J Comp Physiol B (2014) 184:469–482
DOI 10.1007/s00360-014-0808-1

ORIGINAL PAPER

Humic acid and moderate hypoxia alter oxidative and physiological parameters in different tissues of silver catfish (*Rhamdia quelen*)

Ana P. K. Riffel · Etiane M. H. Saccol · Isabela A. Finamor · Giovana M. Ourique · Luciane T. Gressler · Thaylise V. Parodi · Luis O. R. Goulart · Susana F. Llesuy · Bernardo Baldisserotto · Maria A. Pavanato

Received: 21 January 2013 / Revised: 8 December 2013 / Accepted: 17 December 2013 / Published online: 14 February 2014

Abstract Aquatic animals are naturally exposed simultaneously to environments with different concentrations of humic acid (HA) and seasonal or daily variations of dissolved oxygen (DO) levels. This study investigated the effects of simultaneous exposure to different HA and DO levels on plasma ion levels and some hematological and oxidative parameters in different tissues of silver catfish (*Rhamdia quelen*). The fish were exposed to 0, 2.5 or 5 mg L^{-1} HA for 120 h. After this period, each group was divided into two groups: normoxia and hypoxia. Exposure to the different DO levels lasted 96 h, totaling 216 h of experimentation. At the end of the experimental period, blood sampling was performed, and the fish were euthanized prior to the excision of the gills and the brain to evaluate hematological and oxidative parameters. To verify the antioxidant capacity of HA, total phenolic compounds were measured. In general, all tissues of silver catfish exposed simultaneously to hypoxia and different HA concentrations showed a reduction in lipid peroxidation levels, as well as a modulation of the antioxidant system. These effects occurred in an HA concentration-dependent manner. Thus, HA is beneficial to silver catfish exposed to hypoxia. These beneficial effects can be attributed, most likely, to the action of the different HA constituents and functional groups, including phenolic compounds, which have antioxidant properties.

Keywords Fish · Humic acid · Oxidative parameters · Oxygen levels

Abbreviations

CAT	Catalase
CDNB	1-Chloro-2,4-dinitrobenzene
Cl^-	Chloride
DO	Dissolved oxygen
GAE	Gallic acid equivalents
GPx	Glutathione peroxidase
GSH	L-Glutathione reduced
GST	Glutathione-*S*-transferase
H_2O_2	Hydrogen peroxide
HA	Humic acid
HIF-1α	Hypoxia inducible factor 1-alpha
HS	Humic substances
K^+	Potassium
LOOH	Lipid hydroperoxides
LPO	Lipid peroxidation
MCHC	Mean cell hemoglobin concentration
Na^+	Sodium
PMSF	Phenylmethylsulfonyl fluoride
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid

Introduction

Humic substances (HS), which are organic compounds derived from biomass during decomposition, constitute the

Communicated by G. Heldmaier.

A. P. K. Riffel · E. M. H. Saccol · I. A. Finamor · G. M. Ourique · L. T. Gressler · T. V. Parodi · L. O. R. Goulart · B. Baldisserotto · M. A. Pavanato (✉)
Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, Av. Roraima, 1000, Santa Maria, RS 97105-900, Brazil
e-mail: amaliapavanato@yahoo.com.br

S. F. Llesuy
Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires, Buenos Aires, Argentina

largest fraction of dissolved organic carbon found in all aquatic ecosystems (Thurman 1985; Matsuo et al. 2005). HS can be separated into humic acids (HA), fulvic acids and humin (McDonald et al. 2004). HA are compounds of variable molecular weight and consist of polymeric structures with many functional groups; carboxylic and phenolic groups are found in greater quantities, whereas ketones, amines and alkoxyl groups make smaller contributions (Cheng et al. 1999; Fiorentino et al. 2006; Aeschbacher et al. 2012; Uyguner-Demirel et al. 2013).

The effects of HS on aquatic organisms have been extensively discussed (Steinberg et al. 2003; Meinelt et al. 2008). Studies in zebrafish (*Danio rerio*) embryos indicate that HS can exert positive or negative effects depending on their concentration, their source of origin and the physiological condition of the animal. Other studies indicate that HS can modulate the toxicity of pollutants, xenobiotics and the bioavailability of metals (Richards et al. 1999; Paquin et al. 2002; Glover and Wood 2004; Timofeyev et al. 2006; Giacomin et al. 2013) and can alter pH, ionic concentration (McDonald et al. 2004) and enzymatic activity (Timofeyev et al. 2006). Due to these effects and its unusual composition, it is believed that humic acids may act on reactive oxygen species (ROS) and reduce their harmful effects during or following hypoxic episodes in fish.

A decline in the photosynthetic rates and the decomposition of organic matter can change dissolved oxygen (DO) levels, which is a limiting factor for fish and other aquatic organisms. In freshwater plants and cyanobacteria, HS act on the electron transport chain, decreasing photosynthetic rates and, consequently, reducing the release of oxygen into the aquatic environment (Pflugmacher et al. 2006; Steinberg et al. 2008). Hypoxia is characterized by DO levels below 2.8 mg L^{-1} (Diaz and Rosenberg 1995). Hypoxia can also be caused by anthropogenic factors, such as the addition of nutrients and pollutants (Sampaio et al. 2008), or by natural factors, such as high temperatures and vertical stratification (Wu 2002). Some floodplain lakes are normally subjected to temporary hypoxia, mainly in the Amazon region (Affonso et al. 2002). In the Amazon basin, DO levels decline for entire seasons, when stagnant water and decaying organic matter combine (Bickler and Buck 2007). These situations create adverse conditions for aquatic organisms that can alter their physiology (Wu 2002; Wilhelm Filho et al. 2005).

Variations in DO levels are directly related to ROS production in fish, mainly during reoxygenation (Lushchak et al. 2001; Lutz and Nilsson 2004). These ROS are generated in the mitochondria of all aerobic cells by normal breathing and are toxic to cells at high levels, leading to the oxidation of lipids, proteins and DNA, as well as mutagenesis and cell death (Martínez-Alvarez et al. 2005). At low levels, these ROS perform important physiological roles; for example, they can activate and modulate signaling pathways and activate transcription factors that are sensitive to oxidation–reduction reactions, such as the activation of hypoxia inducible factor 1 alpha (HIF-1α) (Semenza 2007; Terova et al. 2008). HIF-1α is a heterodimeric transcription factor responsible for numerous important physiological functions in fish (Wang et al. 1995; Terova et al. 2008), such as gene activation of erythropoietin and glycolytic enzymes (Paul et al. 2004). To regulate ROS concentration in tissues and to combat their deleterious effects, aerobic organisms have developed an antioxidant defense system consisting of enzymatic and non-enzymatic mechanisms. These antioxidant components are located in different cellular compartments and are used to evaluate the antioxidant status in fish as biomarkers for different aquatic environmental conditions (Lushchak et al. 2001; Lushchak and Bagnyukova 2006).

Silver catfish (*Rhamdia quelen*) is a freshwater species with a Neotropical distribution. They are native in places from southeastern Mexico to southern Argentina (Gomes et al. 2000). The lethal concentration of DO levels for this species is 0.52 mg L^{-1} (Braun et al. 2006), which makes it an excellent model for studying DO level variations. Under hypoxic conditions, oxidative and osmoregulatory alterations have been verified in this species (Rosso et al. 2006; Azambuja et al. 2011).

Due to the distribution and peculiarities of silver catfish and its adaptive potential, marketability and wide distribution, the need to know its physiological mechanisms of adaptation to different conditions in its habitat is growing. Furthermore, aquatic animals are naturally exposed to environments with different concentrations of HA and seasonal or daily variations of DO levels simultaneously, but there are no studies that estimate the impact of HA and DO levels on biochemical and physiological parameters in fish. Therefore, the present study aimed to evaluate the effects of both factors on plasma ion levels and specific hematological and oxidative parameters in different tissues of silver catfish. The hypothesis of this work is that HA is beneficial to fish exposed to hypoxia.

Materials and methods

Reagents

HA (CAT: H1, 675-2 Aldrich), which corresponds to 44 % dissolved organic carbon (Matsuo et al. 2005) as well as phenylmethylsulfonyl fluoride (PMSF), 1-chloro-2,4-dinitrobenzene (CDNB), L-glutathione reduced (GSH), epinephrine and glycine were acquired from Sigma Aldrich (USA). Hydrogen peroxide (H_2O_2), trichloroacetic acid (TCA), thiobarbituric acid and albumin were obtained from

Merck KGaA (Germany). All other reagents were of analytical grade.

Determination of total phenolic compounds

The determination of phenolic compounds in HA is an indirect measure of the antioxidant capacity of these compounds (Singleton et al. 1999). The determination of phenolic compounds was performed on HA extracts. Chemical extractions were performed using the method described by Pérez-Jiménez and Saura-Calixto (2005). Total phenolic compounds were determined according to the Folin–Ciocalteu procedure (Singleton et al. 1999). Gallic acid was used as a standard, and the results are expressed as gallic acid equivalents (mg GAE) g per humic acid. The reaction was conducted in triplicate.

Experimental protocol

The fish (143.90 ± 11.7 g, 25.1 ± 0.6 cm) were acclimated to laboratory conditions for 21 days in 250 L tanks under controlled temperature (23 ± 1 °C) and constant aeration (6.5 ± 0.2 mg L^{-1} DO). The fish were then divided into three groups and exposed to different concentrations of HA (0, 2.5 and 5 mg L^{-1}) for 216 h (9 days). At 120 h, each group was further divided into two groups, normoxia (6.7 ± 0.1 mg L^{-1} DO) and hypoxia (2.0 ± 0.2 mg L^{-1} DO), with three replicates each ($n = 8$). The group exposed to 0 mg L^{-1} HA and normoxia was considered to be the control. Decreased aeration and fish oxygen consumption caused a reduction in the levels of oxygen, and hypoxic levels were reached in 6 h. The HA concentrations 2.5 and 5 mg L^{-1} were chosen because they are commonly found in natural environments (McGeer et al. 2002; Steinberg 2003). The experiment was performed in 250 L tanks.

DO levels were controlled by increasing or decreasing the aeration. Feeding was performed daily with commercial feed, ceasing at 96 h. Siphoning and water exchange were also performed daily, followed by verification of the DO levels. To avoid DO fluctuation, water was partially exchanged with the volume of exchanged water reaching up to 40 %. No significant variations in DO levels occurred with water exchange.

Water parameters

DO levels and temperature were measured with a YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA). The pH was verified with a DMPH-2 pH meter (Digimed, São Paulo, SP, Brazil). Nesslerization was used to verify the total ammonia nitrogen levels according to the method of Eaton et al. (2005). Un-ionized ammonia levels were calculated according to the method of Colt (2002). Water hardness was analyzed by the EDTA titrimetric method. Alkalinity was determined according to the method of Boyd and Tucker (1992).

Tissue collection

At the end of the experimental period, the fish were submitted to blood sampling via caudal puncture. The fish were then euthanized by sectioning the spinal cord, and the gills and brain were dissected out and quickly frozen in liquid nitrogen. The tissues were then stocked in a freezer at −70 °C until the determination of the oxidative parameters.

A blood sample was isolated for the measurement of the hematocrit and hemoglobin concentrations. The remainder was centrifuged in heparinized vials at 1,110×*g* for 5 min, and plasma was separated for the determination of plasma ion levels, thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH). The fraction containing the red cells was again separated into two aliquots: one for enzymatic dosages, to which 4 mM magnesium sulfate and 1 mM acetic acid were added, and the other for the determination of non-protein thiols, to which 20 % TCA was added. Both aliquots were centrifuged at 1,110×*g* for 5 min, and the supernatant was separated and then frozen.

The gills and brain were homogenized in a medium containing 120 mM potassium chloride and 30 mM phosphate buffer (pH 7.4) with 1 mM PMSF. The samples were centrifuged at 1,110×*g* in a refrigerated centrifuge for 20 min, and the supernatants were separated for the analyses of oxidative parameters (Riffel et al. 2011).

Hematological parameters

The determination of the hematocrit was performed by the microhematocrit method (Goldenfarb et al. 1971). The percentage of packed red cells was obtained by means of a hematocrit card reader. The hemoglobin concentration was determined using the Drabkin reagent (Kamper and Zijlstra 1964) and expressed as gram per deciliter blood. The mean cell hemoglobin concentration (MCHC) was calculated using the equation [Hb] × 100/Hct and expressed as gram per deciliter.

Plasma ion levels

The concentrations of sodium (Na^+) and potassium (K^+) were determined using the Micronal B262 flame spectrophotometer (Micronal, São Paulo, Brazil). Chloride (Cl^-) concentrations were measured according to the method of Zall (1956) and read in a TP reader Thermo Plate plate reader (Thermo Plate Devices, China) at 480 nm. Standard

solutions were made in distilled water, and five concentrations of the standard solutions were used. The results are expressed in millimole per liter.

Prooxidants assay

Lipid peroxidation (LPO) was monitored through two methods: determination of TBARS and of LOOH. The measurement of the latter was performed with a modified version of the method described by Jiang et al. (1991). This technique can detect the primary products of peroxidation, using Fe^{2+} oxidation by LOOH in an acid medium containing xylenol orange dye, which forms a complex with Fe^{3+}. The results were read at 560 nm and reported as nanomole per milligram protein. In turn, TBARS was determined according to the method of Wills (1987) by measuring the end products of LPO, including malondialdehyde, determined at 535 nm. The results were reported as nanomole per milligram protein.

Protein assay

Tissue proteins were quantified based on the method of Lowry et al. (1951) using bovine serum albumin as the standard. The readings were performed using a spectrophotometer Biospectro SP220 (São Paulo, Brazil) at 625 nm.

Antioxidant defenses assay

Catalase (CAT) activity was evaluated by measuring the decrease in H_2O_2 absorption at 240 nm, as outlined by Boveris and Chance (1973). The results are reported as picomoles per milligram protein.

Total superoxide dismutase (SOD) activity was determined as the inhibition rate of autocatalytic adrenochrome generation at 480 nm. The enzymatic activity is expressed as SOD units milligram per protein. One SOD unit was defined as the amount of enzyme needed for 50 % inhibition of adrenochrome formation, as described by Misra and Fridovich (1972).

Glutathione peroxidase (GPx) activity was measured by monitoring NADPH oxidation at 340 nm. GPx activity is reported as micromoles per minute per milligram protein (Flohé and Gunzler 1984).

Glutathione-*S*-transferase (GST) activity toward CDNB was determined spectrophotometrically at 340 nm according to the method of Habig et al. (1974). One unit of GST activity was defined as the amount of enzyme that catalyzes the conjugation of 1 pmol of CDNB with GSH per minute at 25 °C. The enzymatic activity is expressed as picomoles per minute per milligram protein.

Non-protein thiols were also measured in red blood cells. These groups are non-enzymatic antioxidants and represent an indirect measure of GSH. The method is based on the reaction of GSH with 5,5′-dithio-bis-2-nitrobenzoic acid. The samples were read spectrophotometrically at 412 nm (Ellman 1959). The content of non-protein thiols is expressed as micromoles per milligram protein.

Statistical analysis

The statistical analysis was performed using the software SPSS Statistics® 18. The Cochran and the Bartlett tests were used to verify whether the data were parametric, and two-way ANOVA was used to assess the effects of two factors (oxygen level and humic acid concentration). The total sum of squares of humic acid was partitioned into a linear or a quadratic component. Post hoc analysis was carried out using the *t*-Bonferroni test. The results are expressed as the mean ± S.E.M. The minimum significance level was set at 95 % ($p < 0.05$).

Results

Total phenolic compounds

There were 160.7 ± 4.5 mg GAE g HA^{-1} of total phenolic compounds.

Water parameters

The water parameters remained stable throughout the experimental period. The pH was maintained at 7.1 ± 0.04 and the water temperature at 21.3 ± 0.3 °C. Hardness (20.9 ± 1.6 mg L^{-1} $CaCO_3$), alkalinity (24.1 ± 1.9 mg L^{-1} $CaCO_3$), nitrite (0.87 ± 0.1 mg L^{-1}), total ammonia (2.9 ± 0.3 mg L^{-1}) and non-ionized ammonia (0.04 ± 0.007 mg L^{-1}) were remained in the desired ranges.

Hematological parameters

Hematocrit (Fig. 1a) and hemoglobin levels (Fig. 1b) in silver catfish showed no significant changes between treatments. MCHC levels showed no significant difference between the experimental groups (overall mean 25.7 ± 0.2 g dL^{-1}).

Plasma ion levels

Plasma Na^+ levels (Fig. 2a) in silver catfish were significantly affected by the HA treatment, increasing linearly with increasing HA concentrations in the water. Furthermore, there was a significant interaction between DO and HA in plasma Na^+ levels of silver catfish. In hypoxia, plasma Na^+ levels increased by 43 % in silver

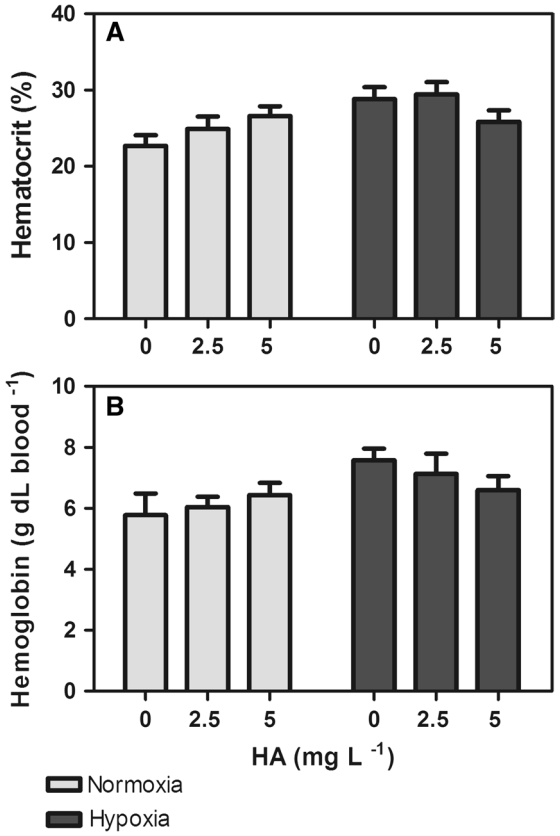


Fig. 1 The hematocrit (**a**) and hemoglobin (**b**) in the blood of *Rhamdia quelen* maintained under hypoxic and normoxic conditions and different humic acid (HA) levels in the water. Data are mean ± SEM

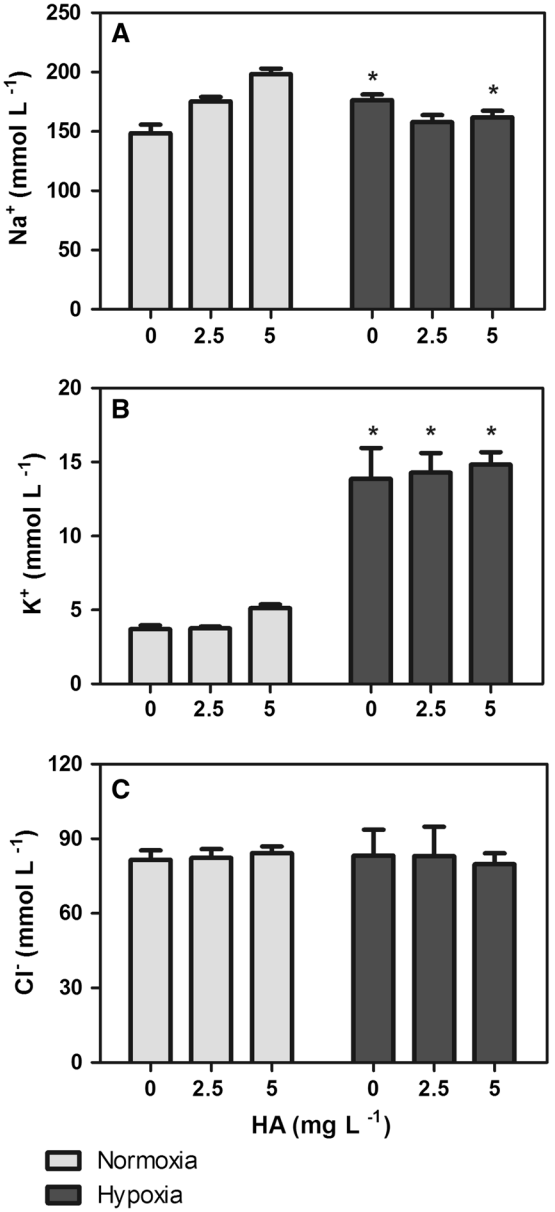


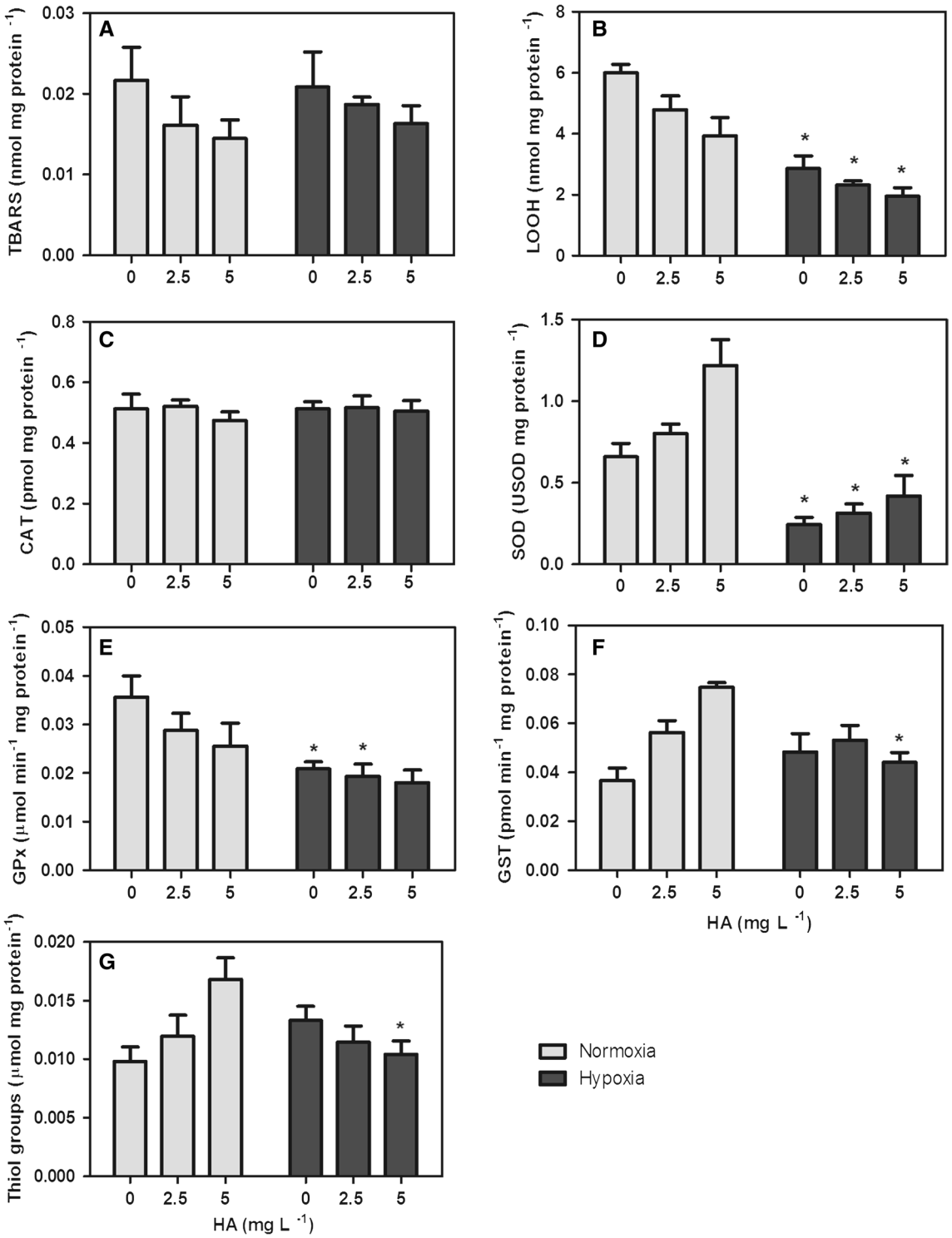
Fig. 2 Plasma ion levels in *Rhamdia quelen* maintained under hypoxic and normoxic conditions and different humic acid (HA) levels in the water. Data are mean ± SEM. *Asterisks* represent significant difference from normoxia group at the same HA concentration, determined by two-way ANOVA and *t*-Bonferroni tests ($p < 0.05$). **a** Plasma Na^+ levels: $F(2,29) = 3.4$ to HA; $F(1,27) = 6.2$ to linearity; $F(2,24) = 16.4$ to interaction. Post hoc $t = 4.3$ and $t = 3.5$–0 and 5 mg L^{-1} HA, respectively. $N = 5$ each group, $p < 0.05$. **b** Plasma K^+ levels: $F(1,24) = 207.01$ to OD. Post hoc $t = 7.6$, $t = 13.9$ and $t = 14.6$–0, 2.5 and 5 mg L^{-1} HA, respectively. $N = 5$ each group. **c** Plasma Cl^- levels. $N = 5$ each group

catfish non-exposed to HA when compared to normoxia, but simultaneous exposure to hypoxia and 5 mg L^{-1} HA decreased plasma Na^+ levels by 18 % compared to the normoxic group exposed to the corresponding HA concentration.

Plasma K^+ levels (Fig. 2b) in silver catfish increased significantly in all hypoxic groups (275, 276 and 195 % at 0, 2.5 and 5 mg L^{-1} of HA, respectively) when compared to normoxic ones. Plasma Cl^- levels (Fig. 2c) showed no significant difference between treatments.

Oxidative parameters

No significant difference was found in LPO levels measured in the plasma by TBARS (Fig. 3a) and in the enzymatic activity of CAT measured in erythrocytes (Fig. 3c). Enzymatic activities of SOD (Fig. 3d) and GST (Fig. 3f) in erythrocytes increased linearly with increasing HA concentrations. In hypoxia, plasma LPO levels measured by LOOH decreased significantly by 52, 55 and 51 % in silver catfish exposed to 0, 2.5 and 5 mg L^{-1} HA, respectively, compared to normoxia with the corresponding HA concentration (Fig. 3b). Likewise, in hypoxia, SOD activity was significantly decreased by 62, 60 and 71 % in erythrocytes of silver catfish exposed to 0, 2.5 and 5 mg L^{-1} HA, respectively, when compared to normoxia with the corresponding HA concentration (Fig. 3d). GPx activity in erythrocytes also decreased in hypoxic conditions by 40 and 30 % in



erythrocytes of silver catfish exposed to 0 and 2.5 mg L^{-1} HA, respectively, when compared to normoxic conditions with the corresponding HA concentration (Fig. 3e). A significant interaction between DO and HA was observed in GST activity (Fig. 3f) and the levels of non-protein thiols (Fig. 3g). In hypoxia, GST activity and the levels of non-protein thiols decreased significantly by 41 and 38 %, respectively, in erythrocytes of silver catfish exposed to 5 mg L^{-1} HA, when compared to the respective normoxic conditions (Fig. 3f, g, respectively).

In the gills, LPO levels measured by TBARS (Fig. 4a) and measured by LOOH (Fig. 4b), as well as activities of

◀**Fig. 3** Oxidative parameters in the blood of *Rhamdia quelen* maintained under hypoxic and normoxic conditions and different humic acid (HA) concentrations in water. Data are mean ± SEM. *Asterisks* represent significant difference from normoxia group at the same HA concentration, determined by two-way ANOVA and *t*-Bonferroni tests ($p < 0.05$). **a** Thiobarbituric acid reactive substances (TBARS): $N = 7$ each group. **b** Lipid hydroperoxides (LOOH): $F(2,30) = 6.6$ to HA; $F(2,35) = 57$ to DO. Post hoc $t = 6.2$, $t = 5.2$ and $t = 3.5$–0, 2.5 and 5 mg L^{-1} HA, respectively. $N = 6$ each group, $p < 0.05$. **c** Catalase (CAT). $N = 7$ each group. **d** Superoxide dismutase (SOD): $F(2,41) = 4.2$ to HA; $F(1,39) = 4.8$ to linearity; $F(1,36) = 57.8$ to DO. Post hoc $t = 4.5$, $t = 4.9$ and $t = 4.1$–0, 2.5 and 5 mg L^{-1} HA, respectively. $N = 7$ each group, $p < 0.05$. **e** Glutathione peroxidase (GPx): $F(1,36) = 13.7$ to DO. Post hoc $t = 3.2$ and $t = 2.3$–0 and 2.5 mg L^{-1} HA, respectively. $N = 7$ each group, $p < 0.05$. **f** Glutathione-*S*-transferase (GST): $F(2,41) = 3.4$ to HA; $F(1,39) = 4.5$ to linearity; $F(2,36) = 8.6$ to interaction. Post hoc $t = 4.2$–5 mg L^{-1} HA, respectively. $N = 7$ each group, $p < 0.05$. **g** Non-protein thiol groups: $F(2,36) = 5.3$ to interaction. Post hoc $t = 3.07$–5 mg L^{-1} HA, respectively. $N = 7$ each group, $p < 0.05$

SOD (Fig. 4d), GPx (Fig. 4e) and GST (Fig. 4f) decreased linearly with increasing HA concentrations.

In hypoxia, LPO levels measured by LOOH decreased significantly by 22 and 23 % in gills of silver catfish exposed to 2.5 and 5 mg L^{-1} HA, respectively, when compared to normoxia with the corresponding HA concentration (Fig. 4b). CAT activity in hypoxia was elevated by 81 % at 2.5 mg L^{-1} HA and 153 % at 5 mg L^{-1} HA when compared to the respective normoxic conditions (Fig. 4c). In hypoxic conditions, SOD activity increased significantly by 24 and 39 % in the gills of silver catfish exposed to 2.5 and 5 mg L^{-1} HA, respectively, when compared to normoxic conditions with the corresponding concentration of HA (Fig. 4d). Furthermore, a significant interaction between DO and HA was observed in CAT activity (Fig. 4c), indicating that HA modifies the effect of hypoxia on this enzyme. Similarly, a significant interaction between DO and HA was observed in GST activity in the gills of silver catfish (Fig. 4f), whose activity in hypoxia decreased by 42 % in the absence of the HA when compared to the respective normoxic group, but in the groups exposed to HA, no difference between normoxia and hypoxia was observed.

In the brain of silver catfish, there was a significantly linear component in LPO levels measured by LOOH (Fig. 5b), SOD activity (Fig. 5c) and GPx activity (Fig. 5d), indicating that these parameters decreased linearly with increased HA concentrations. In hypoxia, LPO levels measured by TBARS were significantly increased by 85 and 95 % in the brain of silver catfish exposed to 0 and 2.5 mg L^{-1} HA, respectively, when compared to normoxic conditions with the corresponding HA concentration (Fig. 5a). GPx activity was elevated by 108 % at 2.5 mg L^{-1} HA and 76 % at 5 mg L^{-1} HA in hypoxia when compared to the normoxic conditions with the corresponding HA concentration (Fig. 5d). HA and DO levels did not significantly modify GST activity (Fig. 5e). The determination of CAT activity in this organ was not possible due to the sensitivity of the method.

Discussion

HS are produced by several sources and show differences in their constitution and functional groups. Thus, their presence in aquatic environments can lead to diverse responses by organisms. The protective ability of HS was associated with a higher aromatic content, which was mainly found in HS derived from the breakdown of lignins, which tend to be optically darker; lignin-derived HS also contain a large fraction of HA (Wood et al. 2011).

HS can directly interact with freshwater organisms, such as phototrophic organisms. These interactions can lead to stress symptoms in these exposed organisms, including a reduction in photosynthetic oxygen release (Pflugmacher et al. 2006; Steinberg et al. 2008). Large changes in oxygen availability in aquatic environments can lead to corresponding wide variations in the production of ROS by animals with aquatic respiration. Increased formation of these species occurs during reoxygenation following periods of hypoxia or anoxia, mainly in mitochondria (Lushchak et al. 2001; Lutz and Nilsson 2004).

Mitochondria are a well-known source of ROS even under normal conditions. In vitro studies showed that HA could prevent the generation of these ROS in liver mitochondria by recapturing superoxide anions with lower efficiency than hydroxyl radicals (Vašková et al. 2011). Nonetheless, data reporting the effects of HA on oxidative parameters in fish are scarce in the literature.

Total phenolic compound concentration

HS behave as natural environmental chemicals and must interact with biotic structures. HS are characterized by many properties due to their composition, which includes various metals, molecular weights of the HS fraction, aromaticity, free organic radicals, functional groups and others (Wood et al. 2011). Aquatic HS are water-soluble compounds comprising carboxylic, carbonyl, phenolic, quinoid and aliphatic groups among others (Meinelt et al. 2007). These authors reported that natural HS with high moieties of high-molecular carbohydrates supported the growth of the fish-pathogenic species *Saprolegnia parasitica*, while synthetic and lignite-derived HS were among the most efficient HS sources to inhibit fungal growth. Growth inhibition was correlated with the molecular weights of the HS fraction, aromatic compounds, COOH groups, C and H. Furthermore, HS with higher molecular weights and

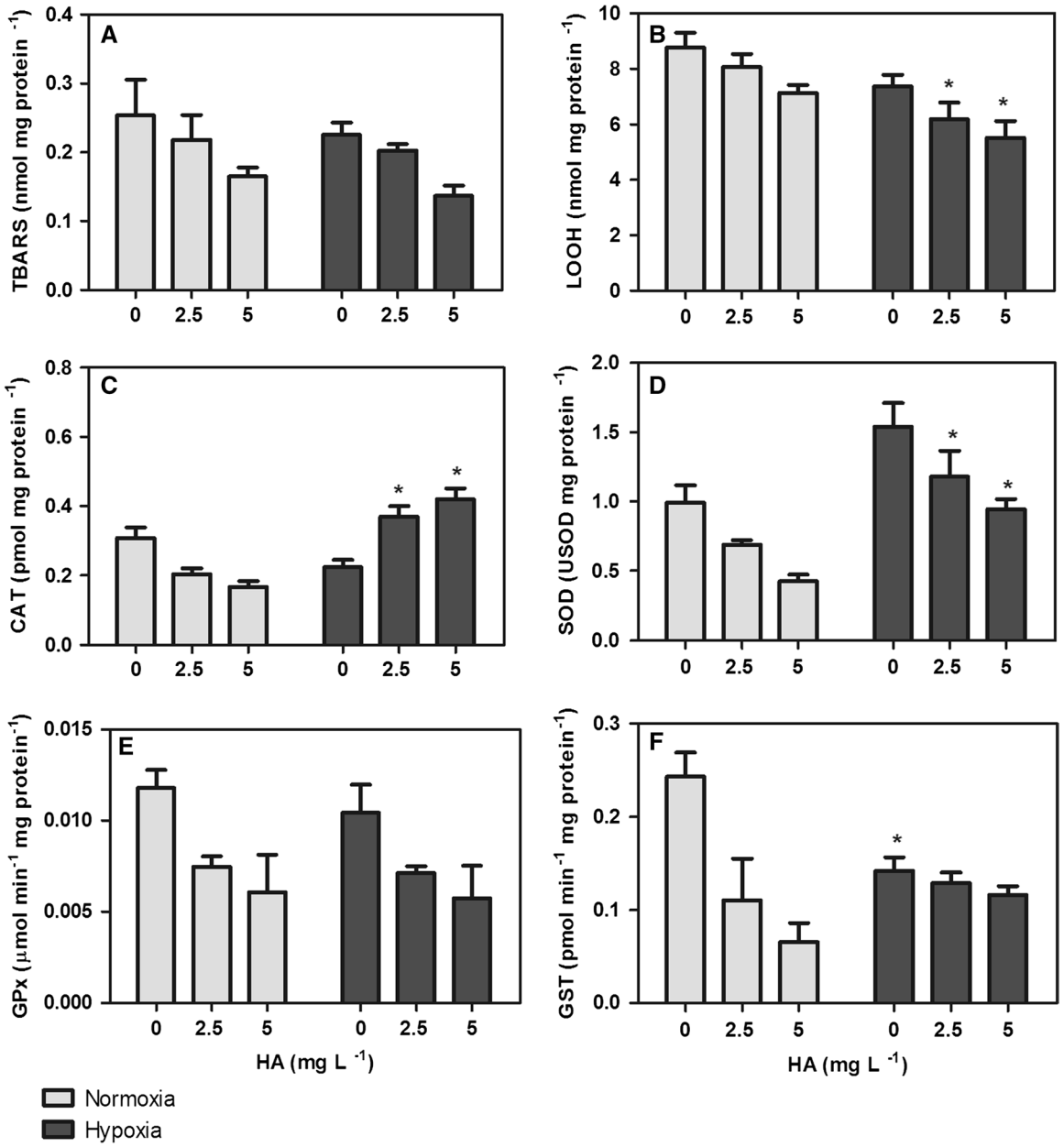


Fig. 4 Oxidative parameters in the gills of *Rhamdia quelen* maintained under hypoxic and normoxic conditions and different humic acid (HA) concentrations in water. Data are mean ± SEM. *Asterisks* represent significant difference from normoxia group at the same HA concentration, determined by two-way ANOVA and *t*-Bonferroni tests ($p < 0.05$). **a** Thiobarbituric acid reactive substances (TBARS): $F(2,39) = 4.73$ to HA; $F(1,37) = 9.3$ to linearity. $N = 8$ each group, $p < 0.05$. **b** Lipid hydroperoxides (LOOH): $F(2,35) = 3.8$ to HA; $F(1,33) = 7.6$ to linearity; $F(1,30) = 14.3$ to DO. Post hoc $t = 2.5$, $t = 2.4$–2.5 and 5 mg L^{-1} HA, respectively. $N = 6$ each group, $p < 0.05$. **c** Catalase (CAT): $F(1,42) = 21.8$ to DO; $F(2,42) = 21.1$ to interaction. Post hoc $t = 4.2$ and $t = 5.4$–2.5 and 5 mg L^{-1} HA, respectively. $N = 8$ each group, $p < 0.05$. **d** Superoxide dismutase (SOD): $F(2,35) = 6.2$ to HA; $F(1,33) = 12.2$ to linearity; $F(1,30) = 21.8$ to DO. Post hoc $t = 3.2$ and $t = 5.8$–2.5 and 5 mg L^{-1} HA, respectively. $N = 6$ each group, $p < 0.05$. **e** Glutathione peroxidase (GPx); $F(2,47) = 8.3$ to HA; $F(1,45) = 16.6$ to linearity. $N = 8$ each group, $p < 0.05$. **f** Glutathione-*S*-transferase (GST): $F(2,41) = 8.3$ to HA; $F(1,39) = 14.5$ to linearity; $F(2,36) = 5.1$ to interaction. Post hoc $t = 3.4$–5 mg L^{-1} HA, respectively. $N = 7$ each group, $p < 0.05$

aromaticity contained a high number of organic radicals and were the most efficient in reducing fungal growth.

Although there may be more functional groups or properties in HS influencing the responses of the organisms, the present research only studied the presence and the effects of the phenolic compounds present in HA on biochemical responses of the silver catfish. The results demonstrated that HA contains phenolic compounds, which

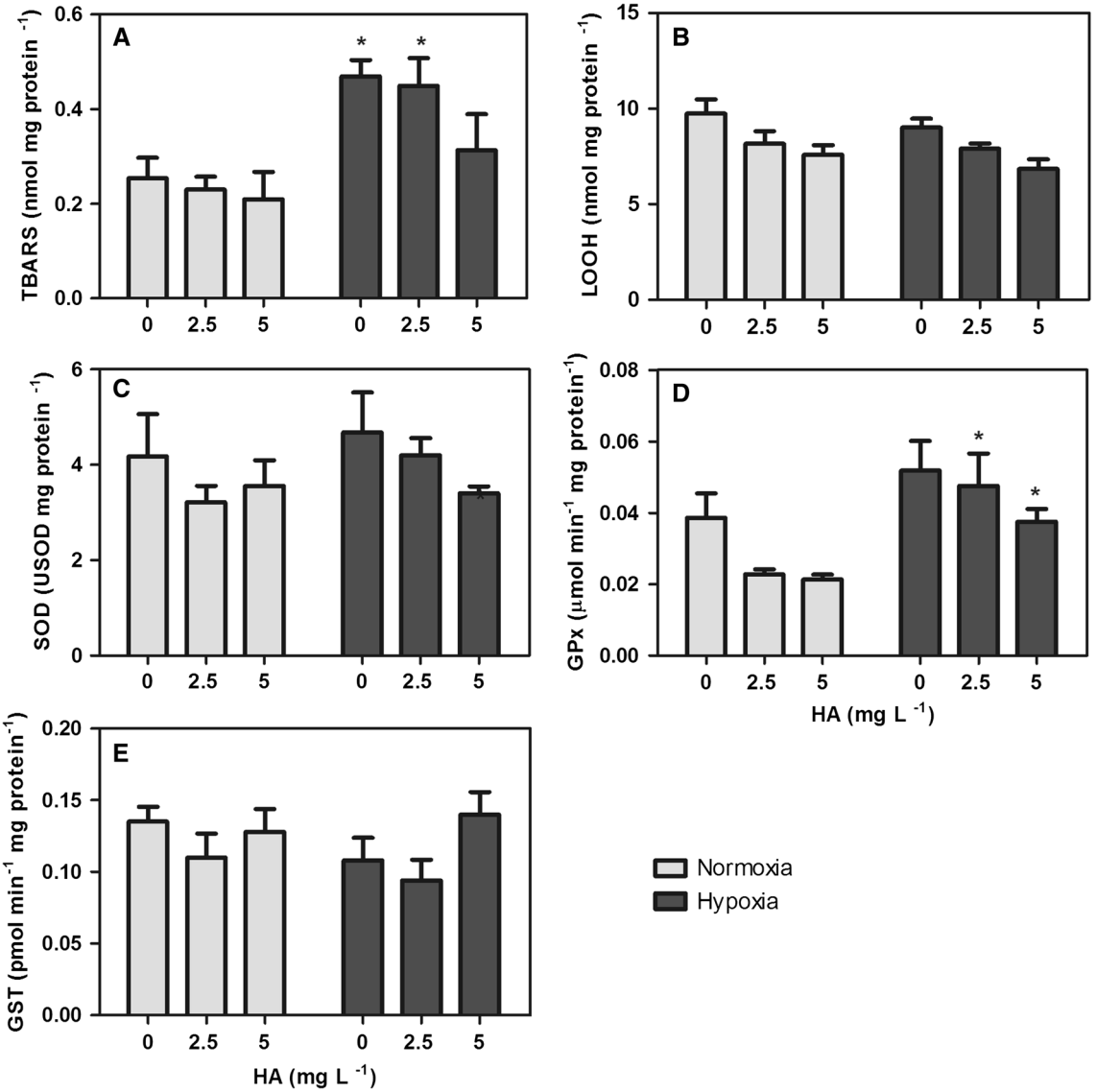


Fig. 5 Oxidative parameters in the brain of *Rhamdia quelen* maintained under hypoxic and normoxic conditions and different humic acid (HA) concentrations in water. Data are mean ± SEM. *Asterisks* represent significant difference from normoxia group at the same HA concentration, determined by two-way ANOVA and *t*-Bonferroni tests ($p < 0.05$). **a** Thiobarbituric acid reactive substances (TBARS): $F(1,24) = 18$ to DO; Post hoc = 3.7 and $t = 3.5$–0 and 2.5 mg L^{-1} HA, respectively. $N = 5$ each group, $p < 0.05$. **b** Lipid hydroperoxides (LOOH): $F(2,29) = 8.16$ to HA; $F(1,27) = 16$ to linearity. $N = 5$ each group, $p < 0.05$. **c** Superoxide dismutase (SOD): $F(2,29) = 3.4$ to HA; $F(1,27) = 5.2$ to linearity; $N = 5$ each group, $p < 0.05$. **d** Glutathione peroxidase (GPx): $F(1,27) = 6.2$ to HA. Post hoc $t = 4.6$, $t = 4.7$–2.5 and 5 mg L^{-1} HA, respectively. $N = 5$ each group, $p < 0.05$. **e** Glutathione-*S*-transferase (GST): $N = 5$ each group

could be involved in the reduction of LPO levels. Plant cell wall metabolites and their degradation products (as HS) contain several bioactive compounds, such as phenolics, which proved to be effective scavengers of ROS (Gali et al. 1992). The antioxidant action of phenolic compounds under in vitro conditions is attributed to their complexing ability (Varanka et al. 2001). Furthermore, phenolic compounds are able to trap alkoxyl radicals, which are the usual initiators of LPO reactions (Millic et al. 1998; Michalak 2006).

Hematological parameters

In this research, hematological parameters of silver catfish were unaffected by treatments, indicating that HA did not alter the number of blood cells as well as their hemoglobin content.

In hypoxia, no alteration in either the hematocrit or hemoglobin level was observed when compared to normoxia, possibly due to the moderate but not severe hypoxic levels employed. Any alteration was most likely not sharp

enough to activate erythropoietin, a mediator of erythrocyte production.

Plasma ion levels

Black waters are poor in ions. HS apparently limit gill permeability and excessive ion loss in these diluted waters by maintaining gill tight junctions (Matsuo and Val 2007). This study observed that HA exposure led to an increase in plasma Na^+ levels and it was directly proportional to the concentration of HA. Thus, it is possible that HA reduced the ion loss via fish gills, consequently increasing the plasma ion levels. In hypoxia, plasma Na^+ levels were increased in silver catfish unexposed to HA when compared to normoxic conditions. Beside this, plasma K^+ levels were increased in all groups in hypoxic conditions compared to normoxic conditions. These elevated levels could be attributed to a shift of fluid into the intracellular compartment to balance the possible increase in the plasmatic lactate concentration from anaerobic pathways (Wood et al. 2007). Such an effect of hypoxia exposure has been previously described for this species (Rosso et al. 2006; Becker et al. 2009). Silver catfish exposed to HA and hypoxia simultaneously showed a decrease in plasma Na^+ levels. It is possible that hypoxia inhibits the effect of HA on fish gills, keeping plasma ion levels near to control values.

Oxidative parameters

ROS are formed during the metabolism of oxygen of all aerobic organisms (including fish) by normal breathing, and ROS concentrations are controlled by antioxidant defenses. These defenses include the enzymes SOD, CAT, GPx and GST. SOD converts the superoxide anion formed from molecular oxygen into H_2O_2, which is converted to water and oxygen by the action of CAT. GPx, in turn, catalyzes the conversion of both H_2O_2 and organic hydroperoxides to less reactive products, employing glutathione GSH in its reduced form as the electron donor (Halliwell and Gutteridge 1999). GST plays the main role in eliminating exogenous substances, and some of these enzymes eliminate organic hydroperoxides. In hypoxia, fish may decrease overall metabolic rates as a strategy to survive this adverse condition. In this situation, phosphorylation is affected (Lushchak and Bagnyukova 2006), changing the redox status of cells, enzymatic processes and signaling molecules (Zhu et al. 2013). These changes in redox status of cells are dependent on the degree of hypoxia (Welker et al. 2013) and the time to exposure, inducing an increase in ROS production within a few hours (Lushchak and Bagnyukova 2006) and a decrease in ROS production after a few days of hypoxia exposure (Wilhelm Filho et al. 2005; Sampaio et al. 2008).

All tissues analyzed by this study showed a reduction in LPO levels, which was inversely proportional to the concentration of HA in the initial stage, measured by LOOH. Thus, HA exerts an antioxidant effect on lipid membranes, which could be attributed to a mixture of heterogeneous organic substances, whose properties, molecular weight and relative composition of functional groups depend on the mixture's source of origin (McDonald et al. 2004) and time of decomposition (Aeschbacher et al. 2012).

Aquatic HA contains lignins and tannins with the ability to donate electrons and to neutralize ROS, which provide HA an antioxidant action, protecting other functional groups from oxidation and ensuring their stability (Rimmer and Abbott 2011). Some research demonstrated that HA isolated from rivers, streams and lakes showed shorter degradation that has been correlated with higher electron donor ability due to the presence of phenol groups (Aeschbacher et al. 2012).

Similarly, in both hypoxia and normoxia, GPx activity decreased linearly in gills and brain in an inversely proportional manner to the concentration of HA. In vitro studies by Vašková et al. (2011) also showed a reduction in GPx activity in liver mitochondria exposed to HA. These authors suggested that the decrease in GPx activity indicates that the electrophilic properties of HA markedly balance mitochondrial redox status. The presence of numerous structures in HA resembling those of quinones predetermines them to transfer the accepted electrons within an HA molecule without involvement of mitochondrial enzymes that otherwise reduce the developing peroxides.

Red blood cells were investigated in the present research because they exhibit specific enzymatic responses of cellular protection against ROS, which are produced in large quantities in these cells (Michalak 2006). It was observed that HA exposure led to an increase in SOD and GST activities in normoxia as well as in the levels of non-protein thiols. Glutathione in its reduced form is one of the most abundant intracellular non-protein thiols and acts as a substrate for GST; thus, its increase could be related to the supply of substrate for the activity of GST. As described in the literature, when an elevation of SOD activity is associated with a rise in GSH levels, the generation of superoxide anion and the subsequent ROS is inhibited in fish erythrocytes (Koppenol 1993; Winterbourn 1993; Marcon and Wilhelm 1999). Measurement of antioxidant properties of the selected natural HA showed that HA displays antioxidant properties against superoxide anions (Vašková et al. 2011). It is known that the antioxidant capacity of phenolic compounds does not extend to all reactive species involved in cell damage (Soobratee et al. 2005). It is believed that the increases in these enzymes, particularly the GST, are associated with the modulation of other reactive species which are not neutralized by the action of phenolic compounds.

Furthermore, the activity of antioxidant compounds is not simply limited by quenching of ROS generation and propagation but also interferes with cell signal transduction by interacting with other signaling enzymes and transcription factors (Leonarduzzi et al. 2010), which could increase the activity of these enzymes.

In contrast to normoxia, an interesting interaction between hypoxia and HA was observed for GST activity in red blood cells and in the levels of non-protein thiols, decreasing their levels in the presence of 5 mg L^{-1} of HA. These results indicate that moderate hypoxia may inhibit the effect of HA on GST and non-protein thiols. It is believed that in red blood cells, the association of metabolic depression and the antioxidant properties of HA effectively control ROS, hydroperoxides and exogenous components, which could inhibit the glutathione-related antioxidants. In hypoxia, the red blood cells studied also displayed a reduction in LOOH levels associated with a decrease in SOD and GPx activities as well as the content of non-protein thiols (the latter only in 5 mg L^{-1} HA) when compared to normoxia. Such observed alterations could be associated with the decreased overall metabolic rate in fish exposed to hypoxia (Lushchak and Bagnyukova 2006).

The gills are the primary target of compounds dissolved in water due to their large contact surface, which facilitates interaction with external factors as well as the gill defense system (Pandey et al. 2008). In addition to the reduction of the LPO levels in the initial stage observed in all tissues, a decrease inversely proportional to the concentration of HA in the gills was also observed in the final phase of LPO, determined by TBARS. The results may be associated to the effect of HA in lipid membranes, which become more pronounced in gills due to the direct interaction with HA.

This study observed that HA exposure resulted in a general reduction of the activity of most enzymatic antioxidants and non-protein thiols in both hypoxia and normoxia, and this reduction was inversely proportional to the concentration of HA. Vašková et al. (2011) suggested that the protective mechanisms performed by the antioxidant defense system are moderately disabled by the HA from the selected sources, which is involved in redox regulation. The exception viewed by the present research was CAT activity in the gills. The results showed that this enzyme is unaffected by HA in normoxia. However, a significant interaction between HA and hypoxia was demonstrated for CAT activity. Furthermore, comparing the hypoxic groups in relation to normoxia, there was an increase in CAT and SOD activities in groups exposed to 2.5 and 5 mg L^{-1} HA in hypoxia. The gills are the major route for eliminating H_2O_2 produced by various organs (Marcon and Wilhelm 1999). As described by Timofeyev et al. (2006), H_2O_2 levels in the cytosol of *Gammarus lacustris* increased after exposure to HA from Lake Schwarzer for 6 days. Thus, the present study suggested that CAT activity was elevated in hypoxic conditions in the presence of humic acid, most likely to reduce the toxicity of H_2O_2 produced in the gills by removing it.

Some studies have shown increases of antioxidant enzyme activity in the gills of aquatic animals that are under metabolic depression (Cooper et al. 2002; Trasviña-Arenas et al. 2013; Welker et al. 2013). Cooper et al. (2002) reported an increase in SOD activity in spot croaker fish (*Leiostomus xanthurus*) exposed to 2 mg L^{-1} oxygen. According to Welker et al. (2013), activation of SOD in the gills may protect from the drastic changes in oxygen availability. In addition, an increase in SOD activity may contribute to the increase of CAT activity in the gills of silver catfish. The interaction between hypoxia and HA in gills also increased GST activity. This increase is attributed to the greater interaction with external factors occurring in gills, requiring the most effective removal of exogenous substances and hydroperoxides in relation to other fish tissues.

Data relating the effect caused by HA in the brain were not available in the literature. This research shows that HA exposure led to a reduction in SOD and GPx activities that were inversely proportional to the concentration of HA. As mentioned above, it most likely occurred as a result of the key role played by HA in disabling the protective mechanisms of the antioxidant defense system by recapturing superoxide anions, neutralizing peroxides and acting in redox regulation (Vašková et al. 2011). In hypoxia, TBARS levels were increased in the brain compared to normoxia. A similar pattern was observed in the brain of silver catfish transported in plastic bags for 7 h (Azambuja et al. 2011). The brain contains high amounts of unsaturated lipids and utilizes approximately 20 % of the total oxygen demand of the body (Stella and Lajtha 1987). In hypoxic conditions, an inadequate oxygen supply to the brain may cause increased levels of LPO because the organism tends to primarily protect the vital organs (Lushchak and Bagnyukova 2007). Nilsson and Renshaw (2004) observed that goldfish (*Carassius auratus*) showed an increased oxygen supply to the brain due to blood flow redistribution, which is regulated by adenosine, whose action may exacerbate the generation of ROS, consequently increasing LPO levels (Hochachka and Lutz 2001). Moreover, ROS produced during hypoxia have a relevant role as signal agents, triggering the metabolic adjustments that may proportionate hypoxia tolerance (Guzy and Schumacker 2006; Hamanaka and Chandel 2009; Welker et al. 2013).

Furthermore, in the present work, an elevation in GPx activity was verified only in the brain tissue of silver catfish exposed to hypoxia and HA when compared to normoxia and HA, suggesting that this enzyme is most likely trying to detoxify the H_2O_2 accumulated during stress because the brain is an organ that is markedly affected by hypoxia.

Conclusion

This work indicates that the presence of HA in hypoxic environments can change biochemical and physiological parameters in silver catfish in a concentration-dependent manner because HA is able to cause alterations in plasma ion levels and oxidative parameters. In general, 2.5 and 5.0 mg L^{-1} of HA are beneficial to silver catfish exposed to hypoxia because it plays a key role in protecting the blood, gills and brain from LPO as well as in modulating the antioxidant system, mainly GPx and GST activities. These effects can be attributed, most likely, to the action of the different HA constituents and functional groups, including phenolic compounds, which have antioxidant properties. Further studies are needed to elucidate the complete mechanism of HA action on silver catfish.

Acknowledgments The authors are grateful to Dr. Carlos Fernando de Mello from Universidade Federal de Santa Maria for useful advice and suggestions. The authors also would like to thank the Conselho Nacional de Desenvolvimento Tecnológico (CNPq) and Amazonas State Research Foundation—FAPEAM Fundação de Amparo a Pesquisa do Estado do Amazonas—INCT ADAPTA and also to the Comissão de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Conflict of interest The authors declare that there are no conflicts of interest.

References

Aeschbacher M, Graf C, Schwarzenbach RP, Sander M (2012) Antioxidant properties of humic substances. Environ Sci Technol 46:4916–4925

Affonso EG, Polez VL, Corrêa CF, Mazon AF, Araújo MR, Moraes G, Rantin FT (2002) Blood parameters and metabolites in the teleost fish *Colossoma macropomum* exposed to sulfide or hypoxia. Comp Biochem Physiol C Toxicol Pharmacol 133:375–382

Azambuja CR, Mattiazzi J, Riffel APK, Finamor IA, Garcia LO, Heldwein CG, Heinzmann BM, Baldisserotto B, Pavanato MA, Llesuy SF (2011) Effect of the essential oil of *Lippia alba* on oxidative stress parameters in silver catfish (*Rhamdia quelen*) subjected to transport. Aquaculture 319:156–161

Becker AG, Garcia LO, Kochhann D, Gonçalves JF, Loro VL, Baldisserotto B (2009) Dissolved oxygen and ammonia levels in water that affect plasma ionic content and gallbladder bile in silver catfish. Cienc Rural 39:1768–1773

Bickler PE, Buck LT (2007) Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. Annu Rev Physiol 69:145–170

Boveris A, Chance B (1973) The mitochondrial generation of hydrogen peroxide. Biochem J 134:707–716

Boyd CE, Tucker CS (1992) Water quality and pond soil analyses for aquaculture. Auburn University/Alabama Agricultural Experiment Station, Auburn

Braun N, De Lima RL, Moraes B, Loro VL, Baldisserotto B (2006) Survival, growth and biochemical parameters of silver catfish, *Rhamdia quelen* (Quoy & Gaimard, 1824) juveniles exposed to different dissolved oxygen levels. Aquac Res 37:1524–1531

Cheng ML, Ho HY, Chiu DT, Lu FJ (1999) Humic acid-mediated oxidative damages to human erythrocytes: a possible mechanism leading to anemia in Blackfoot disease. Free Radic Biol Med 27:470–477

Colt J (2002) List of spreadsheets prepared as a complement. In: Wedemeyer GA (ed) Fish hatchery management. American Fisheries Society, Bethesda, pp 91–186

Cooper RU, Clough LM, Farwell MA, West TL (2002) Hypoxia-induced metabolic and antioxidant enzymatic activities in the estuarine fish *Leiostomus xanthurus*. J Exp Mar Biol Ecol 279:1–20

Diaz RJ, Rosenberg R (1995) Marine benthic hypoxia: review of ecological effects and behavioral responses on benthic macrofauna. Oceanogr Mar Biol Annu Rev 33:245–303

Eaton AD, Clesceri LS, Rice EW, Greenberg AE (2005) Standard methods for the examination of water and wastewater, 21st edn. American Public Health Association, Washington

Ellman L (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77

Fiorentino G, Spaccini R, Piccolo A (2006) Separation of molecular constituents from a humic acid by solid-phase extraction following a transesterification reaction. Talanta 68:1135–1142

Flohé L, Gunzler WA (1984) Assays of glutathione peroxidase. In: Colowick SP, Kaplan ON (eds) Methods in enzymology. Academic Press, San Diego, pp 114–121

Gali HU, Perchellet EM, Klish DS, Johnson JM, Perchellet J-P (1992) Hydrolyzable tannins: potent inhibitors of hydroperoxide production and tumor promotion in mouse skin treated with 12-*O*-tetradecanoylphorbol-13-acetate in vivo. Int J Cancer 51:425–432

Giacomin M, Gillis PL, Bianchini A, Wood CM (2013) Interactive effects of copper and dissolved organic matter on sodium uptake, copper bioaccumulation, and oxidative stress in juvenile freshwater mussels (*Lampsilis siliquoidea*). Aquat Toxicol 144:105–115

Glover CN, Wood CM (2004) Physiological interactions of silver and humic substances in *Daphnia magna*: effects of reproduction and silver accumulation following an acute silver challenge. Comp Biochem Physiol C 139:273–280

Goldenfarb PB, Bowyer FP, Hall E, Brosious E (1971) Reproducibility in the hematology laboratory: the microhematocrit determination. Am J Clin Path 56:35–39

Gomes LC, Golombieski JI, Gomes ARC, Baldisserotto B (2000) Biologia do jundiá, *Rhamdia quelen* (Teleostei, Pimelodidae). Uma revisão Cienc Rural 30:179–185

Guzy RD, Schumacker PT (2006) Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. Exp Physiol 91:807–819

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139

Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine. Int J Biochem Cell Biol 31:1454–1468

Hamanaka RB, Chandel NS (2009) Mitochondrial reactive oxygen species regulate hypoxic signaling. Curr Opin Cell Biol 21:894–899

Hochachka PW, Lutz PL (2001) Mechanism, origin and evolution of anoxia tolerance in animals. Comp Biochem Physiol B 130:435–459

Jiang ZY, Woollard ACS, Wolff SP (1991) Lipid hydroperoxide measurement by oxidation of Fe^{2+} in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. Lipid 26:853–856

Kamper EJ, Zijlstra WG (1964) Standardization of haemoglobinometry. In: De Boroviczény CG (ed) Erythrocytometric methods and their standardization. S. Karger, New York, pp 35–62

Koppenol WH (1993) A thermodynamic appraisal of the radical sink hypothesis. Free Rad Biol Med 14:91–94

Leonarduzzi G, Sottero B, Poli G (2010) Targeting tissue oxidative damage by means of cell signaling modulators: the antioxidant concept revisited. Pharmacol Ther 128:336–374

Lowry OH, Rosebrough MJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin reagent. J Biol Chem 193:265–269

Lushchak VI, Bagnyukova TV (2006) Effects of different environmental oxygen levels on free radical processes in fish. Comp Biochem Physiol B 144:283–289

Lushchak V, Bagnyukova T (2007) Hypoxia induces oxidative stress in tissues of a goby, the rotan *Perccottus glenii*. Comp Biochem Physiol B 148:390–397

Lushchak VI, Lushchak LP, Mota AA, Hermes-Lima M (2001) Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. Am J Physiol Regul Integr Comp Physiol 280:100–107

Lutz PL, Nilsson GE (2004) Vertebrate brains at the pilot light. Respir Physiol Neurobiol 141:285–296

Marcon JL, Wilhelm D (1999) Antioxidant processes of the wild tambaqui, *Colossoma macropomum* (Osteichthyes, Serrasalmidae) from the Amazon. Comp Biochem Physiol C Pharmacol Toxicol 123:257–263

Martínez-Alvarez RM, Morales AE, Sanz A (2005) Antioxidant defenses in fish: biotic and abiotic factors. Rev Fish Biol Fish 15:75–88

Matsuo AY, Val AL (2007) Acclimation to humic substances prevents whole body sodium loss and stimulates branchial calcium uptake capacity in cardinal tetras *Paracheirodon axelrodi* (Schultz) subjected to extremely low pH. J Fish Biol 70:989–1000

Matsuo AY, Wood CM, Val AL (2005) Effects of copper and cadmium on ion transport and gill metal binding in the Amazonian teleost tambaqui (*Colossoma macropomum*) in extremely soft water. Aquat Toxicol 74:351–364

McDonald S, Bishop AG, Prenzler PD, Robards K (2004) Analytical chemistry of freshwater humic substances. Anal Chim Acta 527:105–124

McGeer JC, Szebedinszky C, McDonald DG, Wood CM (2002) The role of dissolved organic carbon in moderating the bioavailability and toxicity of Cu to rainbow trout during chronic waterborne exposure. Comp Biochem Physiol C Toxicol Pharmacol 133:147–160

Meinelt T, Paul A, Phan TM, Zwimmann E, Kruger A, Wienke A, Steinberg C (2007) Reduction in vegetative growth of the water mold *Saprolegnia parasitica* (Coker) by humic substance of different qualities. Aquat Toxicol 83:93–103

Meinelt T, Schreckenbach K, Pietrock M, Heidrich S, Steinberg CEW (2008) Humic substances (review series). Part 1: dissolved humic substances (HS) in aquaculture and ornamental fish breeding. Environ Sci Pollut Res 15:17–22

Michalak A (2006) Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Polish J Environ Stud 15:523–530

Millic BL, Djilas SM, Canadinovic-Brunet JM (1998) Antioxidative activity of phenolic compounds on the metal-ion breakdown of lipid peroxidation system. Food Chem 61:443–447

Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247:3170–3175

Nilsson GE, Renshaw GMC (2004) Hypoxic survival strategies in two fishes: extreme anoxia tolerance in the North European crucian carp and natural hypoxic preconditioning in a coral-reef shark. J Exp Biol 207:3131–3139

Pandey S, Parvez S, Ansari RA, Ali M, Kaur M, Hayat F, Ahmad F, Raisuddin S (2008) Effects of exposure to multiple trace metals on biochemical, histological and ultrastructural features of gills of a freshwater fish, *Channa punctata* Bloch. Chem Biol Interact 174:183–192

Paquin PR, Gorsuch JW, Apte S, Batley GE, Bowles KC, Campbell PGC, Delos CG, Di Toro DM, Dwyer RL et al (2002) The biotic ligand model: a historical overview. Comp Biochem Physiol C 133:3–35

Paul A, Hackbarth S, Vogt RD, Roder B, Burnison BK, Steinberg CE (2004) Photogeneration of singlet oxygen by humic substances: comparison of humic substances of aquatic and terrestrial origin. Photochem Photobiol Sci 3:273–280

Pérez-Jiménez J, Saura-Calixto F (2005) Literature data may underestimate the actual antioxidant capacity of cereals. J Agric Food Chem 53:5036–5040

Pflugmacher S, Pietsch C, Rieger W, Steinberg CE (2006) Dissolved natural organic matter (NOM) impacts photosynthetic oxygen production and electron transport in coontail *Ceratophyllum demersum*. Sci Total Environ 357:169–175

Richards JG, Burnison K, Playle RC (1999) Natural and commercial dissolved organic matter protects against the physiological effects of a combined cadmium and copper exposure on rainbow trout (*Oncorhynchus mykiss*). Can J Fish Aquat Sci 56:407–418

Riffel AP, Garcia LO, Finamor IA, Saccol EM, Meira M, Kolberg C, Horst A, Partata W, Llesuy S, Baldisserotto B, Pavanato MA (2011) Redox profile in liver of *Leporinus macrocephalus* exposed to different dissolved oxygen levels. Fish Physiol Biochem 38:797–805

Rimmer DL, Abbott GD (2011) Phenolic compounds in NaOH extracts of UK soils and their contribution to antioxidant capacity. Eur J Soil Sci 62:285–294

Rosso FL, Bolner KCS, Baldisserotto B (2006) Ion fluxes in silver catfish (*Rhamdia quelen*) juveniles exposed to different dissolved oxygen levels. Neotropical Ichthyol 4:435–440

Sampaio FG, Boijink CDL, Oba ET, Dos Santos LRB, Kalinin AL, Rantin FT (2008) Antioxidant defenses and biochemical changes in pacu (*Piaractus mesopotamicus*) in response to single and combined copper and hypoxia exposure. Comp Biochem Physiol C Pharmacol Toxicol 147:43–51

Semenza GL (2007) Hypoxia-inducible factor (HIF-1) pathway. Sci STKE 407:1–8

Singleton VL, Orthofer R, Lamuela-Raventós RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Methods Enzymol 299:152–178

Soobratee MA, Neergheen VS, Luximon-Ramma A, Aruomab OI, Bahorun T (2005) Phenolics as potential antioxidant therapeutic agents: mechanism and actions. Mutat Res 579:200–213

Steinberg CEW (2003) Ecology of humic substances in freshwaters. Springer, Berlin

Steinberg CEW, Paul A, Pflugmacher S, Meinelt T, Klocking R, Wiegand C (2003) Pure humic substances have the potential to act as xenobiotic chemicals—a review. Fresenius Environ Bull 12:391–401

Steinberg CEW, Meinelt T, Timofeyev MA, Bittner M, Menzel R (2008) Humic substances (review series). Part 2: interactions with Organisms. Environ Sci Pollut Res 15:128–135

Stella AMG, Lajtha A (1987) Macromolecular turnover in brain during aging. Gerontology 33:136–148

Terova G, Rimoldi S, Corá S, Bernardini G, Rosalba G, Saroglia M (2008) Acute and chronic hypoxia affects HIF-1α mRNA levels in sea bass (*Dicentrarchus labrax*). Aquaculture 279:150–159

Thurman EM (1985) Organic geochemistry of natural waters. Kuwler Academic Publishers, Dordrecht

Timofeyev MA, Shatilina ZM, Kolesnichenko AV, Bedulina DS, Kolesnichenko VV, Pflugmacher S, Steinberg CE (2006) Natural organic matter (NOM) induces oxidative stress in freshwater amphipods *Gammarus lacustris Sars* and *Gammarus tigrinus* (Sexton). Sci Total Environ 366:673–681

Trasviña-Arenas CH, Garcia-Triana A, Peregrino-Uriarte AB, Yepiz-Plascencia G (2013) White shrimp *Litopenaeus vannamei*

catalase: gene structure, expression and activity under hypoxia and reoxygenation. Comp Biochem Physiol B 164:44–52

Uyguner-Demirel CS, Birben C, Bekbolet M (2013) Key role of common anions on the photocatalytic degradation profiles of the molecular size fractions of humic acids. Catal Today 209:122–126

Varanka Z, Rojik I, Varanka I, Nemcsók J, Abrahám M (2001) Biochemical and morphological changes in carp (*Cyprinus carpio* L.) liver following exposure to copper sulfate and tannic acid. Comp Biochem Physiol C Toxicol Pharmacol 128:467–478

Vašková J, Veliká B, Pilátová M, Kron I, Vaško L (2011) Effects of humic acids in vitro. In Vitro Cell Dev Biol Anim 47:376–382

Wang GI, Jiang BH, Rue EA, Semenza GL (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-pas heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA 92:5510–5514

Welker AF, Moreira DC, Campos EG, Hermes-Lima M (2013) Role of redox metabolism for adaptation of aquatic animals to drastic changes in oxygen availability. Comp Biochem Physiol A 165:384–404

Wilhelm Filho D, Torres MA, Zaniboni-Filho E, Pedrosa RC (2005) Effect of different oxygen tensions on weight gain, feed conversion, and antioxidant status in piapara, *Leporinus elongatus* (Valenciennes, 1847). Aquaculture 244:349–357

Wills ED (1987) Evaluation of lipid peroxidation in lipids and biological membranes. In: Snell K, Mullock B (eds) Biochemical toxicology: a practical approach. IRL Press, Oxford, pp 127–152

Winterbourn CC (1993) Superoxide as an intracellular radical sink. Free Rad Biol Med 14:85–90

Wood CM, Kajimura M, Sloman KA, Scott GR, Walsh PJ, Almeida-Val VMF, Val AL (2007) Rapid regulation of Na^+ fluxes and ammonia excretion in response to acute environmental hypoxia in the Amazonian Oscar. Am J Physiol Regul Integr Comp Physiol 292:2048–2058

Wood CM, Al-Reasi HA, Smith DS (2011) The two faces of DOC. Aquat Toxicol 105:3–8

Wu RSS (2002) Hypoxia: from molecular responses to ecosystem responses. Mar Pollut Bull 45:35–45

Zall DM (1956) Photometric determination of chlorides in water. Analyt Chem 28:1665–1678

Zhu CD, Wang ZH, Yan B (2013) Strategies for hypoxia adaptation in fish species: a review. J Comp Physiol B 183:1005–1013