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Oxidative stress induced in *Hyalella azteca* by an effluent from a NSAID-manufacturing plant in Mexico

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Abstract Production in the pharmaceutical industry has increased and along with it, the amount of wastewater of various characteristics and contaminant concentrations. The main chemicals in these effluents are solvents, detergents, disinfectants—such as sodium hypochlorite (NaClO)—and pharmaceutical products, all of which are potentially ecotoxic. Therefore, this study aimed to evaluate the oxidative stress induced in the amphipod *Hyalella azteca* by the effluent from a nonsteroidal anti-inflammatory drug (NSAID)-manufacturing plant. The median lethal concentration (72 h-LC_{50}) was determined and *H. azteca* were exposed to the lowest observed adverse effect level (0.0732 %) for 12, 24, 48 and 72 h, and biomarkers of oxidative stress were evaluated [hydroperoxide content (HPC), lipid peroxidation (LPX), protein carbonyl content (PCC), and the activity of the superoxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)]. Statistically significant increases with respect to the control group ($P < 0.05$) were observed in HPC, LPX and PCC in *H. azteca* at all exposure times. Antioxidant enzymes activity SOD, CAT and GPx activity also increased significantly ($P < 0.05$) with respect to the control group. In conclusion, the industrial effluent analyzed in the present study contains NSAIDs and NaClO, and induces oxidative stress in *H. azteca.*

Keywords *Hyalella azteca* · Oxidative stress · Nonsteroidal anti-inflammatory drugs · NaClO · Amphipod

Introduction

Industrial effluents are mixtures of toxic substances and therefore a main source of direct and continuous input of pollutants into aquatic ecosystems. For this reason, the study of the effects of effluent exposure on organisms, populations or communities has high ecological relevance (Silva et al. 2002; Backhaus et al. 2003; Smolders et al. 2004).

Approximately 3000 compounds are used as medicine, and the annual production amount exceeds hundreds of tons (Sarmah et al. 2006; Calisto and Esteves 2009). Once administered, pharmaceuticals are excreted as the parent compound or active metabolites, and can reach the environment at different extents (Zuccato et al. 2005). These products can also enter the water bodies as a result of the disposal of unused or expired medications (Stackelberg et al. 2004). Water treatment plants, hospitals and the pharmaceutical industry are the principal sources of contamination by pharmaceutical agents (Nikolaou et al. 2007).

Production in the pharmaceutical industry has increased and along with it, the amount of wastewater discharges of various characteristics and contaminant concentrations,

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which depend on the nature of the production process and year season. The pharmaceutical industry effluents are mainly produced during the machinery cleaning stage (Balcıoğlu and Ötker 2003). In this process, other compounds such as detergents, solvents and disinfectants, are incorporated to the effluent. Current wastewater treatment systems are not sufficiently effective in reducing and/or removing these contaminants (Petrovic et al. 2009; Radjenović et al. 2009). Thus, nowadays pharmaceutical agents are worldwide of environmental concern, and have been termed "emerging contaminants" (Fent et al. 2006; Richardson 2009).

Such emerging contaminants include nonsteroidal anti-inflammatory drug (NSAIDs), which are one of the most commonly used groups of pharmaceuticals (Gagné et al. 2006; Takagi et al. 2006; Morera et al. 2007). NSAIDs drugs are widely used in humans to prevent, cure, or mitigate diseases. These compounds have been designed to elicit a specific biological action in the body and often resist inactivation prior to inducement of their intended therapeutic effect. These properties being paradoxically responsible for both their bioaccumulation and toxic effect on hydrobionts (Santos et al. 2010).

NSAIDs have diverse antiinflammatory, analgesic and antipyretic properties (Gonzalez-Rey and Bebianno 2011). Chemically, they are a heterogeneous group and are not closely related in terms of structure, although they share diverse therapeutic actions and adverse effects (Hardman et al. 2003). Their mechanism of action is through inhibition of the cyclooxygenase (COX) enzymes: COX-1 (constitutive) and COX-2 (inducible) which convert arachidonic acid to prostaglandins and thromboxanes, mediators involved in diverse homeostatic processes through the body (Hardman et al. 2003; Parolini et al. 2009). The most common members of this group of pharmaceuticals in terms of consumption and biological action are naproxen (NPX), paracetamol (PAR), diclofenac (DCF), ibuprofen (IBP) and acetylsalicylic acid (ASA; Katzung 2007). In Mexico, NSAIDs are among the most frequently sold and used medicinal remedies (Gómez-Oliván et al. 2009). They are marketed in diverse pharmaceutical forms and are immoderately used since they can be obtained without prescription. Environmentally, the importance of NSAIDs lies in their inherent properties of persistence, bioaccumulative nature, water solubility, low volatility and low tendency for adsorption by organic matter, which enables them to remain in the aquatic environment for extended periods favoring their uptake and bioconcentration by hydrobionts (Bendz et al. 2005; Carlsson et al. 2006).

Many investigations have been conducted to understand the occurrence and fate of NSAIDs in wastewater. IBP and DCF have been worldwide detected in water bodies at concentrations ranging from ng L^{-1} to μg L^{-1} (Ferrari et al. 2003; Santos et al. 2010). In Mexico, several studies have reported their presence in effluents and water systems. Siemens et al. (2008) found IBP and DCF at concentrations of 0.12–2.30 μg L^{-1} in Mexico City effluent in the Mezquital Valley; Gibson et al. (2010) detected 0.742–4.824 μg L^{-1} in wastewater from the Tula Valley; while Félix-Cañedo et al. (2013) recorded 0.025–0.1 μg L^{-1} in surface water and 0.001–0.005 μg L^{-1} in ground water in tributaries of the Lerma-Cutzamala system, one of the largest water supply networks in Latin America. A study conducted in Madín Reservoir in State of México, showed that water contained a significant NSAIDs loading, with DCF, IBP, and NPX concentration in the order of μg L^{-1} (González-González et al. 2014).

Recent studies have shown that NSAIDs induce reactive oxygen species (ROS) production in cells (Ruas et al. 2008) and elicit and/or contribute to oxidative stress generation, and consequently, DNA damage (Gómez-Oliván et al. 2012, 2013; Islas-Flores et al. 2013; San Juan-Reyes et al. 2013; Gómez-Oliván et al. 2014a). Also, NSAIDs induce both geno- and cyto-toxicity on aquatic organisms such as *Oryzias latipes*, *Dreissena polymorpha*, *Ruditapes philippinarum* and *Daphnia magna* (Hong et al. 2007; Parolini et al. 2010; Matozzo et al. 2012; Gómez-Oliván et al. 2013), affect reproduction and development in fish, and induce gill alterations as well as hepatotoxicity and nephrotoxicity (Schwaiger et al. 2004; Hoeger et al. 2005; Mehinto et al. 2010).

Toxic response can be demonstrated with the use of an adequate selection of biomarkers. A biomarker is defined as any quantifiable change in biological, physical or chemical response which can be related to exposure to or toxicity induced by environmental chemicals (van der Oost et al. 2003). Oxidative stress biomarkers are becoming increasingly important in the field of ecotoxicology. It has been suggested that they could also be used in environmental monitoring programs (Pandey et al. 2003).

Oxidative stress is defined as disruption of the balance between ROS and the antioxidant systems in the body (Barata et al. 2005). ROS, such as hydrogen peroxide (H_2O_2), the superoxide anion ($O_2{\cdot}^-$) and the hydroxyl radical (HO·), 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 (Halliwell and Chirico 1993). To mitigate the negative effects of ROS, fish and other vertebrates possess an antioxidant defense system that uses both enzymatic and non-enzymatic mechanisms. The most important antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST; Gutteridge 1995). Antioxidants protect the body against oxyradical-induced damage such as breaks in the DNA chain, protein oxidation and LPX

induction (Winzer et al. 2000). A change towards 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).

Current knowledge of the fact that this oxidative damage is induced on aquatic organisms has stimulated the study of the effects of pharmaceutical agents on sentinel organisms (Valavanidis et al. 2006). *Hyalella azteca* is a sentinel organism that is widely used in ecotoxicology laboratories for the asssessment of chemical risk to aquatic environments because of the many advantages it offers, such as the widespread distribution and common occurrence in association with freshwater environments, together with its ecological importance, ease of culturing and handling during testing, rapid growth, short life cycle, sensitivity to diverse xenobiotics in water. *H. azteca* has been used in water-only acute and sublethal toxicity tests with various chemicals (FDA 1987; Borgmann et al. 1989, 1990, 1991, 2005, 2007; Schubauer-Berigan et al. 1993; Phipps et al. 1995). Biological endpoints for these tests have included survival, growth, and reproductive success in partial or full life-cycle studies (ISO 2011).

The present study aimed to evaluate the oxidative stress induced in *H. azteca* by an effluent from an NSAID-manufacturing plant.

Materials and methods

Sampling of industrial effluent

Effluent emanating directly from an NSAID-manufacturing plant in Toluca (State of Mexico) was sampled as stipulated in the official Mexican norm on wastewater sampling (NMX-AA-003-1980). Samples taken from the outlet draining the production area, which is directly connected to the drainpipe exiting plant, were collected in stoppered 20-L polyethylene containers previously washed with 30 % nitric acid (Sigma-Aldrich, Toluca, Mexico) and rinsed with deionized water. Samples were labeled, protected from light, and immediately transported to the lab, where they were stored at 4 °C. It is worth noting that industrial waste receives no treatment and goes directly to Lerma river. Then deleterious effects such as oxidative stress, genotoxicity and cytotoxicity are expected. These effects have been demonstrated in *Cyprinus carpio* (San Juan-Reyes et al. 2013, 2015).

Physicochemical characterization

The physicochemical characteristics of effluent samples [temperature, dissolved oxygen, conductivity, pH, chlorides, fluorides, hardness, ammonia, total suspended solids, total P, total N, biochemical oxygen demand, and sodium hypochlorite (NaClO)] were determined as stipulated in the official Mexican norms NOM-001-SEMARNAT-1996, NOM-073-ECOL-1994 and APHA, AWWA, WPCF 1995. The official Mexican norms set the maximum permissible levels of contaminants in wastewater discharges arising in the pharmaceutical and pharmacochemical industries and entering, respectively domestic waters and resources, and receiving water bodies (Table 1); while APHA, AWWA, WPCF 1995 set the standard methods for the examination of water and wastewater.

Quantification of NSAIDs by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Standards

Standard solutions were prepared in a 60:40 mixture of acetonitrile and ammonium formate at pH 6 (pH was regulated using 1 M HCl). Standards of 10 μg mL^{-1} of DCF, IBP, NPX and PAR were prepared and stored in the dark at −8 °C. A 1000 μg mL^{-1} solution was used for mass spectrometer (MS) tuning, a 200 μg mL^{-1} solution for recovery studies, and solutions containing 1, 2, 10, 50, and 250 μg mL^{-1} of the above NSAIDs for instrument calibration.

Equipment

The high-performance liquid chromatography (HPLC)-MS/MS system used was an Agilent 1290 Infinity HPLC unit (Santa Clara, CA). The RRHD Eclipse Plus C18 chromatography column (2.1 × 50 mm, 1.8-μm) 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, NPX and PAR were quantified on an Agilent 6430 Triple Quadrupole MS equipped with electrospray ionization (ESI). The ESI positive mode was used throughout. Electrospray voltage operated at 4000 V as the MS collected data in the negative ion mode. MS optimization was performed by direct infusion of a 10 μg mL^{-1} standard solution of DCF, IBP, NPX and PAR; thereafter, the ionization mode and precursor ion mode were selected. The retention time, base peak, m/z, and fragmentor voltage were as follows, 24.9, $[M-Na]^{-1}$ 294, and 80 V for DCF analysis; 25.6, $[M-H]^{-1}$ 205, and 80 V for IBP, 20.1, $[M-H]^{-1}$ 229, and 70 V during NPX analysis. In PAR case, MS data were collected in the positive ion mode and the retention time, base peak, m/z, and collision voltage were 23.2, $[M+H]^{+1}$

Table 1 Physicochemical characteristics of the industrial effluent analyzed

Physicochemical characteristics	NOM-001-SEMARNAT-1996[a]	NOM-073-ECOL-1994[b]	Industrial effluent
Temperature (°C)	40	40	15.6
Dissolved oxygen (mg L^{-1})	NI	NI	12.2
Conductivity (μS cm^{-1})	NI	NI	143.2
pH	6.5-8.5	6-9	6.3
Chlorides (mg L^{-1})	Maximum 250	NI	101
Fluorides (mg L^{-1})	0-15	NI	3.8
Hardness (mg L^{-1})	Maximum 500	NI	245.7
Ammonia (mg L^{-1})	NI	NI	0.73
Total suspended solids (mg L^{-1})	60	150	36
Total P (mg L^{-1})	10	10	7.3
Total N (mg L^{-1})	25	NI	18
Biochemical oxygen demand (mg L^{-1})	60	100	33
NaClO (mg L^{-1})	NI	NI	1.0

NI not included in the official norm

[a,b] Official Mexican norms establishing the maximum permissible limits of contaminants in wastewater discharges arising in the pharmaco-chemical and pharmaceutical industries and entering [a] domestic waters and resources, and [b] receiving water bodies

152.3, and 18 V, accordingly. The collision energies were 20–15 for DCF, 10 for IBP, 10–20 for NPX and 15-20 for PAR analysis.

Calibration curves

Calibration curves of each of the NSAIDs were determined using standard solutions of 1, 3, 10, 50 and 250 μg mL^{-1} prepared in a 60:40 mixture of acetonitrile and ammonium formate at pH 6. Linear regression coefficients (R^2) were >0.99 for DCF, IBP, NPX and PAR. The MS/MS detector was maintained according to manufacturer specifications and was regularly cleaned, but when changes >50 % were observed in calibration curve slopes, additional cleaning was conducted. The cleaning involves passing mobile phase through the chromatographic column by 30 min.

Water samples from test systems

Water samples (5 mL) were directly taken from the exposure containers and collected in glass vials and refrigerated at 4 °C for subsequent determination of test concentrations. The samples were vacuum-filtered through 10-μm GF/C glass microfiber filters, followed by 0.45-μm nylon membrane filters (Whatman, Cambridge, UK). A liquid–liquid extraction with 5 mL (1:1, v/v) hexane/ethyl acetate was performed to extract DCF, IBP, NPX and PAR from 1-mL water samples. These samples were centrifuged at 1800×*g* during 10 min. The extraction was repeated and organic layers were combined and evaporated until dryness. The procedure was carried out by quintuplicate. Results were expressed as time-weighted average concentrations of DCF, IBP, NPX and PAR.

Procurement, culturing and maintenance of specimens

Hyalella azteca was collected from its natural habitat in San Miguel de Almaya Lake, municipality of Capulhuac (State of Mexico), and transported to the laboratory in plastic bags with constant aeration. Breeding stock was transported to the laboratory using the source of water in which the organisms were reared. Water used for transporting animals was well oxygenated (90–100 % saturated). Upon arrival at the testing laboratory, the organisms were acclimated gradually to the laboratory holding and testing conditions such that the organisms were not stressed. Test organisms were in good health, and the mortality rate for juvenile *Hyalella* did not exceed 20 % (USEPA 2000). The collected organisms were identified morphologically (Pennak 1978). The morphological characteristics that we find useful to distinguish the species of *H. azteca* worked in this study were: the size of the antennae, the number of setae on the inner plate of maxilla 1, the setae organization on the palp of the maxilliped, the number and organization of setae on the propodus of gnathopod 1, the posterior setation of the basis, the shape of the propodus, and the irregular shape of the palm on gnathopod 2, the shape of the epimeral plates; the structure of uropod 3, especially the setation and the ratio of peduncle to ramus; and the shape and setation of the telson. To eliminate potential differences in sensititivity to contaminants due to

acclimation to local conditions or maternal effects, we used organisms from the same clade that had been in culture under the same feeeding conditions, temperatura and photoperiod for approximately 4 months (third-generation neonates obtained by sexual reproduction). During culture, specimens were maintained in reconstituted water ($NaHCO_3$ = 174 mg L^{-1}, $MgSO_4$ = 120 mg L^{-1}, KCl = 8 mg L^{-1} and $CaSO_4{\cdot}2H_2O$ = 120 mg L^{-1}); all reagents were obtained from Sigma-Aldrich, St. Louis MO) pH 7.5–8.5 at room temperature with constant oxygen (6.4–6.6 mg L^{-1}, O_2) and a 12 h/12 h light/dark photoperiod, and were fed ground lettuce ad libitum. To demonstrate the ability to obtain consistent and precise results using *H. azteca* in toxicity tests, previously in our lab reference toxicants were used [copper sulphate ($CuSO_4$), potassium chloride (KCl) and sodium chloride (NaCl)]. Cultures were observed on a frequent and routine basis (daily). The estimated number of surviving adults and the production of young in each culture chamber, dates of culture renewals, numbers and age classes of transferred individuals, daily feedings, water quality measurements, were documented.

Artificial sediment

The artificial sediment used was 70 % sand (0.2 mm), 20 % kaolinite (<0.002 mm) and 10 % organic matter (0.2 mm). The organic matter source was lamb compost inactivated by dry heating at 55–60 °C for 3 days. The sediment was sterilized with three 15-min autoclave cycles at 121 °C and 15-lb pressure, separated by 1-h intervals (OECD 1984; SETAC 1993).

Determination of the median lethal concentration (LC_{50})

Test systems

Test systems were set up by adding reconstituted water and artificial sediment in a 3:1 ratio to 50-mL polyethylene containers equipped with constant oxygenation and maintained under a 12 h/12 h photoperiod at room temperature. Light intensity adjacent to the surface of the overlying water was 500 lx. The test was conducted at a daily mean temperature (overlying water) of 23 ± 1 °C. Static systems were used, the medium was not replaced and no food was provided to specimens during exposure.

Determination of the LC_{50}

To establish the target concentration to be used in evaluating oxidative stress, the median lethal concentration (LC_{50}) of the industrial effluent was determined. To this end, five experimental systems containing different proportions of industrial effluent (0.71, 0.73, 0.75, 0.76 and 0.78 %) in reconstituted water were added to artificial sediment (3:1 ratio) and a sixth effluent-free control system were set up. Ten amphipods (of 2 days old at start of test) were assigned randomly to each test vessel. These organisms were handled as little and as carefully as possible during their transfer to the test vessels. Amphipods were placed below the air/water interface in the overlying water. Test vessels were covered, the overlying water in each vessel were aerated continuously at a minimal rate. The assay was performed by triplicate. A total of 180 organisms were used in the LC_{50} determination. The mean survival rate for amphipods in control water system was 100 % at the end of the test.

Duration of the exposure period was 72 h. At the end of the 72-h exposure, the number of alive and dead amphipods was recorded for each replicate including the control groups. The 72-h LC_{50} of industrial effluent and its 95 % confidence limits ($P < 0.05$) were estimated by Probit analysis (EPA, v1.5). The obtained data were used to estimate the concentration to be used in the assays for oxidative stress determination.

The concentration–response curve in the acute toxicity test was constructed. The LOAEL is the lowest-observed-adverse-effect-level. This is the lowest concentration of a test substance or material to which organisms were exposed, that caused observed and statistically significant sublethal effects on the *H. azteca*. In order to establish the lowest dilution degree of the studied industrial effluent that causes an alteration, the LOAEL was calculated using the concentration–response curve equation and probit analysis, obtained from the acute assay. The LOAEL determined in this study was 0.07 %.

Sublethal toxicity assays

Sublethal toxicity assays were conducted in order to determine the oxidative stress involved at adding industrial effluent at a concentration equal to the LOAEL (0.07 %), to four test systems with 150 mg wet tissue of *H. azteca*. The assessed exposure times were 12, 24, 48 and 72 h. An effluent-free control system with 150 mg wet tissue of *H. azteca* (entire organisms were used) was set up for each exposure time, and sublethal assays were performed by triplicate.

After exposure, specimens were removed and homogenized in 1 mL Tris buffer solution pH 7. The supernatant was centrifuged at 12,500×*g* for 15 min at −4 °C. The following oxidative stress biomarkers were evaluated: hydroperoxide content (HPC); lipid peroxidation (LPX); protein carbonyl content (PCC) in order to assess oxidized protein levels; and activity of the antioxidant enzymes

superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Total protein content (Bradford 1976) was determined and was used to express the results of the biomarkers evaluated. All biochemical assays were done on the supernatant, except for LPX assessment in which the bud was used.

Determination of HPC

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

Determination of LPX

LPX was determined using the thiobarbituric acid-reactive substances method (Büege and Aust 1978). To 100 mL of supernatant, Tris–HCl buffer solution pH 7.4 (Sigma-Aldrich, St. Louis) was added until a 1-mL volume was attained. Samples were incubated at 37 °C for 30 min; 2 ml TBA-TCA reagent [0.375 % thiobarbituric acid (Fluka-Sigma-Aldrich, Toluca, Mexico) in 15 % trichloroacetic acid (Sigma-Aldrich, St. Louis)] were added and samples were shaken in a vortex. They were then heated up to boiling for 45 min, allowed to cool down, and the precipitate was removed by centrifugation at 3000×*g* for 10 min. Absorbance was read at 535 nm against a reaction blank. MDA content was calculated using the molar extinction coefficient (MEC) of malondialdehyde (MDA) (1.56×10^5 M cm^{-1}). Results were expressed as mM MDA protein^{-1}.

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, 150 µL of 10 mM DNPH in 2 M HCl were added and the resulting solution was incubated at room temperature for 1 h in darkness. Then, 500 µL of 20 % trichloroacetic acid were added and the solution was allowed to rest for 15 min at 4 °C. The precipitate was centrifuged at 11,000×*g* for 5 min. The bud was washed several times with 1:1 ethanol: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. Absorbance was read at 366 nm. Results were expressed as nM reactive carbonyls formed (C=O)/mg protein, using the MEC of 21,000 M cm^{-1}.

Determination of SOD activity

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

Determination of CAT activity

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

Determination of GPx activity

GPx activity was determined by the Gunzler and Flohe-Clairborne (1985) method as modified by Stephensen et al. (2000). To 100 µL of supernatant 10 µL glutathione reductase were added (2 U glutathione reductase, Sigma-Aldrich, St. Louis), plus 290 µL reaction buffer [50 mM K_2HPO_4 (Vetec, St. Louis), 50 mM KH_2PO_4 (Vetec, St. Louis) pH 7.0, 3.5 mM reduced glutathione (Sigma-Aldrich, St. Louis), 1 mM sodium azide (Sigma-Aldrich, St. Louis) and 0.12 mM NADPH (Sigma-Aldrich, St. Louis)] and 100 µL H_2O_2 (0.8 mM, Vetec, St. Louis). Absorbance was read at 340 nm at 0 and 60 s. Enzyme activity was estimated by using the equation: GPx concentration = $(A_0 - A_{60})$/MEC), where the MEC of NADPH = 6.2 mM cm^{-1}. Results were expressed as mM NADPH mg protein^{-1}.

Determination of protein content

To 25 µL of supernatant, 75 µL deionized water and 2.5 mL Bradford's reagent (0.05 g Coommassie Blue dye, 25 mL of 96 % ethanol and 50 mL H_3PO_4, in 500 mL deionized water) were added. The test tubes were shaken and allowed to rest for 5 min prior reading absorbance at 595 nm and interpolation on a bovine albumin curve (Bradford 1976).

Statistical analysis

In the acute toxicity assay (72-h LC_{50} of industrial effluent), Probit analysis was performed and significance assessed by the degree of 95 % LC_{50} overlap, also the value of LOAEL was calculated (EPA Analysis Program v1.5). The χ^2 linear adjustment test was not significant at $P < 0.05$.

In the sublethal toxicity assays, statistical evaluation of results was done with one-way analysis of variance (ANOVA) and differences between means were compared using the Tukey–Kramer multiple comparisons test, with P set at <0.05. Statistical determinations were made with the SPSS v10 software package (SPSS, Chicago, IL).

Results

Physicochemical characterization

Results of physicochemical characterization are shown in Table 1. The physicochemical characteristics of the effluent do not exceed the limits established in the official Mexican norms (NOM-001-SEMARNAT-1996; NOM-073-ECOL-1994). Dissolved oxygen was 12.2 mg L^{-1}, conductivity 143.2 µS cm^{-1}, ammonia 0.73 mg L^{-1} and NaClO 1.0 mg L^{-1}. These four parameters are not taken into account in either of these norms.

DCF, IBP, NPX and PAR quantification

NSAID quantification in the industrial effluent showed that the concentrations in it were DCF (1.04 ± 0.05 mg L^{-1}), IBP (1.0 ± 0.03 mg L^{-1}), NPX (1.72 ± 0.03 mg L^{-1}) and PAR (3.03 ± 0.02 mg L^{-1}).

LC_{50}-72 h determination

The LC_{50} of the industrial effluent as well as their 95 % confidence intervals was 0.732 % (0.725–0.741), and the LOAEL was 0.07 % (Fig. 1). The χ^2 linear adjustment test was not significant at $P \leq 0.05$.

Oxidative stress status

HPC

The HPC induced by industrial effluent is shown in Fig. 2. A significant increase compared to the control group ($P < 0.05$) was observed at all exposure times. These increases were 44.5, 77.8, 130.3 and 105.7 % at 12, 24, 48 and 72 h, respectively.

LPX

The amount of MDA induced by the industrial effluent is shown in Fig. 3. A time-dependent increase compared to the control group ($P < 0.05$) was found at all exposure times. These increases were 270.2, 317.9, 419.7 and 531.9 % at 12, 24, 48 and 72 h, respectively.

PCC

Protein oxidation in specimens exposed to industrial effluent is shown in Fig. 4. Significant increases in this biomarker with respect to control were observed ($P < 0.05$) in all exposure times. These increases were 80.1, 74.2, 71.2, 117.4 % at 12, 24, 48 and 72 h respectively.

SOD activity

Figure 5 shows SOD activity results. The industrial effluent induced significant increases with respect to the control group ($P < 0.05$). The observed increases were 82.0, 91.0, 155.1, 184.4 % at 12, 24, 48 and 72 h respectively.

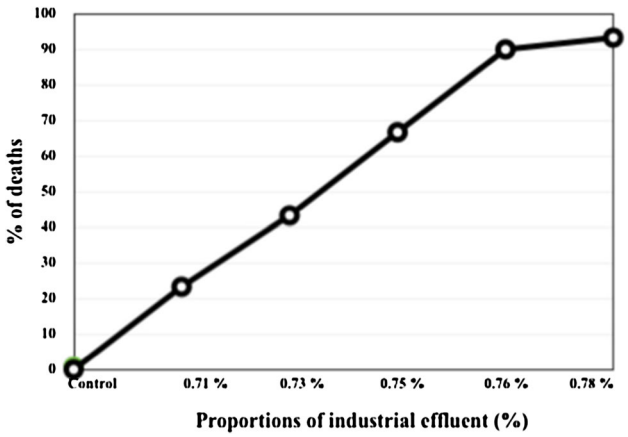

Fig. 1 Median lethal concentration (LC_{50}) of the industrial effluent, and lowest observed adverse effect level (LOAEL) in *H. azteca*. Values are the mean of three replicates ± SE. EPA Analysis Program v1.5

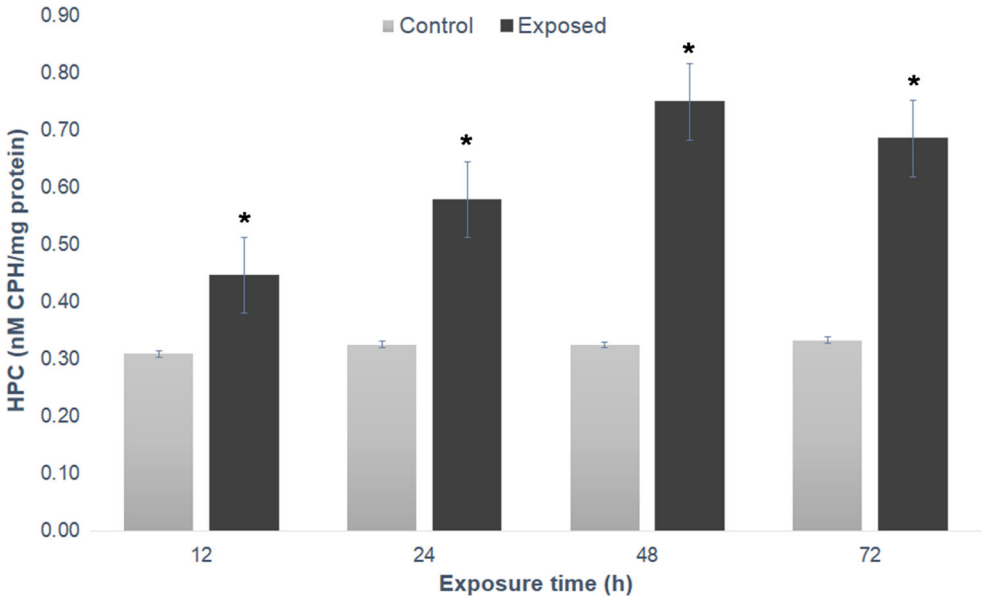

Fig. 2 Hydroperoxide content (HPC) in *H. azteca* exposed to industrial effluent for 12, 24, 48, and 72 h. Values are the mean of three replicates ± SE. *CHP* cumene hydroperoxide. *Significantly different from control values, ANOVA and Tukey–Kramer ($P < 0.05$)

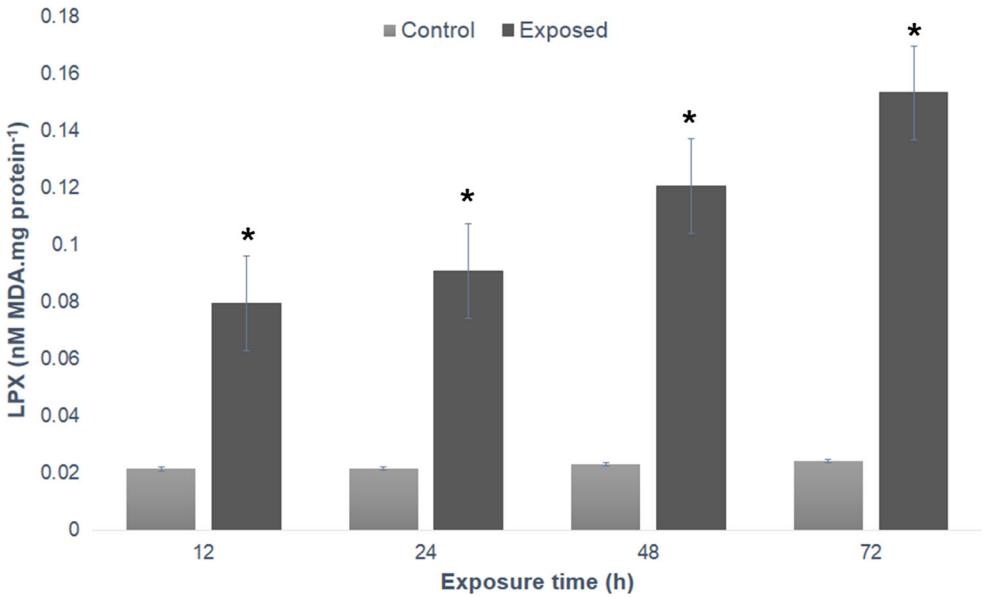

Fig. 3 Lipid peroxidation (LPX) in *H. azteca* exposed to industrial effluent for 12, 24, 48, and 72 h. Values are the mean of three replicates ± SE. *MDA* malondialdehyde. *Significantly different from control values, ANOVA and Tukey–Kramer ($P < 0.05$)

CAT activity

CAT activity, expressed as mM H_2O_2/mg protein, is shown in Fig. 6. The industrial effluent induced a significant increase with respect to the control group ($P < 0.05$). Significant increases occurred at 12, 24, 48 and 72 h were 70.4, 81.4, 129.8 and 178.8 %, respectively.

GPx activity

Figure 7 shows GPx activity results. As regards, the industrial effluent shows a significant increase with respect to the control group ($P < 0.05$). Significant increases occurred at 12, 24, 48 and 72 h were 41.9, 50.6, 74.7 and 120.5 %, respectively.

Discussion

The physicochemical properties of the effluent analyzed in the present study (Table 1) do not exceed the limits established in the official Mexican norms NOM-001-SEMARNAT-1996 and NOM-073-ECOL-1994. The dissolved oxygen, conductivity, ammonia and NaClO are not considered in either of these norms. Ammonia and conductivity are confounding factors that may interfere with

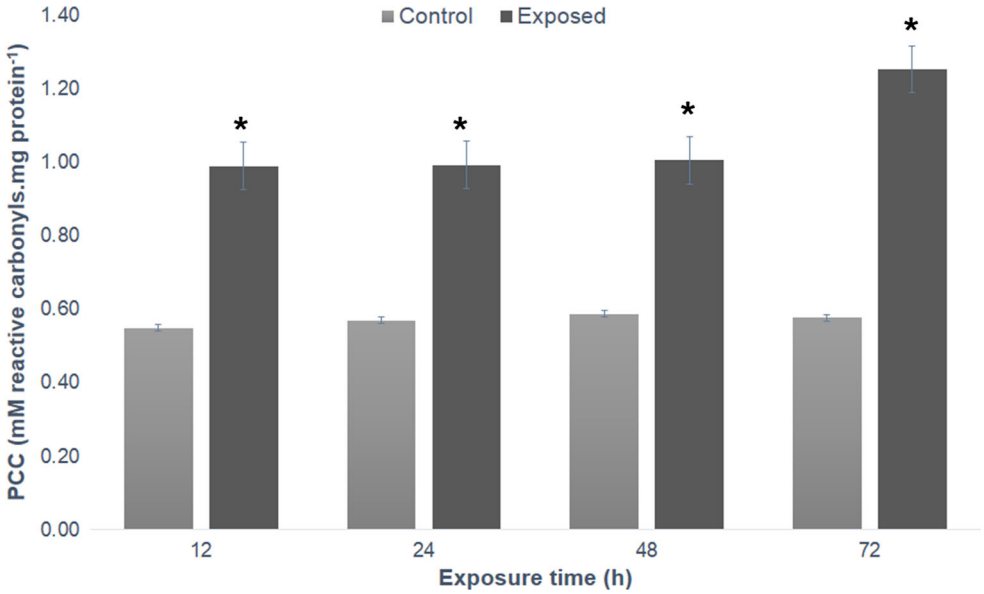

Fig. 4 Protein carbonyl content (PCC) in *H. azteca* exposed to industrial effluent for 12, 24, 48, and 72 h. Values are the mean of three replicates ± SE. *Significantly different from control values, ANOVA and Tukey–Kramer ($P < 0.05$)

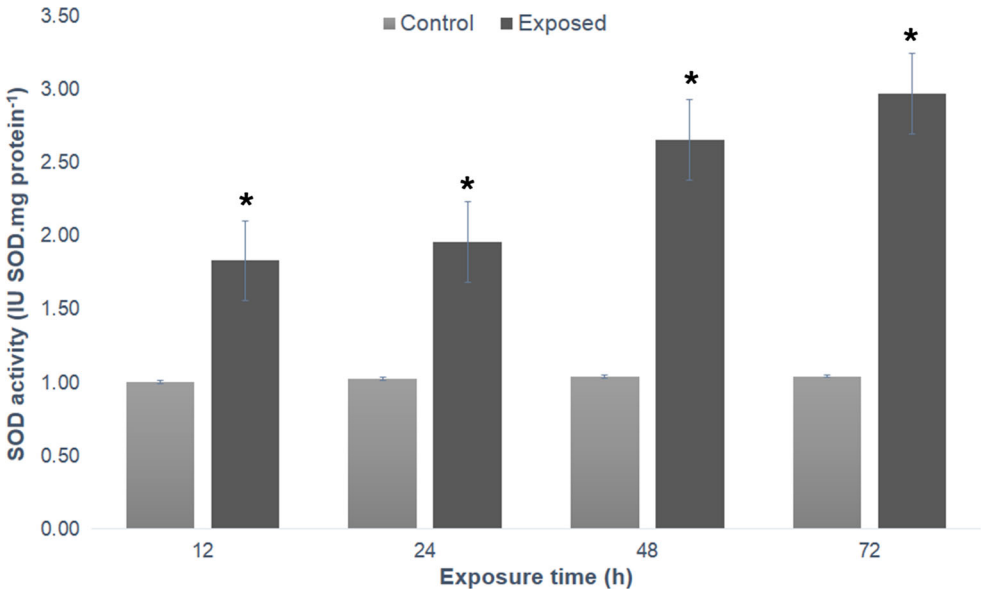

Fig. 5 Superoxide dismutase (SOD) activity in *H. azteca* exposed to industrial effluent for 12, 24, 48, and 72 h. Values are the mean of three replicates ± SE. *Significantly different from control values, ANOVA and Tukey–Kramer ($P < 0.05$)

the bioeffects of micropollutants. Postma et al. 2002 indicate that the highest values at which they observed no significant effects were ammonia 13–60 mg L^{-1} and conductivity <650 µS cm^{-1}. On this basis, ammonia and conductivity in our study (0.73 mg L^{-1} and 143.2 µS cm^{-1}, respectively) are not confounding factors.

Another contaminant identified in the effluent was NaClO in 1.0 mg L^{-1} that is used, together with other substances, as a disinfectant in water chlorination. This type of compounds can form highly toxic products such as haloalkanes, haloacetic acids, haloacetonitriles, haloketones and haloaldehydes (WHO 1996). These are formed by reactions of chlorine with the humic and fulvic acids normally present in surface water (Rook 1977; Cantor 1997; Boorman 1999).

The pharmaceutical plant from where the studied effluent comes from is used exclusively for NSAID manufacture. Since the plant has no wastewater treatment system, the generated effluents contain NSAIDs derived from the manufacturing process. These contaminants are polar pharmaceuticals which gives them the capacity to move through the sediment profile (environmental kinetics) and persistent enough to contaminate the aquifer (Almeida et al. 2013). The NSAIDs detected in the effluent include DCF, IBP, NPX and PAR at concentrations of 1.0–3.03 mg L^{-1}. According to Gibson et al. (2010), the

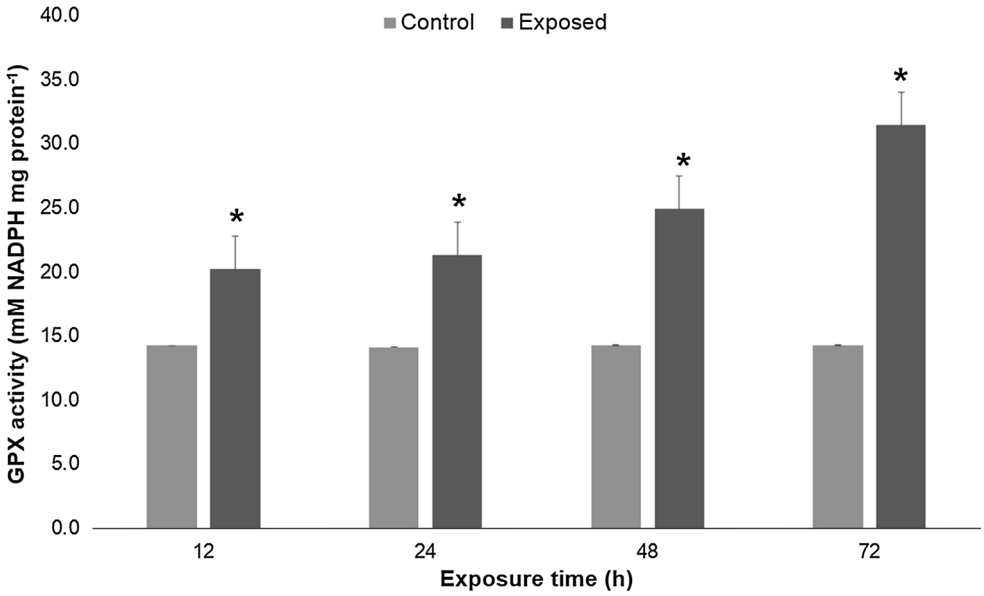

Fig. 6 Catalase (CAT) activity in *H. azteca* exposed to industrial effluent for 12, 24, 48, and 72 h. Values are the mean of three replicates ± SE. *Significantly different from control values, ANOVA and Tukey–Kramer ($P < 0.05$)

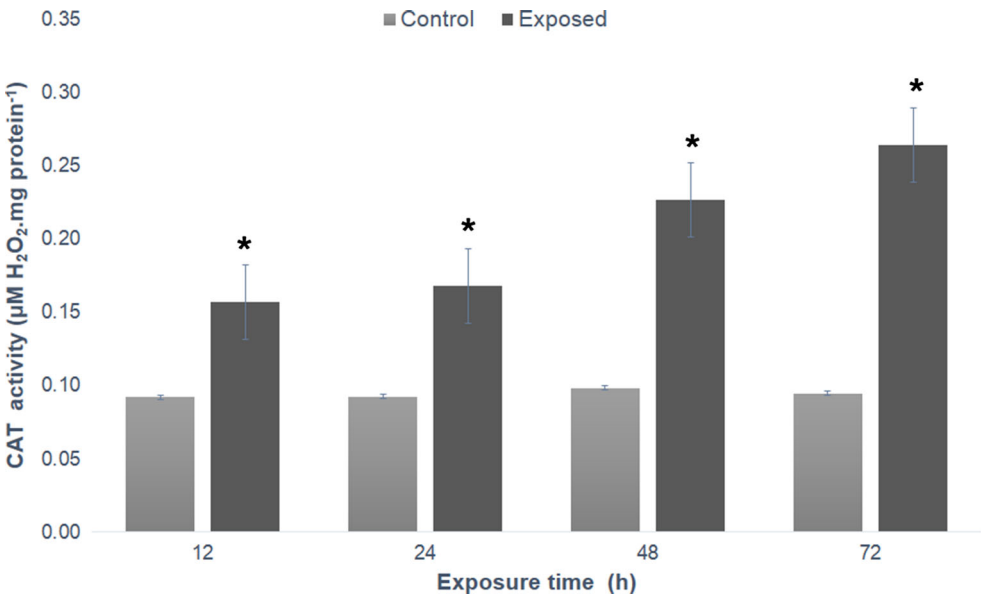

Fig. 7 Glutathione peroxidase (GPx) in *H. azteca* exposed to industrial effluent for 12, 24, 48, and 72 h. Values are the mean of three replicates ± SE. *Significantly different from control values, ANOVA and Tukey–Kramer ($P < 0.05$)

concentrations of IBP, NPX and DCF greater than 1 µg L^{-1} are a risk for groundwater contamination resulting from the wastewater irrigation.

In our study the LC_{50} of the effluent in *H. azteca* was 0.732 %, with a 95 % confidence interval of 0.725–0.741 (Fig. 1). This proportion of the effluent contains: DCF (7.5 µg L^{-1}), IBP (7.2 µg L^{-1}), NPX (12.3 µg L^{-1}) and PAR (21.8 µg L^{-1}). In other species such as *Danio rerio*, the LC_{50} from bulk drug production has ranged from 2.7 to 8.1 %, when the effluent contains NPX, IBP and PAR at concentrations of 4.4, 1.2 and <1 µg L^{-1}, respectively (Carlsson et al. 2010). Based on these findings, the evaluated effluent in this study is more toxic. This may be due NSAIDs concentrations found were higher. On the othe hand, should be considered that the evaluated effluent had 1.0 mg L^{-1} of NaClO. Hypochlorite ion (^{-}OCl) stress is also suggested to generate common deleterious oxidative species which can damage cellular components and organisms (Dunkan and Touati 1996; Dukan et al. 1999). Also, it should be considered other contaminants not analyzed in this study.

Gómez-Oliván et al. (2014b) found values 72 h-LC50 to *H. azteca* of DCF (4.6 mg L^{-1}), IBP (1.7 mg L^{-1}), NPX (7.6 mg L^{-1}) and PAR (7.7 mg L^{-1}) in isolated systems. These results demonstrate that the studied industrial effluent is more harmful to *H. azteca* than isolated AINEs.

The LC_{50} findings in this study may be explained by the fact that NSAIDs act by blocking the enzyme cyclooxygenase. The latter is responsible for catalyzing arachidonic acid degradation in prostaglandin production (Cha et al. 2006; Fortier et al. 2008). These eicosanoids act as autocrine and paracrine messengers and, in invertebrate species such as *H. azteca*, as important mediators during reproduction and in the immune system (Stanley 2000; Fortier et al. 2008). Prostaglandins are also involved in neurotransmission and the transport of ions across cell membranes (Arkhipova et al. 2005). The final result of these actions in the present study might have been inhibition of neurotransmission evidenced by *H. azteca* immobilization.

It is also necessary to indicate that although *H. azteca* has been in culture for over 25 years in laboratories across North America, each laboratory has its own protocol for their particular stock (Environment Canada 2013). Even though standard laboratory methods for culturing *H. azteca* have been published in Canada and the United States, laboratory personnel are given freedom of choice in several aspects of culturing, e.g., food, water sources, substrate, etc. Similarly, standard protocols for sediment and water toxicity tests using *H. azteca* are different in throughout the world. Although standardized toxicity protocols exist, many institutions employ different test conditions. Consequently, it is difficult to compare results (e.g., LC_{50}s) among different publications.

Diverse studies have shown that NSAIDs are unstable and are photodegradable (depending on its structure and physicochemical properties), to metabolites more toxic to aquatic organisms than the unaltered pharmaceuticals (Borgmann et al. 2007; Araujo et al. 2011). The main metabolites present in water bodies as a result of bacterial degradation and abiotic characteristics (pH and light) are as follows: for D, 5,4′-dihydroxy-diclofenac, 3′-hydroxy-diclofenac, 4′-hydroxymethyl diclofenac, 3′-hydroxy-4′-hydroxymethyl diclofenac, 4′-hydroxy- and 5′-hydroxy-diclofenac (Deng et al. 2003); to PCM, *p*-hydroxyacetanilide, *p*-hydroxyacetanilide glucuronide, and *N*-acetyl benzoquinoneimine (Mycek et al. 2004); to IBP, 1-hydroxy and 2-hydroxy ibuprofen and arylcarboxyl ibuprofen (Carballa et al. 2004); and to NPX, naproxen-β-1-*O*-acyl glucuronide and 6-*O*-desmethyl naproxen (Huq 2006). These compounds can deposit as sediment and are able to bind to the humic and fulvic acids in organic matter and be subsequently ingested by amphipods.

Also, the biotransformation of NSAIDs once they enter the body must also be considered. Cytochrome P450 mixed function oxidase (MFO) systems play the major role in oxidation of drugs and others foreign compounds in humans and in a vast variety of species such as bacteria, plants, fish and aquatic invertebrates (Snyder 2000; Rewitz et al. 2006; Gottardi et al. 2016). Different P450 gene families (CYP) have been characterized in fish and invertebrates, such as CYP1, CYP2, CYP3, CYP4, CYP11, CYP17, and CYP19 (Stegeman and Livingstone 1998). The CYP2 family is specifically known to be responsible for NAIDs biotransformation, particularly so in the case of the subfamily CYP2C9 (Blanco et al. 2005; Zanger et al. 2008). Other pathway of biotransformation of carboxylate NSAIDs (ASA, DCF, NPX, and IBP) is glucuronic acid conjugation catalyzed by the uridine diphosphoglucuronosyl transferase superfamily of enzymes, which results in acyl glucuronides (Pritchard 1993). These compounds are reactive intermediates that can undergo acyl migration and hydrolysis. They can also form adducts with nucleophilic amino acid residues. Many NSAID-derived acyl glucuronides, including those obtained from DCF and IBP, have been shown to form covalent bonds with intra and extracellular proteins, with toxicological consequences (Boelsterli 2007).

Molecular biomarkers are used to test for oxidative damage induced in 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). The induction of oxidative stress usually is monitored via registration of products of ROS-induced modification of cellular constituents. ROS-modified lipids, proteins and nucleic acids along with low and high molecular mass antioxidants and antioxidant potential is a battery of indices commonly used to describe oxidative stress (Lushchak 2011).

In the LPX process, polyunsaturated fatty acids react with ROS, particularly the hydroxyl radical (HO·) and the RNS, peroxynitrite ($ONOO^-$), via a chain reaction mechanism. This allows the formation of hydroperoxides that are degraded to low molecular weight products, including MDA (Wilhelm Filho 1996). A time-dependent increase with respect to the control group ($P < 0.05$) was found in HPC (Fig. 2). Also, in Fig. 3 a similar behaviour was observed and a time-dependent level of damage to lipids respect to control is shown in LPX biomarker. These findings may be explained by the fact that in the NSAID biotransformation by CYP2C9, ROS are formed. These ROS can be OH· and oxygenated intermediates like the oxy-cytochrome P450 complex [P450 (Fe^{3+}) $O^{2\cdot}$] as a result of the release of the superoxide anion by reaction decoupling. In both cases, ROS production is increased, which explains the observed increases in LPX and HPC.

Similar effects were observed by Oviedo-Gómez et al. (2010), they indicate that the amphipod *H. azteca* exposed to DCF in 46.7 mg kg^{-1} showed a significant increase ($P < 0.05$) with respect to controls at 12, 24, 48 and 72 h. These authors mention that LPX increase can be explained in terms of formation of 4′-hydroxy DCF and 5′-hydroxy DCF and their subsecuent biotransformation to benzoquinones which increase ROS formation. Also, Gómez-

Oliván et al. 2012, found similar effects when exposed *H. azteca* to PAR at 770 μg kg^{-1}. This increase may be due to the formation of *N*-acetyl *p*-benzoquinonimine which is able to bind to cellular membranes.

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.

Protein damage is induced by oxidation of side chains of amino acids (Reed 1995), which leads to loss of sulfyhydryl groups and changes in the resonance structures of amino acids, altering their function and therefore the integrity of the body (Parvez and Raisuddin 2005). Figure 4 shows a time-dependent increase in PCC with respect to the control group ($P < 0.05$), this may be due to the presence of superoxide ion which rapidly reacts with the nitric oxide (NO) derived from arginine metabolism, forming $ONOO^-$ (Halliwell 1997; Doi et al. 2002; Jifa et al. 2006). The oxidant agent peroxynitrite is known to induce protein oxidation and nitration in absence of GSH, eliciting mitochondrial dysfunction and eventually leading to irreversible damage and severe loss of cellular ATP (Jaeschke et al. 2003).

Another possible explanation for the findings in PCC, is that the hypochlorite ion (^-OCl) is a strong oxidizing agent. HOCl and ^-OCl have been reported to react with a wide variety of biological molecules such as proteins (Hazell et al. 1994; Hawkins and Davies 1998, 1999), amino acids (Nightingale et al. 2000), peptides (Heinecke et al. 1993), lipids (Spickett et al. 2000) and DNA (Prutz 1998) at physiological pH conditions. The Cl atom in HOCl and ^-OCl behaves as Cl^+, a strong electrophile, and combines with a pair of electrons where the substrate exhibits high electron densities. Among biological molecules, the C=C double bond, peptide bond (amide bond), amino groups, and thiol groups are susceptible to the electrophilic attack of Cl^+ (Pereira et al. 1973; Winterbourn and Brennan 1997; Nightingale et al. 2000).

When NaClO is added to water, the solution readily reacts with biomolecules (including proteins and nucleotide bases) to produce a variety of chlorinated organic compounds which are mostly lipophilic, persistent and toxic in aquatic environments (Emmanuel et al. 2004). Under the stress of HOCl, the possible involvement of reactive oxygen species (ROS) generated in microbial cells has been suggested (Dukan et al. 1999). The primary effect of HOCl is either or both (1) the oxidation of sulfhydryl (SH) groups of essential enzymes and antioxidants and (2) deleterious effects on DNA synthesis.

The high number of pollutants in the aquatic ecosystem can disturb the equilibrium between ROS and the antioxidant systems (Jos et al. 2005). The activity of antioxidant enzymes is usually an element of monitoring of oxidative stress (Timofeyev et al. 2006; Valavanidis et al. 2006; Falfushynska and Stolyar 2009).

The antioxidant defense system is essential in the neutralization of ROS and related damage (Regoli et al. 2002). This system is mediated by a cascade of antioxidant enzymes that sequester ROS and convert them to less toxic and reactive species. This group of enzymes includes SOD, CAT and GPx.

The increases in SOD activity (Fig. 5) were induced by release of the anion radical O_2 (Livingstone 2003). This activity alteration is the first mechanism of antioxidant defense since SOD is the main enzyme responsible for offsetting the effects of ROS, particularly the superoxide ion (van der Oost et al. 2003) that is converted to hydrogen peroxide by such an enzyme. Subsequently, H_2O_2 is sequestered and degraded to H_2O by CAT and GPx.

Increased SOD activity may act as a signal of oxidative stress, leading to activation and/or induction of antioxidant enzymes associated with a system of H_2O_2-sequestration such as CAT or GPx (Vlahogianni et al. 2007).

In our study, CAT and GPx activity in *H. azteca* was increased with respect to control group at all exposure times ($P < 0.05$; Figs. 6, 7). The increase observed in CAT activity may be due to higher concentrations of H_2O_2 as a result of SOD activity.

Most chlorine compounds are powerful prooxidants which can affect antioxidant defenses in exposed organisms (Ueno et al. 2000; Pozzetti et al. 2003).

Obtained results are consistent with those obtained by Oviedo-Gómez et al. (2010), who reported increases in the activity of CAT and GPx in *H. azteca* exposed to DCF. Also, similar results were found by Gómez-Oliván et al. (2014a, b) in *Daphnia magna* exposed to IBP and DCF. This result indicates this enzyme is unable to offset ROS-induced. Bagnyukova et al. (2006) state that LPX products may be involved in the regulation of some antioxidant enzymes, so that the LPX increase found in our study may likewise explain the increases observed in the activity of antioxidant enzymes (SOD, CAT, and GPx).

The results found in this study, the widespread distribution and common occurrence of *H. azteca* in association with freshwater sediment, together with its ecological importance, ease of culturing and handling during testing, rapid growth, short life cycle, sensitivity to contaminants in sediment, and extensive use in sediment and water toxicity tests, can help meet requirements related to environmental appraisal and protection However to any further ecological or toxicological studies should confirm the identity of the populations being worked with. Due, *H. azteca* is considered a species complex. The genetic evidence have shown the degree of heterogeneity of the populations within the distributional range of the species (Duan et al. 1997, 2000; Wit and Hebert 2000).

Conclusions

The industrial effluent analyzed in the present study contains NSAIDs and NaClO, and induces oxidative stress in *H. Azteca* used and identified morphologically. The set of assays used in the present study constitutes a reliable early warning biomarker for use in evaluating the toxicity induced by these emerging contaminants in *H. Azteca*. However, should be considered that there are evidence of different sensitivities to contaminants exists among members of the *H. azteca* species complex, and this has important implications for biomonitoring programs.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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