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Metals and Nonsteroidal Anti-inflammatory Pharmaceuticals Drugs Present in Water from Madín Reservoir (Mexico) Induce Oxidative Stress in Gill, Blood, and Muscle of Common Carp (*Cyprinus carpio*)

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Abstract Many toxic xenobiotics that enter the aquatic environment exert their effects through redox cycling. Oxidative stress, which incorporates both oxidative damage and antioxidant defenses, is a common effect induced in organisms exposed to xenobiotics in their environment. The results of the present study aimed to determine the oxidative stress induced in the common carp *Cyprinus carpio* by contaminants [metals and nonsteroidal anti-inflammatory drugs (NSAIDs)] present in Madín Reservoir. Five sampling stations (SSs), considered to have the most problems due to discharges, were selected. Carp were exposed to water from each SS for 96 h, and the following biomarkers were evaluated in gill, blood, and muscle: hydroperoxide content, lipid peroxidation, protein carbonyl content, and the activity of antioxidant enzymes superoxide dismutase and catalase. Results show that contaminants (metals and NSAIDs) present in water from the different SSs induce oxidative stress. Thus, water in this reservoir is contaminated with xenobiotics that are hazardous to *C. carpio*, a species consumed by the local human population.

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Madín Reservoir is located on the Río Tlalnepantla in Mexico; its dam was built downstream from the town of Madín at the point where the municipalities of Naucalpan de Juárez, Atizapán de Zaragoza, and Tlalnepantla de Baz (State of Mexico) meet. This reservoir is used for multiple purposes: It supplies potable water to the municipalities of Naucalpan and Atizapán, and is the site of diverse recreation activities, including sailing and fishing of diverse species, such as the common carp *Cyprinus carpio*.

One of the main sources of water contamination is the high acidity of local rainfall, which ranges in pH from 5.0 to 5.4. This phenomenon is due mainly to the high emissions of contaminants (SO_x, NO_{x-} and Cl_2) generated by both fixed and mobile sources (industry and motor vehicles). In addition, these municipalities do not have the equipment required for adequate treatment of local wastewater. Thus, Madín Reservoir, a water body destined for domestic use, is contaminated with direct sanitary discharges from human settlements in the area. Wastewater, particularly industrial wastewater, contains hazardous elements such as diverse chemicals, heavy metals, pharmaceuticals, solvents, acids, grease, and oils, among others. In addition, households make extensive use of detergents rather than soap, and motor vehicle repair shops commonly throw used oil into the sewer.

Preliminary studies performed to identify contaminants at this site show that water in Madín Reservoir contains a

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considerable metal load, especially for iron (Fe) and aluminum (Al), both of which exceed the permissible levels for aquatic life protection, whereas nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac (DCF), ibuprofen (IBP), and naproxen (NPX), are present at $\mu g\ L^{-1}$ levels. Pesticides, hydrocarbons, and surfactants originating in anthropogenic activities have also been qualitatively detected.

Diverse studies have shown that heavy metals in general can bind to sulfhydryl groups, consequently damaging the diverse structures that contain these groups. Al can indirectly produce reactive oxygen species (ROS) and resultant oxidative stress (Yokel 2000). Fe sustains redox cycling during which there is production of ROS (Stohs and Bagchi 1995), which exogenously contributes to oxidative stress.

Several studies have reported NSAID-induced toxicity in aquatic organisms because these organisms are more susceptible to toxic effects due to continued exposure to wastewater discharges throughout their life cycle (Fent et al. 2006). NSAIDs have been shown to affect fish reproduction and growth and to induce gill alterations as well as hepatotoxicity and nephrotoxicity (Schwaiger et al. 2004; Hoeger et al. 2005; Mehinto et al. 2010). Other investigators have stated that paracetamol and DCF induce oxidative stress on *Hyalella azteca* and *C. carpio*, damaging biomolecules such as lipids, proteins, and DNA (Oviedo-Gómez et al. 2010; Gómez-Oliván et al. 2012; Islas-Flores et al. 2013). Many chemical products induce oxidative stress. These contaminants can stimulate ROS production and induce changes in antioxidant systems (Monteiro et al. 2006; Uner et al. 2006; Slaninova et al. 2009). Cleaning products and their ingredients (anionic surfactants and bleaching agents) are toxic to aquatic life (Ankley and Burkhard 1992). The metabolism of surfactants by aquatic animals can induce ROS formation and oxidative stress on these organisms (Hofer et al. 1995; Livingstone 2003; Jifa et al. 2005; Li 2008).

Oxidative stress occurs through alteration of the balance between ROS and antioxidant systems in the body. ROS, such as hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{-\bullet}$), and the hydroxyl radical ($HO^{\bullet}$), are formed in cells as a result of metabolic processes (Valavanidis et al. 2006). Aerobic organisms produce ROS due to their oxidative metabolism. Hydroxyl radicals may initiate lipid peroxidation (LPX) in body tissues. To mitigate the negative effects of ROS, fish and other vertebrates possess an antioxidant defense system that uses enzymatic and nonenzymatic mechanisms. The most important antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione *S*-transferase (GST). The enzymatic defense system includes vitamins E, C, and A; carotenes; and ubiquinol-10 (Wilhelm Filho 1996). Antioxidants protect the body against oxy-radical-induced damage, such as breaks in the DNA chain, protein oxidation and LPX induction (Winzer et al. 2000). An increase in the active form of molecular oxygen species due to overproduction and/or inability to destroy them can lead to damage of DNA structure and may therefore induce mutations, chromosomal aberrations, and carcinogenesis. A change toward increased oxidant status or any imbalance between ROS production and degradation in animal tissues may induce LPX, plasma membrane alterations, or enzyme deactivation (Anand et al. 2000).

Cyprinus carpio, a fish commonly used in commercial aquaculture, has been proposed as a test organism in toxicological assays due to its economic importance and wide geographic distribution. In addition, it shows adaptive response in contaminated aquatic environments (Oruç and Uner 2002).

A previous study by Galar-Martínez et al. (2010) showed that contaminants present in water and sediment in the Madín Reservoir induced oxidative stress by determination of LPX and activity of the antioxidant enzymes SOD, CAT, and GPx in liver and brain of *C. carpio*. These samples were collected during the warm dry season (May 2007). Therefore, in this study, the impact of pollutants (metal and NSAID concentrations) in the Madín Reservoir was assessed 3 years later, with more biomarkers of oxidative stress and other organs of interest in the common carp, to evaluate the ecosystem deterioration.

The results of the present study aimed to evaluate oxidative stress induced by contaminants (metals and NSAIDs) present in water from Madín Reservoir on gill, blood, and muscle of *C. carpio*.

Materials and Methods

The Study Region

Madín Reservoir is located at 19°31′37″ N and 99°15′33″ W on the Río Tlalnepantla, its dam being downstream from the town of Madín, in the municipalities of Naucalpan de Juárez, Atizapán de Zaragoza, and Tlalnepantla de Baz (State of Mexico; Fig. 1). It has a full capacity of 25 million m^3 but normally stores no more than 13 million m^3 as a precaution against larger flows.

Collection of Water Samples in the Reservoir

Water samples were collected during one sampling event from the cold dry season (February 2013) using the procedure stipulated in the official Mexican norm for wastewater sampling (NMX-AA-003-1980). Sampling was

Fig. 1 Madín Reservoir. The location of SSs is shown. *1* Discharge from the town of Nuevo Madín, *2* entry point of the Río Tlalnepantla tributary, *3* lateral branch, *4* dam, and *5* discharge from the town of Viejo Madín

performed at surface level at five sampling stations (SSs) corresponding to the following: (1) discharge from the town of Nuevo Madín, (2) entry point of the Río Tlalnepantla tributary, (3) lateral branch, (4) dam, and (5) discharge from the town of Viejo Madín (Fig. 1). Plastic bottles were used to collect water. Water samples collected at the SSs were placed in stoppered 20-L polyethylene containers that had been previously washed with nitric acid (Sigma-Aldrich, Toluca, Mexico) 30 % and later with deionized water. Samples were identified, protected from the light, immediately moved to the laboratory, and stored at 4 °C. Chemical and toxicological analyses of water were performed 1 day after sampling. The sampling sites were selected according to Galar-Martínez et al. (2010), because these are considered the most problematic due to the discharges that they received.

Physicochemical Characterization

After sampling, the physicochemical characterization of water exposure at the five SSs was realized. Physicochemical characteristics (temperature, dissolved oxygen, conductivity, pH, chlorides, fluorides, and hardness) were measured as stipulated in the official Mexican norm (NOM-002-SEMARNAT-1996) and American Public Health Association (APHA)/American Water Works Association/Water Pollution Control Federation (1995). This official norm establishes the maximum permissible limits of pollutants in wastewater discharges to sewer systems or municipal urban. The APHA establishes the standard methods for the examination of water and wastewater.

Metal Quantification in Water

Metals [Fe, mercury (Hg), and Al] were quantified per the method proposed by Eaton et al. (1995). Concentrated nitric acid (2 mL) was added to 0.5 mL of sample (water). After digestion for 1 h in an autoclave at 120 °C and 15-lb pressure, samples were filtered and diluted with deionized water, then read on a Varian AA1475 atomic absorption spectrophotometer (Melbourne, Australia). Results were interpolated on an Fe, Hg or Al type curve, an Fe, Hg, or Al atomic absorption standard solution (Sigma-Aldrich, St. Louis, Missouri, USA) was used for each one (1 mg mL^{-1}). The percentage of recovery for all metals ranged between 97 and 100 % (100 % for Fe, 97 % for Hg, and 97 % for Al). The absorption wavelength, detection limit (DL), and quantification limit (QL) were 248.3 nm, 0.0019 ± 0.0003, and 0.007 ± 0.001 mg L^{-1} for Fe, 254.7 nm, 0.0025 ± 0.0004, and 0.008 ± 0.001 mg L^{-1} for Hg, and 309.3 nm, 0.0016 ± 0.0008, and 0.005 ± 0.003 mg L^{-1} for Al, respectively. Metals were quantified in their total forms. Results were expressed as mg L^{-1}.

Calibration Curves of Metals

Calibration curves of each of the metals were performed using standard solutions were prepared at the following concentrations: containing 1, 2, 5, 10, and 25 mg mL^{-1} for Fe, 1, 2, 5, 10, and 50 mg mL^{-1} for Al, and 0.1, 0.5, 1, 2, and 5 mg mL^{-1} for Hg. Linear regression coefficients (R^2) were >0.99 for Al, Hg, and Fe.

DCF, IBP, and NPX Quantification in Water by Liquid Chromatography–Tandem Mass Spectrometry

Standard

Standard solutions were prepared in a 60:40 mix of acetonitrile and ammonium formate at pH 6 (pH was regulated using 1 M of HCl). Standards of concentration of 10 μg mL^{-1} of DCF, IBP, and NPX were prepared. All standards were stored in the dark at −8 °C. Solution of 1,000 μg mL^{-1} was used for mass spectrometry (MS) tuning, a 200 μg mL^{-1} solution for recovery studies, and solutions containing 1, 2, 10, 50, and 250 μg mL^{-1} of NSAIDs for calibrations.

Equipment

The high-performance liquid chromatography (HPLC)–MS/MS system consisted of an Agilent 1290 Infinity HPLC unit (Santa Clara, California, USA). The RRHD Eclipse Plus C18 (2.1 × 50 mm, 1.8 μm) chromatography column

was maintained at 40 °C. The mobile phase was a 60:40 v/v mixture of acetonitrile and ammonium formate (10 mM). Flow rate was 0.3 mL min^{-1}, run time 1.8 min, and injection volume 2 µL. DCF, IBP, and NPX were identified and quantified by means of a mass spectrometer (triple quadrupole 6430; Agilent, Santa Clara, California, USA) fitted with electrospray ionization (ESI). ESI positive mode was used throughout. Electrospray voltage operated at 4,000 V as the MS collected data in the negative ion mode. The retention time, base peak, *m/z*, and fragmentor voltage were as follows: 24.9, $[M–Na]^{-1}$ 294, and 80 V for DCF, 25.6, $[M–H]^{-1}$ 205, and 80 V for IBP, 20.1, $[M–H]^{-1}$ 229, and 70 V for NPX. Mass spectrometer optimization was performed by direct infusion of a µg mL^{-1} standard solution of DCF, IBP, and NPX; thereafter, the ionization mode and precursor ion mode were selected. These pharmaceuticals were selected because they are among the most commonly used drugs in Mexico (Gómez-Olivan et al. 2009).

The method DL (MDL) and method QL (MQL) were defined and determined as the minimum detectable amount of DCF, IBP, and NPX with a signal-to-noise ratio of 3:1 and 10:1, respectively, from SS waters-spiked extract. These data (MDL and MQL) were 30 and 84 ng L^{-1} for DCF, 31 and 86 ng L^{-1} for IBP, and 26 and 72 ng L^{-1} for NPX. Instrumental DLs (IDLs) were determined by direct injection of decreasing amounts of the standard mixture. The IDLs were 27 pg/injected for DCF, 22 pg/injected for IBP, and 26 pg/injected for NPX.

Calibration Curves

Calibration curves of each of the NSAIDs were determined using standard solutions at concentrations of 1, 3, 10, 50, and 250 µg mL^{-1} prepared in 60:40 mix of acetonitrile and ammonium formate at pH 6. Linear regression coefficients (R^2) were >0.99 for DCF, IBP, and NPX. The MS/MS detector was maintained according to the manufacturer's specifications and cleaned regularly, but when changes in the slopes of the calibration curves were observed at >50 %, the detector underwent additional cleaning.

Water

On reception, samples were vacuum-filtered through 1–0 µm glass microfibers filters (GF/C Whatman, UK) followed by 0.45-µm nylon membrane filters (Whatman). Water samples (5 mL) from exposure tanks were collected in glass vials and refrigerated at 4 °C for subsequent test concentration measurements. Results were expressed as time-weighted average concentrations of DCF, IBP, and NPX. A liquid–liquid extraction with 5 mL (1 + 1, v/v) hexane/ethyl acetate was performed to extract DCF, IBP, and NPX from 1-mL water samples. These samples were centrifuged at 1,800 rpm for 10 min, and then the upper organic layer was re-extracted. The extraction was repeated, and organic layers were combined and evaporated to dryness. The water samples of the five SSs were extracted in the same manner.

Specimen Collection and Maintenance

Three-month-old common carp (*C. carpio*) juveniles (18.39 ± 0.31 cm length and 50.71 ± 7.8 g weight) were obtained from the aquaculture facility in Tiacaque, State of Mexico. Fish were safely transported to the laboratory in well-sealed polyethylene bags containing oxygenated water, then stocked in a large tank with dechlorinated tap water (previously reconstituted with salts) and acclimated to test conditions for 30 days before beginning of the experiment. During acclimation, carp were fed Pedregal Silver fish food, and three fourths of the tank water was replaced every 24 h to maintain a healthy environment. The physicochemical characteristics of tap water reconstituted with salts were maintained, i.e., temperature 20 ± 2 °C, oxygen concentration 80–90 %, pH 7.5–8.0, total alkalinity 17.8 ± 7.3 mg L^{-1}, and total hardness 18.7 ± 0.6 mg L^{-1}. A natural light-to-dark photoperiod (12:12) was maintained.

During the aquaculture period fish, were not exposed to any metals or pharmaceuticals. Fe, Hg, and Al concentrations were determined by atomic absorption spectrophotometry and DCF, IBP, and NPX concentrations by LC–MS/MS in pond water from the aquaculture facility. Neither metals (Fe, Hg and Al) nor pharmaceuticals (DCF, IBP, and NPX) were detected in these samples.

Oxidative Stress Determination

Test systems consisted of 120 × 80 × 40-cm glass tanks filled with reconstituted water with the following salts: $NaHCO_3$ (174 mg L^{-1}; Sigma-Aldrich, Toluca, Mexico), $MgSO_4$ (120 mg L^{-1}; Sigma-Aldrich, St. Louis, Missouri, USA), KCl (8 mg L^{-1}; Vetec-Sigma-Aldrich, St. Louis, Missouri, USA), and $CaSO_4{\cdot}2H_2O$ (120 mg L^{-1}; Sigma-Aldrich, Toluca, Mexico) were maintained at room temperature with constant aeration and a natural light-to-dark photoperiod (12:12). Static systems were used, and no food was provided to specimens during the exposure period.

The fish used in sublethal toxicity assays were 4-month-old common carp (*C. carpio*) juveniles (25.6 ± 0.47 cm length and 80.3 ± 6.8 g weight). Sublethal toxicity assays involved adding Madín Reservoir water to five test systems with six carp each. The exposures were performed with full-strength SS waters. One system was used for each SS. The volume of water for each SS system was 40 L.

A reservoir water-free control system with six carp was set up for each SS, and sublethal assays were performed in triplicate. A total of 180 fish were used. The survival of total fish was 100 %. At the end of the exposure period (96 h), fish were removed from the systems and placed in a tank containing 50 mg L^{-1} of clove oil as an anesthetic (Yamanaka et al. 2011). Anesthetized specimens were placed in a lateral position, and blood was removed with a heparinized 1-mL hypodermic syringe by puncture of the caudal vessel performed laterally near the base of the caudal peduncle, at mid-height of the anal fin and ventral to the lateral line.

After puncture, specimens were placed in an ice bath and killed. Gill and muscle of each fish were removed, placed in 1 mL phosphate buffer solution [NaCl (Sigma-Aldrich, Toluca, Mexico) 0.138 M, KCl (Vetec-Sigma-Aldrich, Toluca, Mexico) 0.0027 M, Na_2HPO_4 (Sigma-Aldrich, Toluca, Mexico) 0.01 M, and KH_2PO4 (Sigma-Aldrich, Toluca, Mexico) 0.002 M] pH 7.4 and homogenized. The supernatant was centrifuged at 12,500 rpm and −4 °C for 15 min. Tissue samples were stored at −70 °C before analysis.

Blood samples (150 µL) of each fish were collected in heparinized tubes and placed in 1 mL phosphate buffer solution as described previously and ultrasonicated, then stored at −70 °C before analysis.

The following biomarkers were then evaluated: hydroperoxide content (HPC), LPX, protein carbonyl content (PCC), and activity of the antioxidant enzymes SOD and CAT. All bioassays were performed on the supernatant except LPX.

Determination of HPC

HPC was determined by the ferrous oxidation–xylenol orange method (Jiang et al. 1992). To 100 µL of supernatant (previously deproteinized with 10 % trichloroacetic acid, TCA) was added 900 µL of the reaction mixture [0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange, and 4 mM butyl hydroxytoluene in 90 % (v/v) methanol]; all reagents were obtained from Sigma-Aldrich, St. Louis, Missouri, USA. The mixture was incubated for 60 min at room temperature, and absorbance was read at 560 nm against a blank containing only reaction mixture. Results were interpolated on a cumene hydroperoxide curve and expressed in nM CHP mg^{-1} protein.

Determination of LPX

LPX was determined using the thiobarbituric acid (TBA)-reactive substances method (Büege and Aust 1978). To 100 mL of supernatant Tris–HCl buffer solution (pH 7.4; Sigma-Aldrich, Toluca, Mexico) was added until a 1-mL volume was attained. Samples were incubated at 37 °C for 30 min; 2 mL of TBA–TCA reagent [0.375 % TBA (Fluka-Sigma-Aldrich, Toluca, Mexico) in 15 % TCA (Sigma-Aldrich, Toluca, Mexico)] was added, and samples were shaken in a vortex. They were then heated to boiling for 45 min, allowed to cool, and the precipitate removed by centrifugation at 3,000 rpm for 10 min. Absorbance was read at 535 nm against a reaction blank. Malondialdehyde (MDA) content was calculated using the molar extinction coefficient (MEC) of MDA (1.56×10^5 M cm^{-1}). Results were expressed as mM MDA mg^{-1} protein.

Determination of PCC

PCC was determined using the method of Levine et al. (1994) as modified by Parvez and Raisuddin (2005) and Burcham (2007). To 100 µL of supernatant was added 150 µL of 10 mM DNPH in 2 M HCl, and the resulting solution was incubated at room temperature for 1 h in the dark. Next, 500 µL of 20 % TCA was added, and the solution was allowed to rest for 15 min at 4 °C. The precipitate was centrifuged at 11,000 rpm for 5 min. The bottom was washed several times with 1:1 ethanol and ethyl acetate, then dissolved in 1 mL of 6 M guanidine solution (pH 2.3) and incubated at 37 °C for 30 min. All reagents were obtained from Sigma-Aldrich, St. Louis, Missouri, USA. Absorbance was read at 366 nm. Results were expressed as nM reactive carbonyls formed (C=O) mg^{-1} protein, using the MEC of 21,000 M cm^{-1}.

Determination of SOD Activity

SOD activity was determined by the method of Misra and Fridovich (1972). To 40 µL of supernatant in a 1-cm cuvette was added 260 µL carbonate buffer solution [50 mM sodium carbonate and 0.1 mM ethylene diamine tetraacetic acid (EDTA)], pH 10.2, plus 200 µL adrenaline (30 mM); all reagents from Sigma-Aldrich, St. Louis, Missouri, USA. Absorbance was read at 480 nm after 30 s and 5 min. Enzyme activity was determined using the MEC of SOD (21 M cm^{-1}). Results were expressed as IU mg^{-1} protein.

Determination of CAT Activity

CAT activity was determined by the method of Radi et al. (1991). To 20 mL of supernatant was added 1 mL isolation buffer solution [0.3 M saccharose (Vetec-Sigma-Aldrich, St. Louis, Missouri, USA), 1 mL EDTA (Sigma-Aldrich, Toluca, Mexico), 5 mM HEPES (Sigma-Aldrich, Toluca, Mexico), and 5 mM KH_2PO_4 (Vetec-Sigma-Aldrich, Toluca, Mexico)] plus 0.2 mL of a hydrogen peroxide solution

Table 1 Physicochemical characteristics of water from Madín Reservoir (State of Mexico), Mexico

Physicochemical characteristics	NOM-002-SEMARNAT-1996	SS1	SS2	SS3	SS4	SS5
Temperature (°C)	Maximum 40	18.1	17.2	18.6	17.8	18.6
Dissolved oxygen (mg L^{-1})	NI	5.6	6.3	5.6	5.8	5.3
Conductivity (μs cm^{-1})	NI	143.2	139.7	118.6	123.8	111.6
pH	6.5–8.5	6.3	6.5	6.1	6.4	6.3
Chlorides (mg L^{-1})	Maximum 250	128	132	167	202	186
Fluorides (mg L^{-1})	0–15	3.2	5.1	4.8	3.9	4.1
Hardness (mg L^{-1})	Maximum 500	148.3	153.2	141.9	162.3	128.7

The official Mexican norm (NOM-002-SEMARNAT-1996) establishes the maximum permissible limits of pollutants in wastewater discharges to sewer systems or municipal urban

NI not included

(20 mM; Vetec-Sigma-Aldrich, Toluca, Mexico). Absorbance was read at 240 nm after 0 and 60 s. Results were derived by substituting the absorbance value obtained for each of these times in the following formula—CAT concentration = $(A_0 - A_{60})$/MEC where the MEC of H_2O_2 is 0.043 mM cm^{-1}—and were expressed as μM H_2O_2 mg^{-1} protein.

Statistical Analyses

Results were expressed as the mean ± SEM. After testing for normality (Shapiro–Wilks) and for homogeneity of variances (Bartlett's test), difference among means were determined by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test with a 95 % confidence limit whenever the data difference was significant. Pearson's correlation analysis was used to examine possible associations between heavy metals and NSAIDs in SS water and oxidative stress biomarkers in the different tissues of common carp. Statistical determinations were performed using SPSS v10 software (Chicago, Illinois, USA).

Results

Physicochemical Characterization

The results regarding the physicochemical characterization are listed in Table 1. The physicochemical characteristics at the five SS did not exceed the limits established in the official Mexican norm (NOM-002-SEMARNAT-1996) for the maximum permissible limits of pollutants in wastewater discharges to sewer systems or municipal urban. Dissolved oxygen concentrations ranged from 5.3 to 6.3, and conductivity ranged between 111.6 and 143.2 μs cm^{-1}. In four SS (SS1, SS3, SS$, and SS %), the pH was lower than the Mexican norm.

Quantification of Metals

Regarding the chemical characterization of metals at the different SSs, Fe and Al concentrations at all sites (Table 2) exceed the limits established in the official Mexican norms on the permissible limits of pollutants in wastewater discharges and domestic goods and the permissible levels of heavy metals for aquatic life protection and human consumption (NOM-001-ECOL-1996 and NOM-127-SSA1-1994, respectively). Comparing the concentrations of metals (Fe and Al) and NSAIDs (DCF and IBP) in the five SS showed significant difference ($P < 0.05$; Table 2).

DCF, IBP, and NPX Quantification in Water

Regarding the chemical characterization of NSAIDs in the various SSs, as can be seen in Table 2, DCF, IBP, and NPX concentrations ranged from 0.18 to 4.51 μg L^{-1}.

Determination of HPC

HPC results are shown in Fig. 2. Significant increases with respect to the control group ($P < 0.05$) were observed in gill at SS1 (22.94 %) and in blood at SS1 (101.61 %), SS2 (54.43 %), SS3 (132.55 %), and SS4 (51.34 %), whereas significant decreases occurred in gill at SS5 (29.90 %), in blood at SS5 (27.62 %), and in muscle at SS1 (42.97 %), SS4 (30.96 %), and SS5 (46.69 %).

Determination of LPX

Figure 3 shows LPX results. Significant increases with respect to the control group ($P < 0.05$) were found in gill

Table 2 Metal and NSAID concentrations in water from Madín Reservoir (State of Mexico), Mexico

Pollutants	NOM-001-ECOL-1996[a]	NOM-127-SSA1-1994[b]	SS1	SS2	SS3	SS4	SS5
Fe (mg L^{-1})	0.3	0.3	$1.73 \pm 0.01^{c-e}$	$1.75 \pm 0.01^{c-e}$	$1.51 \pm 0.02^{d-f}$	$1.37 \pm 0.02^{c,e-g}$	$5.10 \pm 0.01^{c,d,f,g}$
Hg (mg L^{-1})	0.01	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Al (mg L^{-1})	0.5	0.2	$6.04 \pm 0.08^{c-e,g}$	$6.70 \pm 0.01^{c-f}$	$6.30 \pm 0.02^{e-g}$	$6.33 \pm 0.02^{e-g}$	$24.45 \pm 0.04^{c,d,f,g}$
DFC (μg L^{-1})	NI	NI	$0.26 \pm 0.02^{c,e}$	0.28 ± 0.02^{c}	$0.20 \pm 0.0^{d-g}$	0.27 ± 0.03^{c}	$0.31 \pm 0.01^{c,f}$
IBP (μg L^{-1})	NI	NI	$4.51 \pm 0.06^{c-e,g}$	$3.61 \pm 0.04^{c-f}$	$3.87 \pm 0.03^{d-g}$	$4.24 \pm 0.02^{c,e-g}$	$3.73 \pm 0.04^{c,d,f,g}$
NPX (μg L^{-})	NI	NI	0.21 ± 0.01	ND	ND	ND	0.18 ± 0.02

Values are the mean of three replicates

ND not detected, *NI* not included

[a] The official Mexican norms on the permissible limits of pollutants in wastewater discharges and domestic goods

[b] The official Mexican norms on the permissible levels of heavy metals for aquatic life protection and human consumption

[c] $P < 0.05$ (significant difference with respect to SS3)

[d] $P < 0.05$ (significant difference with respect to SS4)

[e] $P < 0.05$ (significant difference with respect to SS5)

[f] $P < 0.05$ (significant difference with respect to SS1)

[g] $P < 0.05$ (significant difference with respect to SS2)

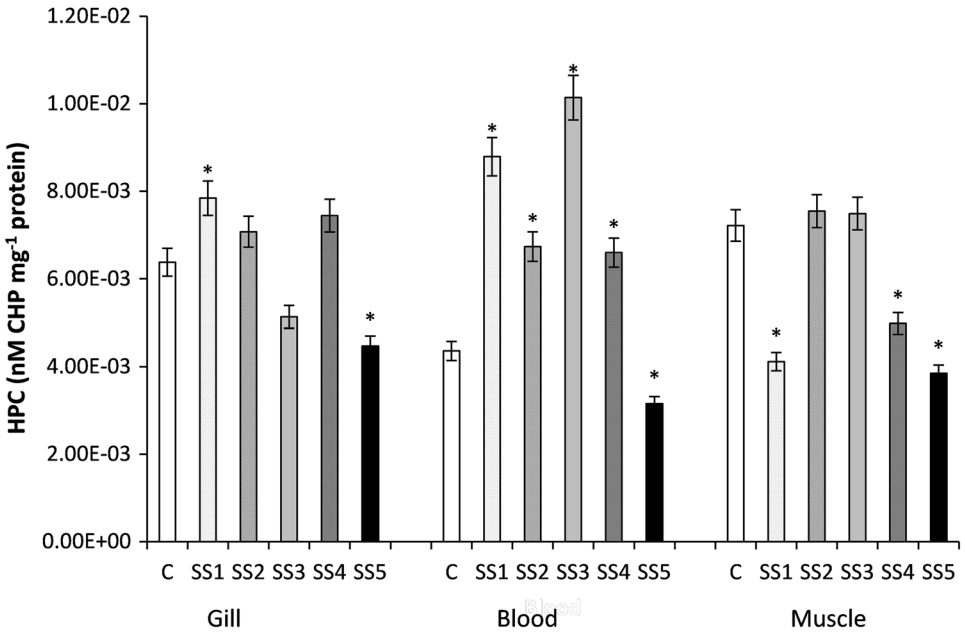

Fig. 2 HPC in gill, blood, and muscle of *C. carpio* exposed to Madín Reservoir water. Values are the mean of three replicates ± SEM. *CHP* cumene hydroperoxide. *Significantly different from control values, ANOVA and Bonferroni ($P < 0.05$)

at SS1 (87.22 %) and SS4 (68.98 %), in blood at SS1 (70.49 %) and SS3 (66.27 %), and in muscle at SS2 (18.76 %), whereas significant decreases were seen in muscle at SS1 (19.58 %) and SS5 (35.36 %).

Determination of PCC

PCC results are shown in Fig. 4. Significant increases with respect to the control group ($P < 0.05$) were observed in blood at SS3 (79.67 %) and in muscle at SS4 (66.28 %), whereas a significant decrease was found in muscle at SS3 (33.36 %).

Determination of SOD Activity

Figure 5 shows SOD activity results. Significant increases with respect to the control group ($P < 0.05$) were found in gill at SS2 (71.54 %) and in blood at SS1 (92.84 %), whereas significant decreases occurred in gill at SS1 (57.99 %) and in muscle at SS1 (22.66 %) and SS5 (45.77 %).

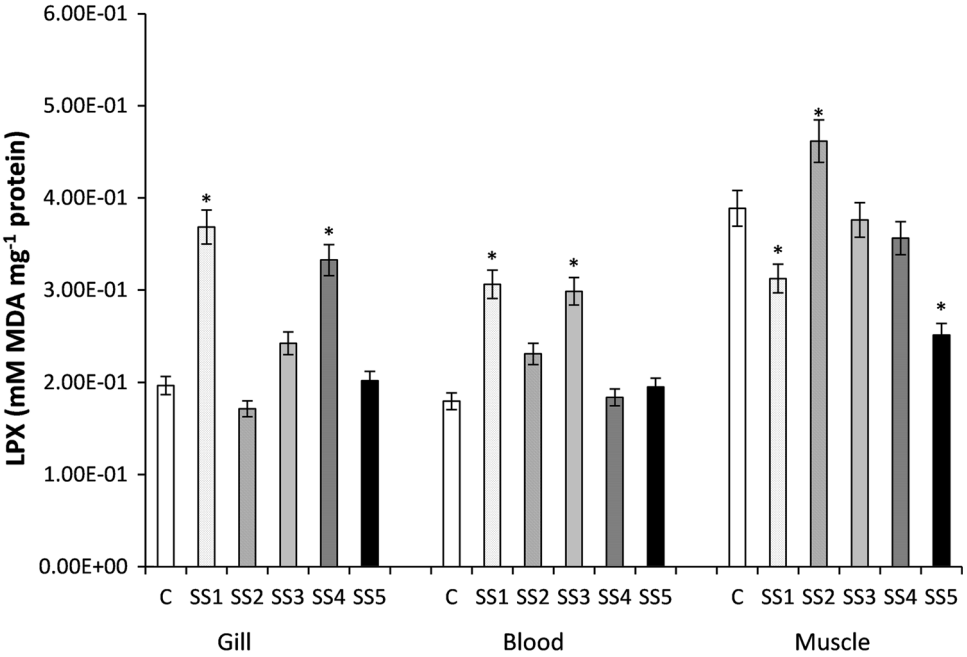

Fig. 3 LPX in gill, blood, and muscle of *C. carpio* exposed to Madín Reservoir water. Values are the mean of three replicates ± SEM. *Significantly different from control values, ANOVA and Bonferroni ($P < 0.05$)

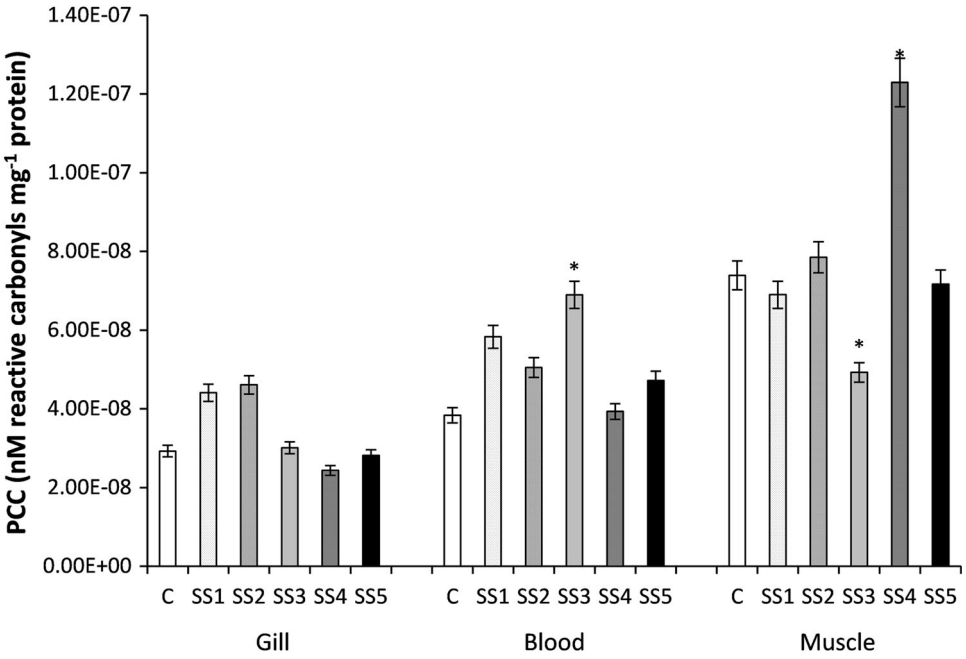

Fig. 4 PCC in gill, blood, and muscle of *C. carpio* exposed to Madín Reservoir water. Values are the mean of three replicates ± SEM. *Significantly different from control values, ANOVA and Bonferroni ($P < 0.05$)

Determination of CAT Activity

CAT activity results are shown in Fig. 6. Significant increases with respect to the control group ($P < 0.05$) were observed in gill at SS1 (386.81 %), SS2 (195.80 %), SS4 (961.10 %), and SS5 (287.55 %), in blood at SS1 (198.69 %), SS2 (148.67 %), and SS5 (111.56 %), and in muscle at SS3 (35.46 %). Significant decreases in this activity occurred in muscle at SS1 (65.29 %) and SS4 (61.16 %).

Table 3 lists the results of the correlation of biomarkers of oxidative stress and concentrations of metal and NSAIDs. As shown, there are close correlations between contaminants in the five SS and biomarkers evaluated.

Discussion

Most of the water-quality conditions measured in this study (Table 1) appeared to be adequate for supporting common

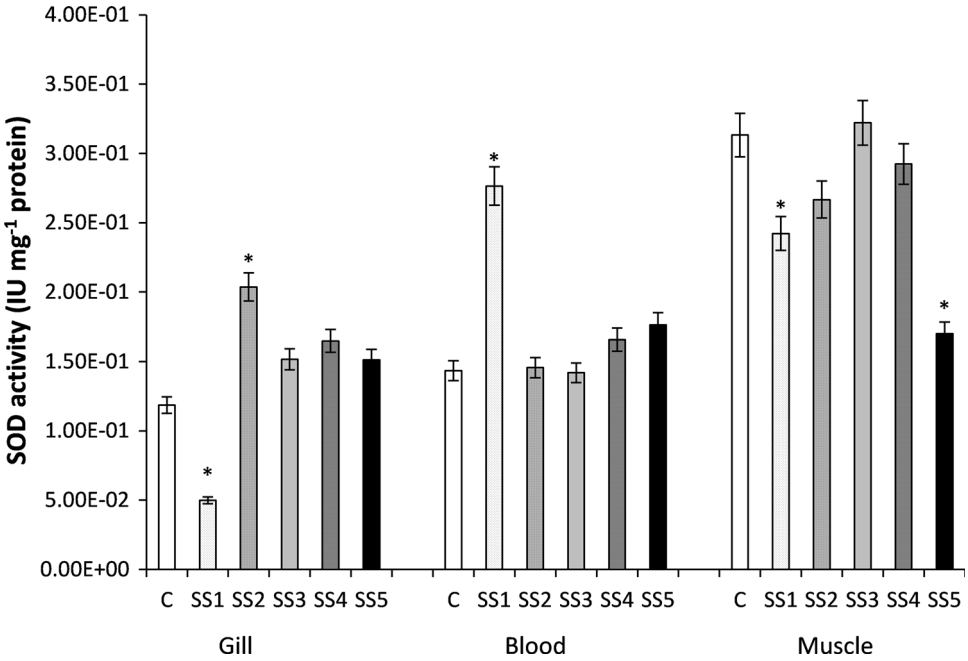

Fig. 5 SOD activity in gill, blood, and muscle of *C. carpio* exposed to Madín Reservoir water. Values are the mean of three replicates ± SEM. *Significantly different from control values, ANOVA and Bonferroni ($P < 0.05$)

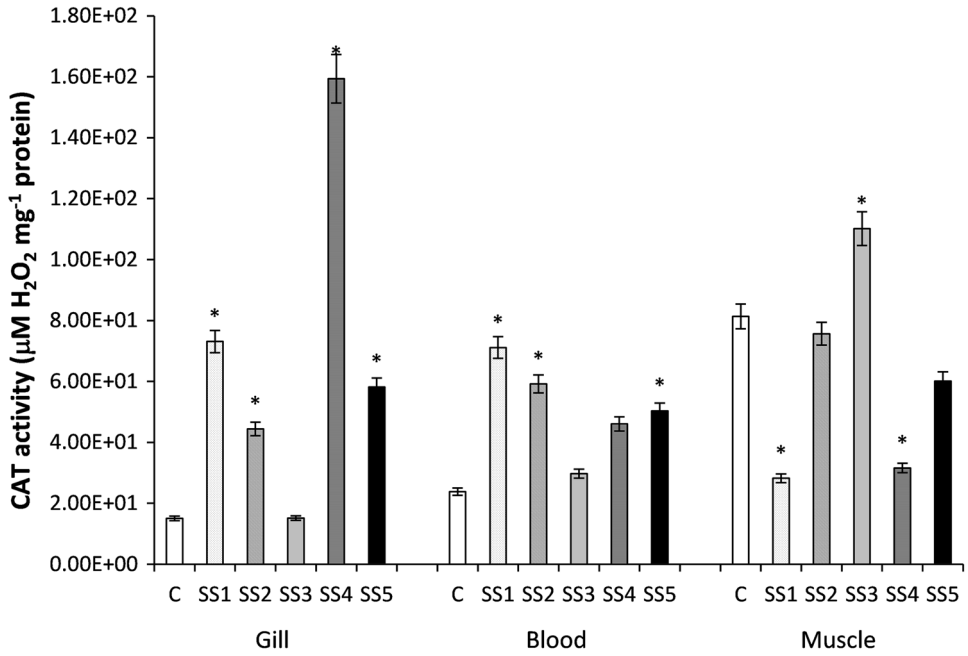

Fig. 6 CAT activity in gill, blood, and muscle of *C. carpio* exposed to Madín Reservoir water. Values are the mean of three replicates ± SEM. *Significantly different from control values, ANOVA and Bonferroni ($P < 0.05$)

carp culture. Distribution and transport of metals in water depends on the species of these metals present in the water column as well as on environmental characteristics. The metals are most soluble to pH <6.5 (Coz et al. 2004). The pH of the five SS was <6.5; thus Al and Fe were in their soluble forms being available for uptake by *C. carpio*.

In contrast, molecular biomarkers are used to test for oxidative damage induced on macromolecules by ROS and reactive nitrogen species (RNS; Valavanidis et al. 2006). These species are essential for cell function in body systems and are constantly produced in cells (Halliwell and Gutteridge 1999). During LPX, polyunsaturated fatty acids with double bonds react with ROS, particularly the hydroxyl radical ($HO^{\bullet}$) and the RNS peroxynitrite ($ONOO^{-}$), through a chain reaction mechanism. This permits the formation of hydroperoxides that are degraded to low molecular-weight products, including MDA (Wilhelm Filho et al. 2005). As can be seen in Fig. 2, an increase in HPC occurred in gill (SS1) as well as blood (SS1–4), with blood displaying the highest value for this

Table 3 Pearson's correlation among pollutant concentrations and oxidative stress biomarkers in gill, blood, and muscle of *C. carpio*

Biomarkers	Tissues	SS	Pollutants				
			Fe	Al	DCF	IBP	NPX
HPC	Gill	SS1	0.030	−0.030	−0.030	−0.030	0.159
		SS2	**0.897**	**0.897**	**0.897**	**0.858**	ND
		SS3	0.401	−0.401	−0.401	−0.324	ND
		SS4	**−0.515**	**−0.515**	**−0.515**	**−0.515**	ND
		SS5	**−0.940**	**0.940**	**0.940**	**0.940**	**0.940**
	Blood	SS1	**−0.989**	**0.989**	**0.989**	**0.989**	**0.999**
		SS2	**0.827**	**0.827**	**0.827**	**0.778**	ND
		SS3	**0.937**	**−0.937**	**−0.937**	**−0.962**	ND
		SS4	**0.629**	**0.629**	**0.629**	**0.629**	ND
		SS5	−0.265	0.265	0.265	0.265	0.265
	Muscle	SS1	−0.370	0.370	0.370	0.370	**0.539**
		SS2	0.242	0.242	0.242	0.321	ND
		SS3	**−0.999**	**0.999**	**0.999**	**0.999**	ND
		SS4	**0.909**	**0.909**	**0.909**	**0.909**	ND
		SS5	**−0.893**	**0.893**	**0.893**	**0.893**	**0.893**
LPX	Gill	SS1	**−0.965**	**0.965**	**0.965**	**0.965**	**0.897**
		SS2	−0.358	−0.358	−0.358	−0.280	ND
		SS3	0.004	−0.004	−0.004	−0.086	ND
		SS4	−0.247	−0.247	−0.247	−0.247	ND
		SS5	**0.985**	**−0.985**	**−0.985**	**−0.985**	**−0.985**
	Blood	SS1	**−0.816**	**0.816**	**0.816**	**0.816**	**0.692**
		SS2	**−0.999**	**−0.999**	**−0.999**	**−0.993**	ND
		SS3	**0.999**	**−0.999**	**−0.999**	**−0.999**	ND
		SS4	**0.811**	**0.811**	**0.811**	**0.811**	ND
		SS5	**0.875**	**−0.875**	**−0.875**	**−0.875**	**–0.875**
	Muscle	SS1	0.011	−0.011	−0.011	−0.011	0.178
		SS2	**0.975**	**0.975**	**0.975**	**0.990**	ND
		SS3	**0.505**	**−0.505**	**−0.505**	−0.433	ND
		SS4	**0.661**	**0.661**	**0.661**	**0.661**	ND
		SS5	**−0.911**	**0.911**	**0.911**	**0.911**	**0.911**
PCC	Gill	SS1	**−0.704**	**0.704**	**0.704**	**0.704**	**0.557**
		SS2	**0.935**	**0.935**	**0.935**	**0.903**	ND
		SS3	**0.904**	**−0.904**	**−0.904**	**−0.866**	ND
		SS4	−0.151	−0.151	−0.151	−0.151	ND
		SS5	**0.905**	**−0.905**	**−0.905**	**−0.905**	**−0.905**
	Blood	SS1	**−0.837**	**0.837**	**0.837**	**0.837**	**0.718**
		SS2	**−0.934**	**−0.934**	**−0.934**	**−0.901**	ND
		SS3	**0.998**	**−0.998**	**−0.998**	**−1.000**	ND
		SS4	−0.449	−0.449	−0.449	−0.449	ND
		SS5	**−0.636**	**0.636**	**0.636**	**0.636**	**0.636**
	Muscle	SS1	**−0.944**	**0.944**	**0.944**	**0.944**	**0.989**
		SS2	0.142	0.142	0.142	0.223	ND
		SS3	**0.996**	**−0.996**	**−0.996**	**−1.000**	ND
		SS4	0.038	0.038	0.038	0.038	ND
		SS5	0.378	−0.378	−0.378	−0.378	−0.378

Table 3 continued

Biomarkers	Tissues	SS	Pollutants				
			Fe	Al	DCF	IBP	NPX
SOD	Gill	SS1	**0.650**	**−0.650**	**−0.650**	**−0.650**	−0.495
		SS2	−0.325	−0.325	−0.325	−0.246	ND
		SS3	**0.663**	**−0.663**	**−0.663**	**−0.599**	ND
		SS4	**0.957**	**0.957**	**0.957**	**0.957**	ND
		SS5	−0.371	0.371	0.371	0.371	0.371
	Blood	SS1	**−0.850**	**0.850**	**0.850**	**0.850**	**0.735**
		SS2	**0.585**	**0.585**	**0.585**	**0.516**	ND
		SS3	0.216	−0.216	−0.216	−0.135	ND
		SS4	**0.844**	**0.844**	**0.844**	**0.844**	ND
		SS5	**−1.000**	**1.000**	**1.000**	**1.000**	**1.000**
	Muscle	SS1	0.414	−0.414	−0.414	−0.414	−0.234
		SS2	**−0.987**	**−0.987**	**−0.987**	**−0.970**	ND
		SS3	**0.866**	**−0.866**	**−0.866**	**−0.822**	ND
		SS4	0.006	0.006	0.006	0.006	ND
		SS5	0.292	−0.292	−0.292	−0.292	−0.292
CAT	Gill	SS1	**0.874**	**−0.874**	**−0.874**	**−0.874**	**−0.950**
		SS2	**−0.648**	**−0.648**	**−0.648**	**−0.583**	ND
		SS3	**−0.574**	**0.574**	**0.574**	**0.639**	ND
		SS4	**0.522**	**0.522**	**0.522**	**0.522**	ND
		SS5	**−0.713**	**0.713**	**0.713**	**0.713**	**0.713**
	Blood	SS1	**−0.554**	**0.554**	**0.554**	**0.554**	**0.701**
		SS2	**−0.666**	**−0.666**	**−0.666**	**−0.725**	ND
		SS3	0.067	−0.067	−0.067	−0.148	ND
		SS4	**0.552**	**0.552**	**0.552**	**0.552**	ND
		SS5	**−0.963**	**0.963**	**0.963**	**0.963**	**0.963**
	Muscle	SS1	**0.511**	**−0.511**	**−0.511**	**−0.511**	**−0.664**
		SS2	0.188	0.188	0.188	0.268	ND
		SS3	**0.998**	**−0.998**	**−0.998**	**−1.000**	ND
		SS4	**−0.924**	**−0.924**	**−0.924**	**−0.924**	ND
		SS5	**0.768**	**−0.768**	**−0.768**	**−0.768**	**−0.768**

Correlations coefficients are significant when they are greater than 0.5 (bold coefficients)

ND not detected

biomarker. Furthermore, the level of damage to lipids is shown in Fig. 3, where increased LPX is evident in gill (SS1 and -4), blood (SS1 and -3), and muscle (SS2). These results may be explained by the presence of NSAIDs (DCF, IBP, and NPX) and their degradation or biotransformation metabolites at all five SSs.

NSAIDs undergo both abiotic transformations by photodegradation and biotic transformations by cytochrome *P450* (CYP)-mediated biotransformation, which in many cases result in the formation of metabolites that are more toxic than the original pharmaceutical agent (Lam et al. 2004).

The main products of the photodegradation of DCF include 5,4-dihydroxy-DCF, 3-hydroxy-DCF, 4-hydroxymethyl-DCF, 3-hydroxy 4-hydroxymethyl-DCF, 4-hydroxy-DCF, and 5-hydroxy-DCF (Deng et al. 2003; Stülten et al. 2008), whereas those of IBP and NPX are 4-isobutyl acetophenone 1-(6-methoxy-2-naphthyl) ethanol, and 2-acetyl-6-methoxy naphthalene (Miranda et al. 1991).

The main metabolites of biotransformation in mammals and in some fishes are, in the case of NPX, 6-*O*-desmethyl-NPX, NPX-acyl glucuronide, and *O*-desmethyl-NPX acyl glucuronide (Vree et al. 1992); in that of DCF they are DCF-1-*O*-acyl glucuronide, 4-hydroxy-DCF, 5-hydroxy-DCF, 5-hydroxy-DCF *p*-benzoquinoneimine, and 4-hydroxy-DCF *p*-benzoquinoneimine (Grillo et al. 2003; Stülten et al. 2008; Islas-Flores et al. 2013); and in the case of IBP they are carboxy-IBP, 3-carboxy-IBP, *p*-carboxy-2-propionate, hydroxy-

IBP, and carboxy hydratropic acid (Zwiener et al. 2002; Graham and Williams 2004).

Benzoquinones formed in NSAID photodegradation and biotransformation are highly electrophilic molecules with a high affinity for binding to lipids, proteins, and DNA (Baillie 2006; Wilhelm et al. 2009) and altering the function of these macromolecules. They are also persistent in the aquatic environment and highly toxic to organisms living therein (Oviedo-Gómez et al. 2010).

NSAIDs toxicity is mediated by ROS formation as a result of biotransformation of these compounds through redox cycling (Ahmad et al. 2000; Abdollahi et al. 2004). CYP is known to produce an oxygenated intermediate—the oxy-CYP complex [*P450* (Fe^{3+}) $O_2^{-\bullet}$]—during the biotransformation of NSAIDs, such as DCF, IBP and NPX, with subsequent release of the superoxide anion by reaction decoupling (Doi et al. 2002). In the present study, increased production of the superoxide anion radical is likely responsible for increases in LPX and HPC in carp exposed to water from the various SSs.

In contrast, the increases in LPX and HPC observed in Figs. 2 and 3 may be due to the fact that metal concentrations exceed those established by official Mexican norms on permissible levels of heavy metals for aquatic life protection and human consumption (NOM-001-ECOL-1996 and NOM-127-SSA1-1994, respectively). Fe catalyzes the reaction of the superoxide anion with hydrogen peroxide to produce hydroxyl radicals by way of the Fenton reaction (Winston and Di Giulio 1991).

Different mechanisms have been proposed as the means through which Al induces ROS formation, e.g., Al displaces the Fe present in various biomolecules, thereby increasing intracellular Fe content and promoting the Fenton reaction (Amador et al. 2001; Dua and Gill 2001; Yousef 2004). This metal is also able to damage the mitochondrion directly and affect electron transport in the respiratory chain (García-Medina et al. 2010). In both cases, ROS production is increased, which explains the increases in LPX and HPC in our study.

Formation of carbonyl groups results from the direct oxidation of amino acid side chains by metals or ROS as well as from protein modification by oxidation-derived secondary products, such as LPX products (Pantke et al. 1999; Grune 2000; Requena et al. 2003). As can be seen in Fig. 4, PCC increased in blood (SS3) and muscle (SS4).

Asensio et al. (2007) reported that NSAID-induced inhibition of glucose-6-phosphate dehydrogenase elicits damage through the oxidation of protein thiols. This may not be the only process of protein carbonyl production: formation of free radicals during the biotransformation of these pharmaceuticals may be one of the sources of oxidation of this biomolecule.

Ingested NSAIDs (DCF, IBP, and NPX) are known to enter in contact with the vasculature where they acetylate the enzyme COX-2 present in endothelium or circulating leukocytes to produce 15-epi-lipoxin A4, which promotes NO synthesis mediated by endothelial and inducible NO synthase (Paul-Clark et al. 2004). When the superoxide anion and NO bind, they may form an RNS (peroxynitrite) through a diffusion-limited reaction (Huie and Padmaja 1993). The oxidant peroxynitrite is known to induce protein oxidation and nitration in the absence of GSH, thus eliciting mitochondrial dysfunction and eventually leading to irreversible damage and severe loss of cellular adenosine triphosphate (Jaeschke et al. 2003).

Levels of protein carbonylation were significantly greater in our study indicating that fish sustained oxidative stress as a result of exposure to metals. Metals are known to directly induce the formation of protein carbonyls by way of metal-catalyzed oxidation reactions (Stadtman and Oliver 1991).

ROS and RNS can remove protons from methylene groups in amino acids leading to the formation of carbonyls that tend to ligate protein amines and also induce damage to nucleophilic centers, sulfhydryl group oxidation, disulfide decrease, peptide fragmentation, modification of prosthetic groups, and protein nitration. These modifications lead to a loss of protein function (Cabiscol et al. 2000; van der Oost et al. 2003; Glusczak et al. 2007) and therefore also of body integrity (Parvez and Raisuddin 2005).

Blood is susceptible of oxidative damage because, in addition to fulfilling diverse functions, such as the transport of proteins (mostly albumin and hemoglobin) and other biomolecules to all body tissues, it also transports xenobiotics throughout the body (Eaton 2006; Halliwell 2006). The gills are likewise known to effect the oxidative metabolism of many toxic agents, thereby promoting production of the ROS responsible for LPX in the present study (Islas-Flores et al. 2013).

Diverse environmental contaminants can induce antioxidant defenses (Vlahogianni et al. 2007). SOD is the first mechanism of antioxidant defense and the main enzyme responsible for offsetting the effects of ROS, particularly the superoxide ion (van der Oost et al. 2003), which is converted to hydrogen peroxide by SOD with H_2O_2 being subsequently sequestered and degraded to H_2O by CAT and GPx. Increases in SOD activity in gill (SS2) and blood (SS1; Fig. 5) were induced by release of the superoxide anion radical (Livingstone 2003).

Because NSAIDs (including DCF, IBP, and NPX) affect the mitochondrion and consequently oxidative phosphorylation, increased ROS production, particularly of $O_2^{-\bullet}$, may occur. As a result, there is an increase in SOD activity and hydrogen peroxide levels (Asensio et al. 2007; Salgueiro-Pagadigorria et al. 2004) as seen in our study with exposure of *C. carpio* to these compounds.

Valko et al. (2005) mentioned that increased SOD activity may be a means to compensate the effect of some xenobiotics, such as heavy metals, i.e., an adaptive response of the organism to induced stress. In contrast, Guecheva et al. (2001) observed similar effects in planaria exposed to Cu and attributed this increase to de novo protein synthesis as a potential adaptive mechanism.

As stated previously, SOD activity increased in the present study in *C. carpio* exposed to metals and NSAIDs, the main contaminants in Madín Reservoir, thus leading to increased hydrogen peroxide formation, which may act as a signal for CAT bioactivation to convert this highly toxic free radical to less toxic compounds. Similar responses have been found in different aquatic organisms exposed to other NSAIDs (Oviedo-Gómez et al. 2010; Gómez-Oliván et al. 2012; Islas-Flores et al. 2013).

Various investigators state that enzymes such as CAT are activated in blood cells by in vitro exposure to Al (Kiss and Hollósi 2001). In addition, exposure of the freshwater fish matrinxã (*Brycon amazonicus*) to sublethal concentrations of $HgCl_2$ for 96 h in a static system resulted in increases activity of SOD, CAT, GPx, GST, and glutathione reductase (Monteiro et al. 2010).

Figure 6 shows that CAT activity increased in gill (SS1, SS2, SS4, and SS5), blood (SS1, SS2, and SS5), and muscle (SS3). These increases can be attributed to the antioxidant capacity of organisms to offset H_2O_2-induced oxidative damage. In contrast, a decrease was observed in CAT activity in muscle of common carp exposed to SS1 and -4 water. Similar results were found by Gómez-Oliván et al. (2014) in *Daphnia magna* exposed to NPX. This drug is present in SS1. This result indicates this enzyme is unable to offset ROS-induced damage.

Bagnyukova et al. (2006) stated that LPX products appear to be involved in the upregulation of several antioxidant enzymes such as SOD, CAT, and GPx. Thus, LPX increases in the present study may also explain the observed increased activity of antioxidant enzymes.

Similar results to those of this study were found by Galar-Martínez et al. (2010). They observed a significant increase in the degree of LPX from SS1, SS3–5 in liver and brain. SOD was increased in liver for SS2 and -3. We observed an increase in CAT from the five SSs in liver and from SS4 in brain. Regarding SS3–5, increases in GPx were observed in liver and brain. This shows that the measures employed to improve the Madín Reservoir have not been very effective.

Conclusion

In conclusion, all five SSs in Madín Reservoir are contaminated with metals and NSAIDs as well as with other possible pollutants that induce oxidative stress on gill, blood, and muscle of *C. carpio*. However, the changes found in the biomarkers used in the present study exhibit differences among SSs and among tissues. The results show that Madín Reservoir is impacted by the presence of various contaminants and that this poses a risk to the animals that inhabit this reservoir.

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