Comparative Biochemistry and Physiology, Part C 148 (2008) 136–142

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Effects of phenol in antioxidant metabolism in matrinxã, *Brycon amazonicus* (Teleostei; Characidae)

Ive Marchioni Avilez [a], Tiago Silvestre Fernandes Hori [b], Luciana Cristina de Almeida [a], Araceli Hackbarth [a], Jayme da Cunha Bastos Neto [c], Vera Lucia Freire da Cunha Bastos [c], Gilberto Moraes *

[a] Department of Genetics and Evolution, Federal University of Sao Carlos, (UFSCar), SP, Brazil
[b] Ocean Sciences Center, Memorial University of Newfoundland, St John's, NL, Canada
[c] Department of Biochemistry, State University of Rio de Janeiro, (UERJ), RJ, Brazil

ARTICLE INFO

Article history:
Received 15 February 2008
Received in revised form 22 April 2008
Accepted 24 April 2008
Available online 1 May 2008

Keywords:
Antioxidant embolism
Fish
Oxidative stress
Phenol

ABSTRACT

Parameters of the antioxidant defense systems of *Brycon amazonicus* (matrinxã — a neotropical fish) exposed to phenol for 96h plus the recovery over 1 and 2weeks were studied in erythrocytes and liver. Hematocrit increase was observed during phenol exposure and recovery for 1week. Total superoxide dismutases (SOD), glutathione peroxidase (GPx) and reduced glutathione (GSH) did not change during phenol exposure. Erythrocyte glucose-6-phosphate dehydrogenase (G6PDH) increased during that period while catalase (CAT) activity decreased during phenol exposure and recovery for 2weeks. In the liver, SOD and CAT did not change, whereas GPx increased in the first week of recovery and decreased after 2weeks. A late response was observed for G6PDH activity which increased only at the second week. Ascorbate concentration in the brain decreased during phenol exposure and increased over recovery. From our results it appears that the oxidative stress was limited in matrinxã exposed to phenol, but seemed to occur during the recovery period.

1. Introduction

Since the second half of the last century, the environment has been contaminated by numerous xenobiotics, amongst these phenol is of special concern. This xenobiotic is produced by industries of chemicals, dyes, iron, steel, wood, plastics, synthetic fibers, detergents, pesticides, explosives, textiles, petroleum refinery and pulp mills. It can also result from natural processes, such as biotransformation of benzene (Bruce at al., 1987; Jennings et al 1996), tyrosine synthesis and reactions in the digestive system of vertebrates (Tsuruta et al., 1996). Phenol is among the first compounds described as toxic by the US Environmental Protection Agency (EPA), and due to its relevance as an ecotoxin it has been maintained in the priority list. The Current National Recommended Water Quality Criteria from EPA-US advises phenol concentrations lower than 300μg/L in order to protect aquatic organisms, or lower than 1μg/L to prevent the tainting of fish flesh. In Brazil, the National Council of Environment (CONAMA) established the same limits to protect the aquatic fauna (Brasil, 2005). Unfortunately, phenol is often found in the water bodies of Brazil at concentrations above 300μg/L (CETESB, 2003).

Oxidative stress occurs when reactive oxygen species (ROS), such as superoxide ion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and singlet oxygen (O_1) react with lipids, proteins or nucleic acids resulting in several biochemical injuries (Yu and Anderson, 1997; Pinchuk and Lichtenberg, 2002; Valvanidis et al., 2006). Detoxification of ROS is one of the prerequisites of aerobic life (McCord, 2000), and many defenses have evolved providing an antioxidant system which is able to prevent, intercept and repair damages. It consists of non-enzymatic ROS scavengers as: ascorbic acid, reduced glutathione, α-tocopherol, flavonoids, β-carotene and urate, and also of an enzymatic system that includes superoxide dismutase, glutathione peroxidase, catalase, peroxidase, NADPH quinine oxidoredutase, DT-diaphorase, epoxide hydrolase, glucose-6-phosphate dehydrogenase and a few conjugation enzymes (Sies, 1991; Valvanidis et al., 2006).

Many environmental pollutants, such as phenol, may cause oxidative stress in aquatic organisms by inducing ROS production (Sayeed et al., 2003; Oruc et al., 2004). When the pro-oxidants are prevalent over the anti-oxidants the increase of ROS may occur. In biological systems, the balance between both endogenous and exogenous pro-oxidant factors versus antioxidant defenses can be used to assess oxidative damage induced by different classes of chemical pollutants (Valvanidis et al., 2006). Changes of antioxidant enzymes activity may depict a change in the ROS within the cells. Therefore, these enzymes can be used as biomarkers for oxidative stress (Roche and Boge, 2000; Valvanidis et al., 2006). Aquatic organisms are usually more sensitive than terrestrials and may be

* Corresponding author. Department of Genetics and Evolution, Federal University of Sao Carlos, (UFSCar), Rod. Washington Luís, km 235, CEP 13565-905, Sao Carlos, SP, Brazil. Tel.: +55 1633518376; fax: +55 1633518377.
E-mail address: gil@power.ufscar.br (G. Moraes).

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doi:10.1016/j.cbpc.2008.04.008

better experimental subjects to evaluate subtle effects of oxidative stress (Ahmad et al., 2000; Valvanidis et al., 2006).

Phenol is the least lipophilic compound from phenol derivatives such as nitrophenols, alkylphenols and chlorophenols, (Kishino and Kobayashi, 1995). Those derivatives can be found in tissues and induce acute or chronic intoxication. At cellular level, they notably affect mitochondrial respiration, membrane potential or synaptic transmission, which explains their acute neurotoxicity (Kaila, 1982). Phenol and its derivatives are also genotoxic (Jagetia and Aruna, 1997; Yu and Anderson, 1997), carcinogenic (Tsutsui et al., 1997) and immunotoxic (Taysse et al., 1995). In humans, they cause hemolysis and methemoglobinization (Bukowska and Kowalska, 2004), oxidize the normal oxygen-carrying Fe^{2+} hemoglobin into the non-oxygen-carrying Fe^{3+}. Phenol can be more toxic to fish than bacteria and unicellular green algae (Tisler and Zagorc-Koncan, 1997). In fish, phenol is passively diffused through the gills (Kishino and Kobayashi, 1995). Some fish exposed to phenol showed not only decrease in food consumption, weight and fertility (Saha et al., 1999), but also changes in carbohydrate and protein metabolism (Gupta et al., 1983, Hori et al., 2006).

Studies on hepatic biotransformation of phenol have shown that rats metabolize it into hydroquinone and catechol (Sawahata and Neal, 1982). Also in the rainbow trout hepatic microsomal biotransformation of phenol into hydroquinone and catechol has been observed (Kolanczyk and Schmieder, 2002). Moreover, the presence of phenyl sulphate and phenyl glucuronide in bream, goldfish, guppy, minnow, perch, roach, rudd and tench (Layiwola and Linnecar, 1981; Nagel and Urich, 1983) suggests that several freshwater fishes may have enzymes for conjugating phenol. Particularly noteworthy is the fact that biotransformation could result in more toxic compounds. In carp, *Cyprinus carpio*, comparative studies with phenol, hydroquinone and catechol showed that hydroquinone is the most immunotoxic compound (Taysse et al., 1995).

Phenolic compounds and phenol "in vitro" cause cytotoxicity in erythrocytes of the sea bass *Dicentrarchus labrax*, increase of peroxidase activity and hemolysis (Bogé and Roche, 1996). Intra-abdominal injections of phenol and phenolic compounds in sea bass for 3 to 15days decrease total hemoglobin, hematocrit and catalase, but increase cortisol, Mn-superoxide dismutase, glutathione peroxidase and peroxidase activities (Roche and Bogé 1996; Roche and Bogé 2000). In these cases phenol clearly causes changes in antioxidant enzymes activities and leads to oxidative stress (Roche and Bogé, 1996; Roche and Bogé, 2000; Bukowska and Kowalska, 2004). In carp, lipid peroxidation resultant from sub-lethal effects of phenol is also found in phospholipid composition of erythrocyte membranes with subsequent alterations in membrane fluidity and permeability (Kotkat et al., 1999).

The understanding of the toxic actions of phenol may be very important for conservation. However, studies related to this pollutant on Neotropical fish are scarce. Matrinxã, *Brycon amazonicus*, exposed to phenol shows increases of protein catabolism and decreases in carbohydrate catabolism (Hori et al., 2006). Stress cellular responses in matrinxã to phenol exposure are also reported (Hori et al., 2008). This Neotropical fish from the Amazon basin is a well-adapted species to aquaculture presenting a good performance (Castanholli, 1992; Hackbarth and Moraes, 2006). In large areas of the Brazilian basins, deforestation, dams and water pollution are the main threats to several species, particularly matrinxã (Mendonça, 1996). The aim of the present study was to evaluate the antioxidant adaptive responses of matrinxã to environmental phenol exposure as well as its ability to recover. This was done through determination of the antioxidant defense system and the hematological parameters.

2. Materials and methods

Juveniles of *B. amazonicus* from the Aguas Claras Fish Farm, Mococa, Sao Paulo, Brazil, were acclimated previously to experimentation in 2000L dark tanks with dechlorinated tap water for 1month, under natural photoperiod (12:12h), constant temperature (25 ± 1°C) and constant aeration (5.0mg/L). Water quality was daily monitored (dissolved O_2, pH, hardness, conductivity, temperature, alkalinity, ammonia and phenol by APHA method (1980)) during this period and the fish were fed *ad libitum* with commercial food pellets (28% protein). After acclimation, 120 fish (N = 120) with an average mass of 129.5g ± 39.2 (± SD) and average length of 22.5cm ± 2.45 (± SD) were equally divided into twelve 250L dark tanks (n = 10) in a dynamic system. Fish were kept in such new condition for 1week. The tanks were divided into three groups: (E) exposure to sub-lethal phenol concentration (2mg/L corresponding to 10% of the LC50-96h) for 96h and its control; (R1) recovery for 1week after the same phenol exposure and its control; and (R2) recovery for 2weeks and its control. The experiments, and respective controls, were done with replicates. Prior to exposing fish to phenol, they were starved for 24h. The system water was renewed every 24h (semi-static) to preserve the initial water quality. Feeding was discontinued and phenol concentration was adjusted every 12h (Table 1). Control groups were subjected to the same protocol, but free of phenol.

Table 1
Water quality parameters

Period	pH		Conductivity (µS/cm^3)		Dissolved oxygen (mg/L)		Temperature (°C)		Alkalinity (mg de CO_3^-/L)		Hardness (mg de $CaCO_3$/L)		Ammonia (mg/L)		Phenol (mg/L)	
	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E
0 h	7.39	7.58	80.2	80.5	5.87	4.6	24.3	24.3	110	110	37	37	0.27	0.25	0	0
0 h'	7.48	7.53	80.	81.3	5.7	4.7	24.5	24.4	109	109	37	37	0.42	0.37	0	1.91
24 h	7.59	7.70	82	81	4.33	5.08	23.2	23.1	100	100	38	38	0.53	1.42	0	1.67
24 h'	7.75	7.73	80	80	5.5	4.45	23.2	23.2	105	105	38	38	0.21	0.15	0	1.95
48 h	7.30	7.53	79	78	5.66	5.74	23.0	23.1	100	100	40	40	0.40	0.79	0	1.52
48 h'	7.40	7.60	79	78	5.5	5.32	24.8	24.9	100	100	40	40	0.09	0.06	0	2.03
72 h	7.60	7.70	80.5	80.5	4.93	5.0	24.6	24.6	100	100	40	40	0.18	0.09	0	1.59
72 h'	7.60	7.70	80.5	80.5	5.56	5.15	25.5	25.2	100	100	40	40	0.16	0.16	0	1.98
96 h	7.42	7.52	79.6	78.7	4.23	4.99	25.8	25.6	100	100	40	41	0.23	0.16	0	1.45
Recovery (1 week)	7.60	7.60	80.5	80.5	6.0	6.36	25.8	26.0	105	105	38	39	0.14	0.14	0	0
Recovery (2 weeks)	7.5	7.50	80.5	80.5	6.90	6.65	23.8	23.5	105	105	40	40	0.15	0.15	0	0

These water parameters were determined in the experimental systems of exposure of matrinxã to phenol for 96 h plus recovery for 1 and 2 weeks, wherein: C = control; E = exposure; 0 h = prior to the experiment (without phenol); 0 h' = start of the experiment (with phenol); (24 h, 48 h, 72 h and 96 h) = the water quality parameters before renewing the water; (24 h', 48 h' and 72 h') = the water parameters after renewing the water. In some cases phenol concentration was also adjusted.

Table 2
Erythrocytic anti-oxidants of matrinxã exposed to phenol

	Control	Exposure	Recovery control	Recovery	Recovery control	Recovery
	(96 h)	(96 h)	(1 week)	(1 week)	(2 weeks)	(2 weeks)
SOD	0.15±0.07	0.18±0.12	0.36±0.14	0.29±0.10	0.41±0.11	0.45±0.28
CAT	64.93±19.20	39.47±13.11*	79.01±25.22	78.79±34.97	122.05±55.18	78.79±39.79*
GPx	989.13±184.63	809.38±210.10	705.52±152.06	718.02±188.91	418.93±191.07	346.11±123.04
G6PDH	2.63±0.77	4.33±1.72*	2.66±0.59	2.60±0.63	3.11±1.45	3.71±1.56
GSH	14.17±2.52	13.60±3.98	9.80±2.11	9.41±2.36	8.46±1.53	9.95±3.68

Fish were exposed to 2 mg/L of phenol for 96 h and recovered for 1 and 2 weeks in free-phenol water. SOD (superoxide dismutase) is expressed in U/mg total Hb; CAT (catalase) is expressed in mol/min/g total Hb (hemoglobin) and GPx (glutathione peroxidase) and G6PDH (glucose 6, phosphate dehydrogenase) are expressed in mmol/min/g total Hb; GSH (reduced glutathione) is expressed in nmol/mg total Hb. The values are followed by (mean±SD) for $n=12$; significant differences are marked by (*) for $P<0.05$ as compared to the respective control.

At the end of 96h, twelve fish from E considered replicate (n = 12), plus control (n = 12) were taken for blood sampling, immediately killed by transecting the spinal cord, and biometry was done. Liver and brain were quickly excised, rinsed with 0.9% NaCl solution, immediately frozen in liquid nitrogen and stored at − 80° C for biochemical analyses. Blood samples were divided into aliquots for different analyses. Fish from recovery tanks (R1 and R2) remained in a dynamic system (continuously renewed water), under the same water conditions, but were not fed over the recovery period (Table 1). After recovery, twelve fish from R1 (n = 12) plus controls (n = 12) and R2 plus controls, were taken for blood and tissues sampling and biometry as for E.

2.1. Blood parameters

Hematocrit (Hct), total hemoglobin (Total Hb) and red blood cell count (N_{RBC}) were done in total blood. Hematocrit was determined on blood samples centrifuged at 12,000*g* for 3min in capillary tubes. Total hemoglobin was determined photometrically at 540nm with 10μL of blood in 2.0mL of Drabkins' solution. Red blood cells (N_{RBC} mm^{-3}) were counted under a light microscope with a Neubauer chamber. Mean corpuscular volume (MCV) was calculated as MCV=Hct·10/N_{RBC}; mean corpuscular hemoglobin (MCH) content as MCH=Total Hb·10/N_{RBC} and mean corpuscular hemoglobin concentration (MCHC) as MCHC=Total Hb·10/ Hct.

2.2. Liver homogenate

Liver samples were thawed quickly, weighed and homogenized with an IKA Turrax, in 100mM K-phosphate buffer (pH 7.0) plus 0.25M sucrose (at ratio of 1:10w/v) for 1min at 4000rpm. Samples were centrifuged at 15,000*g* for 10min at 4°C, and the supernatant was used as enzyme source. Total protein was usually near 4mg/mL (Bradford, 1976). Tissue extracts were diluted in 100mM K-phosphate buffer (pH 7.0) with 0.25M sucrose with the following ratios: 10 times for SOD and G6PDH determinations; 100 times for GPX; and 200 times for CAT. Enzyme activities were determined photometrically in a Beckman DU-520. SOD, as reported below, was expressed in enzyme unity/mg of protein; G6PDH was expressed in mmol NADP consumed/min/mg of protein; GPx was expressed in mmol of NADPH consumed/min/mg of protein and CAT in mmol H_2O_2 consumed/min/mg of protein.

2.3. Hemolysate

Blood aliquots of 1mL were centrifuged at 12,000*g* for 3min. The packed red cells were washed three times with saline solution (0.9%), centrifuged at 1000*g* for 10min at 4°C, white cells removed by suction from the upper layer and the red cells were re-suspended into 1mL Tris–HCl buffer (5mM, pH 8.0). The erythrocytes were centrifuged at 1000*g* for 10min at 4°C and the hemolysate (± 0.8 Hb total g/100mL) was used as enzyme source (Beutler, 1984). Activity of erythrocyte G6PDH was expressed in mmol NADP consumed/min/g of total Hb; GPx was expressed in mmol of NADPH consumed/min/g of total Hb; SOD in enzyme unity/mg total Hb and CAT in mol H_2O_2 consumed/min/g total Hb.

2.4. Enzymatic defense antioxidant system

2.4.1. Superoxide dismutase — SOD

The SOD assay was based on the ability of the enzyme to inhibit the auto-oxidation of a 10mM pyrogallol–HCl solution (Beutler, 1984). To determine erythrocyte SOD activity, 0.1mL of hemolysate was added to 0.7mL of water and then transferred to 0.2mL of ethanol plus 0.12mL of chloroform. The mixture was centrifuged for 5000*g* for 10min at 4°C and the supernatant was used as enzyme source. Hepatic SOD was determined in liver homogenate diluted 1:10. The enzyme assay was performed in 1M Tris–HCl buffer (pH 8.0), an aliquot of enzyme source, and 10mM pyrogallol–HCl. Oxidation was measured kinetically at 420nm for 1min. An inhibition curve was done for each fish and 1 unit of SOD was calculated to 50% of inhibition of pyrogallol oxidation.

Table 3
Hepatic anti-oxidants of matrinxã exposed to phenol

	Control	Exposure	Recovery control	Recovery	Recovery control	Recovery
	(96 h)	(96 h)	(1 week)	(1 week)	(2 weeks)	(2 weeks)
SOD	17.30±3.35	20.50±4.78	25.33±8.85	21.79±3.88	17.54±5.45	20.19±5.64
CAT	591.58±187.12	620.83±207.85	854.09±167.00	836.95±309.48	800.96±283.26	774.24±223.71
GPx	13.57±3.61	13.36±3.78	11.26±2.62	21.42±8.89*	11.12±3.62	7.72±2.18*
G6PDH	0.069±0.02	0.061±0.02	0.055±0.02	0.050±0.01	0.044±0.01	0.029±0.01*
Ascorbic acid	2.54±0.90	1.71±0.61*	1.80±0.61	1.50±0.43	1.57±0.52	1.28±0.31

Fish were exposed to 2 mg/L of phenol for 96 h and recovered for 1 and 2 weeks in free-phenol water. SOD (superoxide dismutase) is expressed in U/mg protein; CAT (catalase), GPx (glutathione peroxidase) and G6PDH (glucose 6, phosphate dehydrogenase) are expressed in mmol/min/mg; ascorbic acid is expressed in μmol/g of wet tissue. The values are followed by (mean±SD) for $n=12$; significant differences are marked by (*) for $P<0.05$ compared to the respective control.

Table 4
Ascorbic acid levels in the brain of matrinxã exposed to phenol

	Control (96 h)	Exposure (96 h)	Recovery control (1 week)	Recovery (1 week)	Recovery control (2 weeks)	Recovery (2 weeks)
Ascorbic acid	0.80±0.23	0.91±0.37	0.72±0.17	0.94±0.25*	0.87±0.28	1.24±0.48*

Fish were exposed to 2 mg/L of phenol for 96 h and recovered for 1 and 2 weeks in free-phenol water. Ascorbic acid is expressed in μmol/g of wet tissue. The values are followed by (mean±SD) for $n=12$; significant differences are marked by (*) for $P<0,05$ compared to the respective control.

2.5. Catalase — CAT

CAT activity was determined following the H_2O_2 concentration decrease at 230nm (Beutler, 1984). One aliquot of 20μL of hemolysate was firstly diluted at a 1:2 ratio in beta-mercaptoethanol–EDTA solution followed by dilution in water at a 1:30 ratio and finally was added 20μL of 100% ethanol. This alcoholic solution was used as enzyme source. Liver homogenate was diluted 1:200 and 20μL of 100% ethanol was added. The CAT activity was measured following the decay in absorbance of a reaction mixture containing: 0.1M Na-phosphate buffer pH 7.0 and 45mM H_2O_2 (for erythrocyte), or 22.5mM H_2O_2 (for liver) and an adjusted volume of enzyme. The molar extinction coefficient used for H_2O_2 was 0.071mM^{-1} cm^{-1}.

2.6. Glutathione peroxidase — GPx

Activity of GPx was determined through the auxiliary enzyme glutathione reductase (GR). This enzyme reduces glutathione, the reaction product of GPx, and oxidizes NADPH which is kinetically followed at 340nm (Beutler 1984). The blood GPx enzyme source was a hemolysate preparation diluted 1:1 in beta-mercaptoethanol–EDTA solution and 1:5in 5mM Tris–HCl buffer pH 8.0. Liver homogenate was diluted 1:100. The reaction mixture was 0.05M Tris–HCl buffer (pH 8.0), 1mM GSH, 1U GR, 4.8nM Na-azide, 0.105mM *t*-butyl hydroperoxide and an enzyme source aliquot. The molar extinction coefficient for NADPH at 340nm was 6.2mM^{-1} cm^{-1}.

2.7. Glucose 6-P dehydrogenase — G6PDH

Activity of GP6DH was determined kinetically through the increase in absorbance at 340nm due to NADPH formation (Beutler 1984). Erythrocytic GP6DH enzyme source was a hemolysate preparation diluted 1:1 in beta-mercaptoethanol–EDTA solution and 1:5 in 5mM Tris–HCl buffer (pH 8.0). Liver homogenate was diluted 1:10. The reaction mixture was: 0.1M Tris–HCl buffer (pH 7.0), 0.2mM NADP, 0.01mM $MgCl_2$, 0.1M G6P and proper aliquot of enzyme source. The molar extinction coefficient used for NADPH was 6.2mM^{-1} cm^{-1}.

2.8. Non-enzymatic anti-oxidants

2.8.1. Reduced glutathione — GSH

GSH was determined by colorimetric method. Total blood sample (50μL) was hemolyzed in 500μL water, and 750μL of a precipitation solution (1.67g metaphosphoric acid, 0.2g EDTA, 30g NaCl, for 100mL of distilled water) was added. After this, the mixture was centrifuged at 11,300*g* for 3min. One aliquot (500μL) was drawn from the supernatant and 2mL of 0.3M Na_2PHO_4 plus 250μL of 0.02% 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) were added. GSH was read at 412nm, compared with a standard solution containing 100nmol of the GSH, and expressed in nmol/mg total hemoglobin (Beutler, 1984).

2.9. Ascorbic acid

Ascorbic acid in brain and liver was determined at 524nm. Briefly, brain and liver samples were homogenized (1:10w/v) in 20% TCA, and centrifuged at 11,300*g* for 3min. Samples (250μL) of the supernatant were mixed with 250μL of water plus 25μL 0.02% 2,6-dichlorophenolindophenol and incubated for 1h at room temperature. After that, 250μL of a 2% thiourea and 5% metaphosphoric acid solutions plus 250μL of 0.2% dinitrophenylhydrazine in 12M sulfuric acid were added. The reaction tubes were incubated in a water bath at 60°C for 3h. Following the incubation, 500μL of 18M sulfuric acid was added and the tubes were centrifuged at 500*g* for 10min. Absorbance was read, compared to a standard containing 100nmol of ascorbic acid and expressed in μmol/g of tissue (Carr et al., 1983).

2.10. Statistical analyses

All parameters are expressed as mean ± SD of 12 individuals (n = 12). Normalization of data was initially verified with the Kolmogoroff–Smirnoff test followed by a parametric test (unpaired t test), to compare the treatments versus respective controls. The confidence limit was established at $P < 0.05$. Statistical analyses were performed with Graphpad Instat (version 3.0 for Windows).

2.11. Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA) and Merck.

3. Results

Enzymes from red blood cells implicated in oxidation defenses responded distinctly in matrinxã exposed to phenol. The superoxide dismutase and glutathione peroxidase activities were neither affected by exposure nor by recovery (Table 2). However, during exposure to

Table 5
Hematological parameters of the matrinxã exposed to phenol

	Control (96 h)	Exposure (96 h)	Recovery control (1 week)	Recovery (1 week)	Recovery control (2 weeks)	Recovery (2 weeks)
Hct	28.5±3.34	31.33±2.68*	26.79±3.42	31.16±2.81*	29.29±3.02	26.72±2.90*
Total Hb	8.42±1.16	8.96±1.01	8.12±1.15	8.90±1.15	8.95±1.30	8.19±1.30
NRBC	1.94±0.52	2.23±0.36	1.91±0.26	2.07±0.21	2.19±0.39	1.92±0.34
MCV	158.61±55.66	142.95±20.23	152.40±23.67	152.05±22.49	135.82±16.69	141.92±24.96
MCH	46.92±17.40	40.92±6.58	46.40±7.13	43.36±6.35	41.63±7.28	43.38±8.14
MCHC	2.96±0.36	2.86±0.20	3.03±0.22	2.87±0.40	3.05±0.28	3.08±0.52

Fish were exposed to 2 mg/L of phenol for 96 h and recovered for 1 and 2 weeks in phenol-free water. Hct (Hematocrit) is expressed in %; Total Hb (total hemoglobin) is expressed in g/100 mL; N_{RBC} (Red Blood Cell) is expressed in 10^6 cells mm^3; MCV (mean corpuscular volume) is expressed in fL; MCH (mean corpuscular hemoglobin) is expressed in pg and MCHC (mean corpuscular hemoglobin concentration) is expressed in g dL^{-1}. The values are followed by (mean±SD) for $n=12$; significant differences are marked by (*) for $P<0.05$ compared to the respective control.

phenol blood catalase decreased 40% and glucose 6-P dehydrogenase increased 64%. CAT activity also decreased 36% during the second week of recovery, and G6PDH returned to the basal levels. Reduced glutathione level were unaffected by phenol exposure and remained constant even during recovery.

The liver enzymes related to the antioxidant system in matrinxã seemed to be distinctly responsive to phenol (Table 3). In the liver, the activities of SOD and CAT were unaltered by phenol exposure. However, GPx, which did not change after exposure, increased 90% after the first week of recovery, decreasing 30% after 2weeks. A late response was observed for G6PH activity. It did not alter after exposure or even after the first week of recovery, but decreased 34% after the second week. Ascorbic acid decreased 32% during phenol exposure and it was not altered during recovery in liver. Unchanged concentrations of ascorbic acid were observed in the brain after phenol exposure; however, ascorbic acid increased 30% and 42% after the first and second weeks of recovery, respectively (Table 4).

Hematological responses due to stress caused by phenol were clearly observed in matrinxã. Hematocrit values (Table 5) increased 10% after exposure and 16% after 1 week of recovery, decreasing to 9% at the second week. No changes were observed in the other hematological parameters.

4. Discussion

The antioxidant metabolism of matrinxã was responsive to the environmental presence of phenol. Liver and red blood cells of matrinxã presented enzyme activity alterations after both exposure and recovery.

Superoxide Dismutase catalyzes the reduction of superoxide anions to hydrogen peroxide. One might expect changes in the activity of this enzyme when cells are exposed to oxidative stress. Red blood cells from *Leporinus elongatus* (Wilhelm-Filho et al., 2005) submitted to hypoxia and from *Oreochromis niloticus* (Bainy et al., 1996) dwelling in the highly eutrophic and polluted Billing's reservoir water showed increased SOD activities. In the red blood cells from matrinxã, total SOD activity was unaltered after phenol exposure or recovery. This was also observed in *D. labrax* red blood cells exposed to phenol "in vitro" (Bogé and Roche, 1996) or after intra-abdominal injections of *D. labrax* with phenol and phenol derivatives (Roche and Bogé, 1996; Roche and Bogé, 2000), while the activity of Mn-SOD was reduced. Human erythrocytes did not present SOD activity alteration when exposed to phenol (Bukowska and Kowalska, 2004). However, that work refers to human erythrocytes which are destitute of nucleus and were performed 'in vitro'. Therefore, there is no possibility to synthesise high amounts of the enzyme in contrast to fish; examined "in vivo' and presenting nucleated erythrocytes. Considering total SOD activity, phenol seemed to result in non-oxidative stress in red blood cells of matrinxã.

The only red blood cell enzyme that increased in matrinxã after phenol exposure was glucose-6-P dehydrogenase (G6PDH). This enzyme activity supplies NADPH to several anabolic processes and to glutathione reductase, which produces GSH from GSSG. Since GSH is GPx electron donor, the level of GSH may follow the activity of GPx in a converse relation. However, the levels of GSH, which decreased in human erythrocytes exposed to high concentrations of phenol (>250 mg/L) (Bukowska & Kowalska, 2004), and the activity of GPx did not change, neither after exposure of matrinxã to phenol nor after recovery. Interestingly, GPx and G6PDH increased while GSH decreased in *O. niloticus* from the Billing's reservoir (Bainy et al., 1996). Also, activity of GPx increased in sea bass submitted to intra-abdominal phenol injections (Roche and Bogé, 1996; Roche and Bogé, 2000). Since G6PDH activity in human erythrocytes exposed to phenol did not change (Bukowska and Kowalska, 2004) it seems that there is not a direct effect of phenol on these activities.

In addition to the set of enzymes involved in the avoidance of oxidative stress, some substances, as vitamins and GSH, are also involved. Vitamin E is absent in the red blood cells of matrinxã, but high concentration of GSH is present (Wilhelm-Filho, 1996). We did not detect ascorbic acid in the plasma of matrinxã. Accordingly, GSH might be highly important in the response of matrinxã to oxidative stress. However, since GSH was unaltered in erythrocytes of matrinxã, it is possible that phenol does not provoke oxidative stress in these cells of matrinxã.

CAT and GPx activities are fundamental to remove hydrogen peroxide from cytoplasm, however, only the CAT activity was decreased in the blood of matrinxã exposed to phenol. Other phenolic compounds have also been reported to reduce CAT activity, as observed in sea bass (Roche and Bogé, 1996). The low CAT activity might be due to decrease of free radicals from a direct scavenger effect of phenol or from a phenol interaction with the enzyme molecule. This enzyme is a hemoprotein and works also as a NADPH store (Kirkman and Gaetani, 1984). A consequential reduction of the NADPH reservoir would increase G6PDH activity in order to provide this reducing coenzyme pool. Considering that the present parameters are a good set of red blood cell biomarkers to analyze oxidative stress (Roche and Bogé, 2000) and no changes were observed in such biochemical profile, even after recovery, it showed that environmental phenol did not cause any oxidative stress in the red blood cells of matrinxã neither worked as antioxidant.

With exception of the hematocrit, all other hematological parameters did not change under environmental phenol exposure. This response is typical of some types of stressing condition. Hematocrit of sea bass *D. labrax* exposed to phenol did not change, regardless of total hemoglobin and cortisol increases (Roche and Bogé, 2000). Hematocrit of rainbow trout increases when exposed to phenol for 24 h (Swift 1981; Swift 1982). In that case plasma cortisol and glucose also increase. However, the level of plasma glucose decrease in matrinxã exposed to 2 mg/L of phenol for 96 h (Hori et al., 2006). Therefore, the increase of hematocrit in matrinxã is likely due to either increase metabolic demand or gill damages resulting in impairment of oxygen transport, or both. In fact, we have observed drastic changes of the gill ultra-structure in the present trials (on going experiments).

Hepatic responses of matrinxã to phenol exposure were observed just during recovery. These were restricted to increase of GPx and G6PDH activities, while SOD and CAT remained unaltered. These enzymes were expected to increase in the presence of oxidant agents or free radical generators. GPx activity increased in matrinxã after the first week of recovery but decreased after the second week. Nevertheless, there is a lack of data about phenol effects on anti-oxidants enzymes in liver of fish. GPx activity increased during exposure to phenol derivatives, as observed in rainbow trout exposed to hexachlorobenzene (Lindström-Seppä and Roy, 1996) and to pulp mill (Ahmad et al., 2000), in *Channa punctatus* exposed to deltamethrin (Sayeed et al., 2003) and in *Carassius auratus* chronically exposed to 2,4-dichlorophenol (Zhang et al., 2004). G6PDH is less studied in fish that endured oxidative stress. One case reports a decrease of this enzyme in tilapia exposed to waters from Billings' Reservoir, wherein high levels of numerous xenobiotics are reported (Bainy et al., 1996). However, the Cyprinidae fish, sampled from Seyhan Dan Lake polluted waters in Turkey, presented increased G6PDH activity (Güll et al., 2004).

Activities of SODs and CAT were also expected to increase. It is important to bear in mind that SODs comprises a multitude of enzymes, such as Mn-SOD, Cu–Zn SOD, with different responsiveness patterns. Increase of SOD and CAT in liver is reported in some fish species under oxidative stress (Bainy et al., 1996; Sayeed et al., 2003; Güll et al., 2004; Zhang et al., 2004; Nam et. al, 2005; Wilhelm-Filho et al., 2005).

In the liver of matrinxã exposed to phenol, the profile of responses to the enzymes studied suggests that phenol did not act primarily as an oxidant or a producer of free radicals. However, metabolism of phenol by cytochrome P450 should produce derivatives capable of causing the oxidant stress responses observed in matrinxã exposed to phenol after 1 and 2 weeks of recovery. This response, only observed in liver, depict its role in detoxification process of phenol.

Ascorbic acid has been studied in fish due to its antioxidant properties (Dabrowski, 2001). Increases in ascorbic acid utilization by fish exposed to a variety of stressors have been reported (Benitez and Halver, 1982; Tucker and Halver, 1986) and different responses are observed in *C. punctatus* exposed to different pollutants (Sayeed et al., 2003). Lower SODs activities observed in fish as compared to guinea pig and primates can result from higher demand of fish for ascorbate supplementation under conditions of free radical generation (Nandi et al., 1997). SOD activity in the liver of matrinxã exposed to phenol for 96 h was not altered, but the ascorbate concentration decreased as compared to control. This decrease can be ascribed to consume of ascorbic acid by free radicals generated when phenol was metabolized by monooxygenases (cytochrome P450). The concentration of ascorbate was slightly reduced in liver of control and treated fish after 1 and 2 weeks of recovery, probably because of starvation. The progressive increase of ascorbate observed in the brain at 1 and 2 weeks of recovery remains to be investigated.

Most studies on toxicity of phenol in fish were carried out with phenol derivatives, and the set of data on recovery of fish exposed to phenol are scarce. In our laboratory, the effect of this toxicant in warm water fish was recently reported on metabolic adaptive responses in matrinxã (Hori et al., 2006; Hori et al., 2008). However, the cellular machinery of this species to cope with oxidative stress caused by phenol has not yet been studied. Noteworthy, our findings indicated that the biochemical strategies developed during exposure of fish to water contaminated with phenol seem to be different from the responses found during intra-abdominal injection or "in vitro" studies. In conclusion, we can say that the primary effects of phenol on matrinxã cells were not as a free radical generator. This pollutant seemed to act mainly as a radical scavenger; however, in the course of recovery free radical should be produced as consequence of phenol metabolism, which should explain the liver antioxidant response.

Acknowledgements

We are indebted to the Department of Genetics and Evolution at Federal University of Sao Carlos for facilities and technical support that allowed us to investigate the effects of phenol, in matrinxã. In particular we would like to acknowledge the assistance of Antônio Donizete Aparecido da Silva, Luis Antônio Kioshi Inoue and all colleagues of the Laboratory of Adaptive Biochemistry. We also thank CAPES, CNPq and FAPESP for financial support.

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