugated. GST induction occurs in several tissues at different periods of exposure to inducers, this depending on the type of tissue and nature of the inducer (Ahmad et al., 2005; Maria et al., 2009; Monteiro et al., 2010; Oliveira et al., 2010a; Printes et al., 2011).

Metallothioneins (MT) are known to be over-expressed in organisms from environments with high metal concentrations. Metal metabolism involves formation of GSH–metal complexes, from which the metal is further transferred to MT apoproteins (Atli et al., 2006). Levels MT were considerably elevated in liver and gill of fish collected in Monjolinho River as compared to their references. In the current study, MT concentrations were higher at the months displaying the highest metal concentrations, supporting its usefulness in environmental monitoring even in complex environments where interference of other xenobiotics can be found. MT has a physiological role in the protection of cells against the toxic effects of free oxygen radicals and must be taken into account before elevated MT levels can be interpreted as a sign of metal exposure in aquatic organisms (Viarengo et al., 1999; Oliveira et al., 2010b). Increased levels of MT in gills can be because the gills are the first route of metal uptake. In addition, lower liver MT levels suggesting a MT synthesis reduction that may be associated with increased demand of cysteine residues for GSH synthesis during detoxification of contaminants (Roméo et al., 1997), present in Monjolinho River. The gills are more susceptible to the immediate

(acute) effects of exposure to waterborne contaminants and livers are subjected to the more prolonged (chronic) effects of accumulated contaminants and their, often more toxic, metabolites (Cerqueira and Fernandes, 2002).

Moreover, induction of MT showed a different pattern, with highest mean MT levels in liver of fish in January and November. The variability observed in the MT content of liver may reflect region-wide changes in the bioavailability of metals responsible for MT formation. Kock et al. (1996) also demonstrated that the seasonal patterns of metal accumulation in arctic char *Salvelinus alpinus* were related to increasing metabolic rates during summer and did not correlate with seasonal variations in the metal concentrations in lake water or in the diet. According Schiedek et al. (2006) seasonal variations with higher MT levels in winter than in spring/summer have also been reported for bivalves and the authors related this to fluctuations in body weight. Falfushynska and Stoliar (2009) reported higher levels of Cu and Cd in MT in liver of the carp, *Cyprinus carpio*, in spring, were reflected at site rural probably as the result of the permitting pollution here. Trace metals like Cd, Cu and Zn have a high affinity for sulfur and nitrogen containing functional groups (Voets et al., 2009), but can bind to any molecule with an affinity for that metal.

Our data about the metal concentrations in water demonstrated higher levels of Zn, Mn and Fe in April and higher levels of Cr, Cd and Fe in July and November. The intracellular storage of Zn, Cu, and Cd is connected to the function of MT (Falfushynska and Stoliar, 2009) and the extremely high ability of MT to bind Cd is well known. In our study, the elevation of the level MT and content metals, especially in January, July and November, probably reflect the selective affinity and the low ability to eliminate this metal and can reflect the response to compounds other than metal compounds typical for the mixed urban and industrial pollution. In addition, according Karin et al. (1980) zinc may have been displaced and have allowed an induction of MTs since zinc has been shown to be a primary inducer of MTs.

Moreover, the MT levels higher in liver (January and November), and gills (November) and, decreased of the GPx activity and increased of the GST activity in liver and gills in tilapia may be explained by induction of MT and, the higher tolerance by metals for these fishes. Bouraoui et al. (2008) verified increase of MT levels in the liver of the *S. aurata* exposed to Cd chloride (200 $\mu g\ kg^{-1}$) and showed the same behavior as GST activation. Monteiro et al. (2010) observed increase of MT and GST in liver, gills and red muscle, whereas no changes were detected in the white muscle MT levels of the *B. amazonicus* exposed to $HgCl_2$.

In addition, this study indicated a protective role of MT by scavenging dangerous oxyradical species and probably the inactivation of metals by binding MT. Among those defences, MT is considered as the most suitable biomarker for metal exposure and may have prevented ROS production in the tissues of *O. niloticus*. In this perspective, this study become highly relevant, considered as a beginning to study the effects on acute exposure to single and mixed pollution on fish.

In this study, the results indicate a sensitive biochemical indicator of chemical pollution and the impact of a mixture of contaminants oxidative stress status of fish inhabiting sites with contrasting water quality conditions. Fish exposed to complexes mixtures of the metal (Cu^{2+}, Cd^{2+}, Cr^{3+}, Zn, Mn and Fe) show a tendency toward decreased antioxidant enzyme activity. In this study, enzymatic activities in liver (SOD and GPx) and gills (CAT and GPx) were decreased by affected metal toxication in the fish. Metal toxicity led to free radicals and oxidative damage on tissue. It has been reported by Talas et al. (2008) which described the antioxidative effects on the liver of fish at several levels of biological organization that include changes in biochemistry of animals exposed to metals like Cd^{2+} and Cr^{3+}.

The present study reveled that fish developed tissue-specific enzyme responses, such as increase in CAT activity in liver and seldom in muscle and increase of the SOD activity in gills to encounter the pollution exposure. This data are compatible with lower antioxidant defense and higher susceptibility of fish to oxidative stress damage. The determination of the extent and severity of water contamination by pollutants is often difficult. The results of the current study suggest continued monitoring of Monjolinho River.

Acknowledgments

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