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Short and long-term exposure to diclofenac alter oxidative stress status in common carp *Cyprinus carpio*

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Abstract Diclofenac (DCF) has been detected in significant amounts in municipal treated wastewater effluent. Diverse studies report that trace concentrations of DCF may induce toxic effects on different aquatic organisms as well as developmental, reproductive and renal damage. This study aimed to determine whether short and long-term exposure to DCF alter the oxidative stress (OS) status in blood, muscle, gills, brain and liver of common carp *Cyprinus carpio*. The median lethal concentration of DCF at 96 h (96-h LC_{50}) and subsequently the lowest observed adverse effect level were determined. Carp were exposed (short and long-term) to the latter value for different exposure times (4 and 24 days) and the following biomarkers were evaluated in gill, brain, liver and blood: hydroperoxides content (HPC), lipid peroxidation (LPX), protein carbonyl content (PCC) and the activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Also, the DCF was determined by LC–MS/MS. Significant increases in HPC, LPX and PCC were observed respect to control ($P < 0.05$) particularly in blood, muscle, gill, brain and liver. SOD, CAT and GPx activity also increased in these organs, with respect to controls ($P < 0.05$). DCF concentrations decreased and increased in water system and carp, respectively. *Cyprinus carpio* exposed to DCF was affected in OS status during the initial days of the study (at 4 days), exhibiting an increased response at 24 days in blood and liver. In contrast, a decrease was observed in muscle, gills and brain at 24 days with respect to 4 days. In conclusion, DCF induces OS on blood, muscle, gills, brain and liver in the carp *C. carpio* in short and long-term exposure. The biomarkers employed in this study are useful in the assessment of the environmental impact of this agent on aquatic species.

Keywords Diclofenac · Oxidative stress · Short and long-term exposure · *Cyprinus carpio*

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Introduction

Pharmaceutical drugs are widely used in human and veterinary medicine, to prevent, cure, or mitigate disease in diverse organisms. These agents and their metabolites can enter water bodies by different pathways such as the effluent from domestic, industrial and hospital wastewater. Diverse studies have shown a remarkable increase worldwide in the levels of these contaminants in surface and groundwater, eliciting toxicity problems in diverse hydrobionts (Montforts et al. 2007; Spindler et al. 2007).

Some pharmaceuticals are designed to have a specific mode of action and many of them for some persistence in the body. Once they enter the aquatic environment, persist in it, damaging the health of organisms living in these

ecosystems and even human health. Therefore, these products are currently of worldwide environmental concern and have been called "emerging contaminants" (Richardson et al. 2007). These chemicals includes nonsteroidal anti-inflammatory drugs (NSAIDs), a heterogeneous group with anti-inflammatory, analgesic and antipyretic effects, which act as selective inhibitors of the enzyme cyclooxygenase (COX), inhibiting cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) which are responsible for production of prostaglandins, prostacyclins and thromboxanes. The most common members of this group, based on prescriptions number and sales are acetylsalicylic acid (ASA), diclofenac (DCF), ibuprofen (IBP) and naproxen (NPX).

Diclofenac has been detected in significant amounts in municipal treated wastewater effluent. Ternes et al. (1999) report its presence at concentrations above 1 μg L^{-1} in treatment plant water and lower concentrations in surface water. Other studies have reported concentrations between 10 and 2,200 ng L^{-1} in effluent from wastewater treatment plants in diverse European countries (Stülten et al. 2008; Letzel et al. 2009). In Mexico, there are no reports of the presence of contaminants of this type in water bodies apart from a study by Siemens et al. (2008), who found DCF at concentrations of 0.25–0.50 μg L^{-1} in the Mexico City-Mezquital Valley irrigation system.

Studies by Memmert et al. (2013) suggest that DCF has little or no adverse effect in fish species as rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) up to 320 μg L^{-1}. Unlike other studies report that trace DCF concentrations may induce toxic effects on different aquatic organisms, also developmental, reproductive and renal damage (Marques et al. 2004; Brun et al. 2006). DCF-exposure in aquatic species, particularly, fish, induces acute and/or chronic toxicity, affecting survival, swimming and feeding behavior, growth, embryonic development and reproduction (Fent et al. 2006; Nassef et al. 2010; Lee et al. 2011). Eades and Waring (2010) demonstrate that DCF (10 ng L^{-1}) impaired osmoregulatory ability of a green shore crab *Carcinus maenas*. The authors suggested that this NSAIDs caused the increase in the hemolymph osmolality recorded for the crab. In other studies, LPX was used as a biomarker for oxidative stress (OS). Quinn et al. (2011) recorded significant increments on LPX when zebra mussels (*Dreissena polymorpha*) were exposed to 1 μg L^{-1} of DCF. Feito et al. (2012) reported a reduction of LPX in zebrafish embryos already at a concentration of 0.03 1 μg L^{-1} and at 90 min. These authors also reported phytotoxicological effect by DCF exposure. An increase in mitochondrial activity was reported for fern *Polystichum setiferum* after a 48-h exposure to DCF concentration of 0.3 μg L^{-1}. Chronic effects for DNA of fern, was noted already at a concentration of 0.03 μg L^{-1}. In other study, Brozinski et al. (2013) demonstrated that DCF could be detected in the biles of bream and roach caught from a lake that receives municipal wastewater effluents. DCF concentrations in the lake ranged from 22 to 302 ng L^{-1} whereas the concentration in the bile of bream and roach were up to 95 and 148 μg L^{-1}, respectively, i.e. roughly 1,000 times higher that the aqueous concentrations.

Various studies suggest that in DCF hydroxylation metabolism by the P450 complex, diverse ROS such as the superoxide anion radical (O_2*) may be produced in different organ systems such as common carp *Cyprinus carpio* (Islas-Flores et al. 2013). Also, DCF metabolites 4-OH-diclofenac and 5-OH-diclofenac can be oxidized to intermediates of a reactive quinone imine that reacts with protein nucleophilic groups to form adducts. There is also formation of DCF cationic radicals to nitroxides and quinolone imines, both of which are associated with the redox cycle (Hoeger et al. 2008).

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. 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). Antioxidants protect the body against oxyradical-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 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).

It is well known that bioassay tests can be used to establish the maximum acceptable concentration of any environmental contaminant causing any adverse effects on the organisms (Joss et al. 2005). Embry et al. (2010) reported that aquatic toxicity data on acute and chronic responses to anthropogenic chemicals by fish plays a very important role in environmental monitoring of the xenobiotics. Due to continuous entry of pharmaceuticals into aquatic environments, it is necessary to perform chronic studies to observe the long-term effects of drugs on

non-target organisms (Galus et al. 2013). Further, biomonitoring using chronic toxicity assay may sensitively indicate the stress posed by the pollutants (Zhou et al. 2008).

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. Also, it shows adaptive response in contaminated aquatic environments (Oruç and Uner 2002).

The aim of this study was to determine whether short and long-term exposure to DCF alter the OS status in blood, muscle, gills, brain and liver of common carp *C. carpio.*

Materials and methods

Unless otherwise specified, reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Test substance

The pharmaceutical agent DCF (CAS number 15307-86-5, >99 % purity) $C_{14}H_{11}Cl_2NO_2$, 296 Da. Stock solutions were prepared by dissolving 1 g DCF in deionized water. All concentrations reported were measured. The analysis was performed by LC/MS/MS.

Diclofenac quantification in water and carp by liquid chromatography-tandem mass spectrometry (LC–MS/MS)

Standard

Standard solution was prepared in a 60:40 mix acetonitrile and ammonium formate at pH 6 (pH was regulated using 1 M HCl). Standard of concentration of 10 μg mL^{-1} of DCF was prepared. DCF standard was stored in the dark at −18 °C. Solution of 1,000 μg mL^{-1} was used for MS tuning, a 200 μg mL^{-1} solution for recovery studies and solutions containing 1, 2, 10, 50 and 250 μg mL^{-1} of DCF for calibrations.

Equipment

The HPLC–MS/MS system consisted of an Agilent 1290 Infinity HPLC unit (Santa Clara CA). 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 was identified and quantified by means of a mass spectrometer (Triple quadrupole 6430, Agilent, Santa Clara, CA. USA) fitted with electrospray ionization (ESI). The 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: 24.9, $[M–Na]^{-1}$, 294 and 80 V, respectively. Mass spectrometer optimization was conducted by direct infusion of a 10 μg mL^{-1} standard solution of DCF; thereafter the ionization mode and precursor ion mode was selected.

The method detection limit (MDL) and method quantitation limit (MQL) were defined and determined as the minimum detectable amount of DCF with a signal to noise ratio of 3:1 from DCF waters spiked extract, in mRM mode. These data (MDL and MQL) were 30 and 84 ng mL^{-1} respectively. Instrumental detection limits (IDL) was determined by direct injection of decreasing amounts of the DCF standard. The IDL was 27 pg/injected for DCF.

Calibration curve

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

Water

Upon reception, samples were vacuum filtered through 1–0 μm glass microfiber filters (GF/C Whatman, UK), followed by 0.45 μm nylon membrane filters (Whatman, UK). 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 DCF concentration. A liquid/liquid extraction with 5 mL (1 + 1) (v/v) hexane/ethyl acetate was conducted to extract DCF from 1-mL water samples. These samples were centrifuged at 1,800×*g* 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 were extracted the same form.

Plasma

Frozen plasma samples were brought to room temperature followed by the addition of 5 mL of ice-cold acetone. After thorough mixing on a vortex mixer, contents were centrifuged at 2,000×*g* for 10 min. Supernatant was separated,

evaporated to 0.5 mL, back-extracted into the extraction solvent, and finally evaporated to dryness. Samples were analyzed for DCF by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Specimen procurement and maintenance

Three-month-old common carp (*Cyprinus carpio*) juveniles 19.05 ± 0.51 cm in length and weighing 73.4 ± 7.8 g were obtained from the aquaculture facility in Tiacaque (State of Mexico), safely transported to the laboratory in well-sealed polyethylene bags containing oxygenated water, stocked in a large tank with dechlorinated tap water previously reconstituted with salts and acclimated to test conditions for 30 days prior to the experiment. During acclimation, Pedregal Silver™ fish food was provided and 3/4 of the tank water was replaced every 24 h. The physicochemical characteristics of tap water (previously reconstituted with salts; see section 2.4) and acclimated to test conditions for 30 days prior to the experiment. During acclimation, carp were fed Pedregal Silver™ fish food, and three-fourths of the tank water was replaced every 24 h in order 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}, total hardness 18.7 ± 0.6 mg L^{-1}. A natural light/dark photoperiod (12:12) was maintained. During aquaculture period the fish were not exposed to any pharmaceuticals. DCF concentrations were determined by LC-MS/MS in both pond water from the aquaculture facility in Tiacaque, and neither DCF nor other NSAID was detected in water.

Median lethal concentration (LC_{50}) determination at 96 h

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

To establish the target concentration to be used for OS evaluation, the median lethal concentration at 96 h (LC_{50}-96 h) of DCF was determined. To this end, five experimental systems to which different nominal concentrations of DCF were added (9.5, 18.9, 37.7, 75.2, 150 mg L^{-1}) plus an sixth DCF-free control system were set up, and ten carp randomly selected from the stock (using the random number method) were placed in each system. The assay was performed in triplicate. Three replicates were made, using a total of 540 fish.

Duration of the exposure period was 96 h, at the end of which the number of dead specimens in each system was counted. The 96-h LC_{50} of DCF and its 95 % confidence limits ($P < 0.05$) were estimated by Probit analysis (US-EPA 2009). The data obtained were used to estimate the concentration to be used in the assays for OS determination.

Short and long-term toxicity studies

Short and long-term assays involved adding DCF at a concentration nominal equal to the lowest observed adverse effect level (LOAEL), i.e. 7.098 mg L^{-1}, to four experimental systems with six carp each. The exposure periods were 4 days (short-term exposure) and 24 days (long-term exposure). An third and fourth DCF-free control system were set up for each exposure period. The assay was performed in triplicate. Three replicates were made, using a total of 216 fish.

During short-term toxicity studies, healthy fish were taken from the stock and were maintained in the glass tank. Two days prior to experiments and during the experimental period feeding was discontinued. DCF concentration (7.098 mg L^{-1}) was added in each glass aquaria (120 cm × 80 cm × 40 cm) containing 60 L of water. The assay was performed in triplicate. Three replicates were made and 54 fish of equal size and weight were introduced. A concurrent control of 54 fish in three glass aquaria were also maintained under identical conditions. No mortality was observed during the study period. At the end of 4 days period fish from the control and DCF treated groups were taken for OS analysis.

For long-term studies, DCF concentration (7.098 mg L^{-1}) was added in each glass aquaria (120 cm × 80 cm × 40 cm) containing 60 L of water. The assay was performed in triplicate. Three replicates were made and 54 fish of equal size and weight were introduced. Water was changed daily in order to avoid accumulation of faecal matter and excess feed and renewed with DCF. A concurrent control of 54 fish in three glass aquaria were also maintained under identical conditions. No mortality was observed during the study period. At the end of 24 days period fish from the control and DCF treated groups were taken for OS analysis.

During long-term studies, some physicochemical parameters were evaluated every third day in the control and DCF systems. The main parameters evaluated were: temperature (15 ± 1 °C), pH (7.5 ± 0.4), dissolved oxygen (10 ± 1.3 mg L^{-1}), hardness (104.6 ± 2.4 mg L^{-1}), ammonia (0.73 ± 0.008 mg L^{-1}), CO_2 (8.6 ± 0.4 mg L^{-1}) and nitrite concentration was not detected.

The sublethal assays was performed in triplicate. Three replicates were made, using a total of 216 fish in the short and long-term studies. At the end of the exposure period, fish were removed from the systems and placed in a tank containing 50 mg L^{-1} of clove oil as an anaesthetic (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.

Oxidative stress evaluation

After puncture, specimens were placed in an ice bath and sacrificed. The muscle, gill, liver and brain were removed, placed in phosphate buffer solution (PBS) [NaCl (Sigma-Aldrich, Toluca) 0.138 M; KCl (Vetec-Sigma-Aldrich, Toluca) 0.0027 M] pH 7.4 and homogenized. The supernatant was centrifuged at 12,500×*g* and −4 °C for 15 min. Tissue samples were stored at −70 °C prior to analysis.

Blood samples were collected in heparinized tubes, placed in PBS, ultrasonicated, and stored at −70 °C prior to analysis.

The following biomarkers were evaluated: hydroperoxide content (HPC), malondialdehyde (MDA) content, PCC, and SOD and CAT activity. All bioassays were done on the supernatant except MDA content.

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]. 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 type curve and expressed as nM cumene hydroperoxide mg $protein^{-1}$.

Determination of LPX

LPX was determined by the TBA (thiobarbituric acid)-reactive substances method (Büege and Aust 1978). To 100 mL of supernatant was added Tris–HCl buffer solution pH 7.4 until a 1-mL volume was attained. Samples were incubated at 37 °C for 30 min; 2 mL TBA-TCA reagent [0.375 % TBA (Fluka-Sigma-Aldrich, Toluca) in 15 % TCA] was added prior to shaking in a vortex. Samples were then heated to boiling for 45 min, allowed to cool and the precipitate removed by centrifugation at 3,000×*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 MDA (1.56×10^5 M cm^{-1}). Results were expressed as mM MDA mg $protein^{-1}$.

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 was added 150 µL of 10 mM DNPH in 2 M HCl prior to incubation 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×*g* for 5 min. The bud was washed thrice 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 prior to reading absorbance at 366 nm. Results were expressed as nM reactive carbonyls formed (C=O) mg $protein^{-1}$, using the MEC of 21,000 M cm^{-1}.

Determination of SOD activity

superoxide dismutase activity was determined by the Misra and Fridovich (1972) method. 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 EDTA) pH 10.2, plus 200 µL adrenaline (30 mM). 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 mM 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, 5 mM HEPES 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

Glutathione peroxidase activity was determined by the Gunzler and Flohe-Clairbone (1985) method as modified

by Stephensen et al. (2000). 10 µL glutathione reductase (2 U glutathione reductase), 290 µL reaction buffer [50 mM K_2HPO_4, 50 mM KH_2PO_4 pH 7.0, 3.5 mM reduced glutathione, 1 mM sodium azide and 0.12 mM NADPH and 100 µL H_2O_2 (0.8 mM) were added to 100 µL of supernatant. Absorbance was read at 340 nm at 0 and 60 s. Enzyme activity was estimated using the equation: GPx activity = (A_0-A_{60})/MEC), where the MEC of NADPH = 6.2 mM cm^{-1}. Results were expressed as mM NADPH min^{-1} mg^{-1} protein.

Determination of total protein

Total Protein was determined by the Bradford (1976) method. To 25 µL of supernatant were added 75 µL deionized water and 2.5 mL Bradford's reagent [0.05 g Coomassie Blue dye, 25 mL of 96 % ethanol and 50 mL H_3PO_4 in 500 mL deionized water]. The test tubes were shaken and allowed to rest for 5 min prior to the reading of absorbance at 595 nm and interpolation on a bovine albumin curve. The amount of total protein was used to express the results of OS biomarkers.

Statistical analysis

To estimate 96-h LC_{50} values and their 95 % confidence limits a probit method using EPA Analysis Program v1.5; US-EPA 2009, which was also used for constructing the dose–response curves. In order to evaluate the lowest concentration of DCF that causes an alteration, the LOAEL was calculated using the concentration–response curve and probit analysis, obtained from the acute assay.

In a short and long-term toxicity assays, results of OS biomarkers were statistically evaluated by one-way analysis of variance (ANOVA) and differences between means were compared using the Bonferronís multiple comparisons test, with P set at <0.05. Pearson's correlation analysis was used to examine possible associations between DCF at 4 and 24 days and OS biomarkers in blood, muscle, brain, gill and liver of *C. carpio*. Statistical determinations were made with the SPSS v10 software package (SPSS, Chicago IL, USA).

Results

DCF quantification

Table 1 shows both DCF concentrations in the water system and carp. As can be seen, the DCF concentration in the water system are decreasing over time and increasing in the carp at 4 and 24 days. The increase being more evident after 4 days.

Table 1 DCF concentrations in the exposure times

Exposure time (days)	DCF in water system (µg L^{-1})	DCF in plasma of carp (µg L^{-1})
0	7,098.4 ± 354.9	0
4	1,392.6 ± 58.7	3,416 ± 80.3
24	1,228 ± 46.2	2,044 ± 75.3

Table 2 Median lethal concentration (LC50) of DCF at 96 h in *Cyprinus carpio*

DCF exposure concentration (mg L^{-1})	Fish used in the test	Dead fish in the test
9.5	90	0
18.9	90	0
37.7	90	9
75.2	90	36
150	90	90
Control	90	0
Total	540	135

LC_{50} at 96 h in *Cyprinus carpio* was 70.98 mg L^{-1} with a 95 % confidence interval of (51.66–98.14)

Determination of LC_{50}

Table 2 shows the 96-h LC_{50} of DCF in *C. Carpio* was 70.98 mg L^{-1} with a 95 % confidence interval of (51.66–98.14). The χ^2 linear adjustment test was not significant at $P < 0.05$.

Oxidative stress evaluation

Hydroperoxides content

Hydroperoxides content results are shown in Fig. 1. Significant increases with respect to the control group ($P < 0.05$) were observed in blood at 24 days (23.6 %), in muscle, gills, brain and liver at 4 days (30.5, 283.5, 16,649.0 y 3,248.3 % respectively) and at 24 days (58.7, 96.1, 60.5 y 250.0 %, respectively). At 4 days in blood was observed a significant decrease of 12.7 %. Comparing hydroperoxides content at 4 versus 24 days of exposure a significant increases in blood of 65.6 % and in liver of 150 % were observed; and a significant decreases in muscle (6.4 %), brain (6,463.8 %) and gills (988.3 %) were observed (Table 3).

Lipid peroxidation level

Figure 2 shows LPX results. Significant increases with respect to the control group ($P < 0.05$) were found in brain (71.5 %) at 24 days; in muscle, gills and liver at 4 days

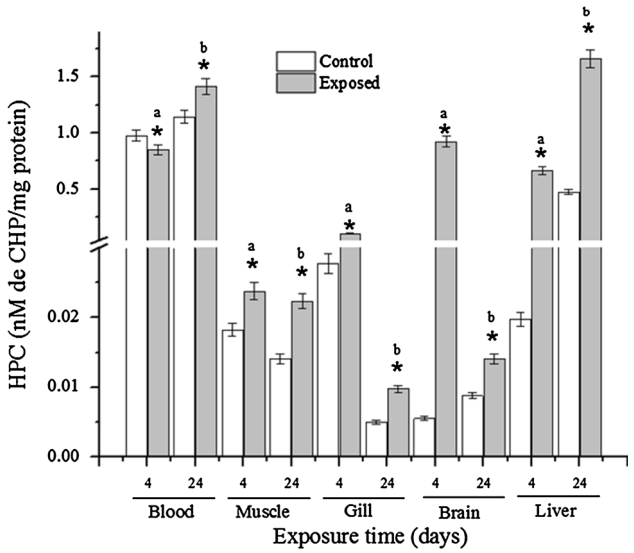

Fig. 1 Hydroperoxide content (HPC) in blood, muscle, gill, brain and liver of *C. carpio* exposed to DCF in short-term exposure (4 days) and long-term exposure (24 days). Values are the mean of three replicates ± SE. CHP = cumene hydroperoxide. Significantly different from: *control group; *a* 4 days; *b* 24 days (ANOVA and Bonferroni's multiple comparisons, $P < 0.05$). $N = 216$ fish

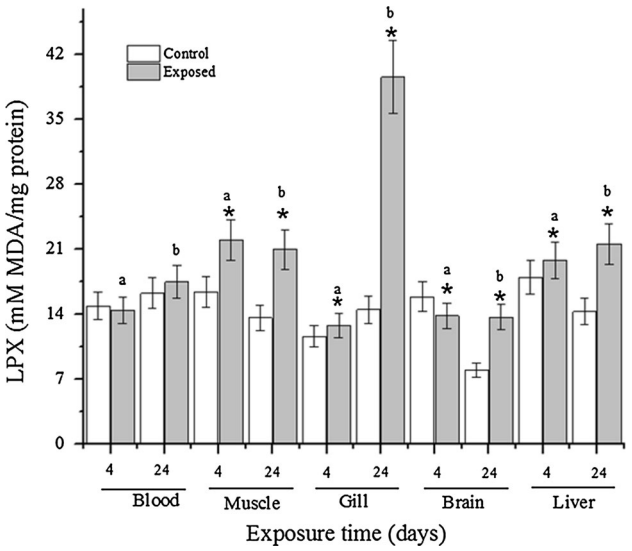

Fig. 2 Lipid peroxidation (LPX) in blood, muscle, gill, brain and liver of *C. carpio* exposed to DCF in short-term exposure (4 days) and long-term exposure (24 days). Values are the mean of three replicates ± SE. PT = protein. Significantly different from: *control group; *a* 4 days; *b* 24 days (ANOVA and Bonferroni's multiple comparisons, $P < 0.05$). $N = 216$ fish

(34.1, 9.7, 10.0 %, respectively) and at 24 days (53.7, 172.8 y 50.8 % respectively). At 4 days in brain a significant decrease was observed (12.8 %). Comparing LPX at 4 versus 24 days, a significant increases in blood, gills and liver (20.7, 209.5 and 9.2 % respectively) and a significant decreases of 5.1 % in muscle and 1.2 % in the brain were observed (Table 3).

Protein carbonyl content

Protein carbonyl content results are shown in Fig. 3. Significant increases with respect to the control group *($P < 0.05$)* were observed in muscle, gills, brain and liver at 4 days (15.8, 202.6, 466.8 and 150 % respectively) and at 24 days in gills, brain and liver (399, 16.3, 89.7 % respectively). In blood, significant decreases were observed at 4 and 24 days exposure (64.98 and 26.53 %). Comparing PCC at 4 versus 24 days of exposure, a significant decreases in muscle, gills and brain (18, 64.9, 387.4 %, respectively) and a significant increases in blood (109.8 %) and liver (31.8 %) were observed (Table 3).

SOD activity

Superoxide dismutase activity results are shown in Fig. 4. Significant increases with respect to the control group *($P < 0.05$)* were observed in gills and brain (195.6 and 63.3 %) at 4 days, in gills (603.4 %) at 24 days. In contrast, a statistically significant decreases were observed in blood (41.1 %) and muscle (57.3 %) at 4 and 24 days in blood, muscle, and brain (64.5, 63.5 and 14.3 % respectively) were observed. Comparing SOD activity at 4 versus

Table 3 Increases and decreases in oxidative stress biomarkers comparing 4 and 24 days

Organ or tissue	Oxidative stress biomarker					
	HPC (%)	LPX (%)	PCC (%)	SOD (%)	CAT (%)	GPx (%)
Blood	↑ 65.6	↑ 20.7	↑ 109.8	↑ 0.04	↑ 341.8	↓ 7.7
Muscle	↓ 6.4	↓ 5.1	↓ 18	↓ 31.3	↑ 9.4	↑ 41.3
Gill	↓ 988.3	↑ 209.5	↓ 64.9	↓ 4,300,438	↓ 11,247.7	↑ 160.6
Brain	↓ 6,463.8	↓ 1.2	↓ 387.4	↓ 1,236.5	↓ 69,417.6	↓ 37.9
Liver	↑ 150	↑ 9.2	↑ 31.8	↓ 19,765,315.5	↓ 9,241.8	↑ 722

CHP hydroperoxide content, *LPX* lipid peroxidation level, *PCC* protein carbonyl content, SOD superoxide dismutase, CAT catalase, GPx glutathione peroxidase

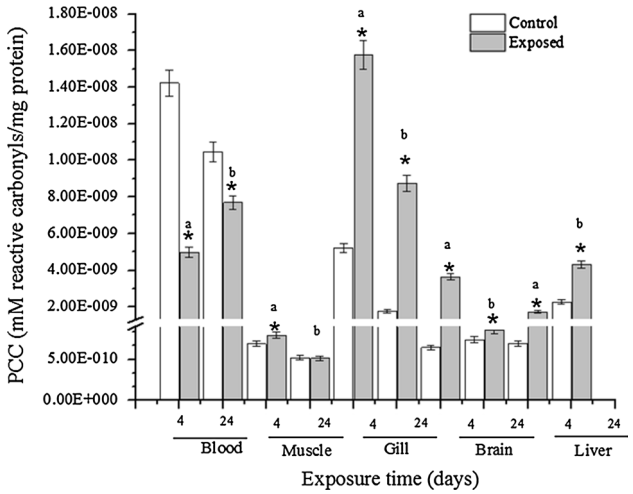

Fig. 3 Protein carbonyl content (PCC) in blood, muscle, gill, brain and liver of *C. carpio* exposed to DCF in short-term exposure (4 days) and long-term exposure (24 days). Values are the mean of three replicates ± SE. PT = protein. Significantly different from: *control group; *a* 4 days; *b* 24 days (ANOVA and Bonferroni's multiple comparisons, $P < 0.05$). $N = 216$ fish

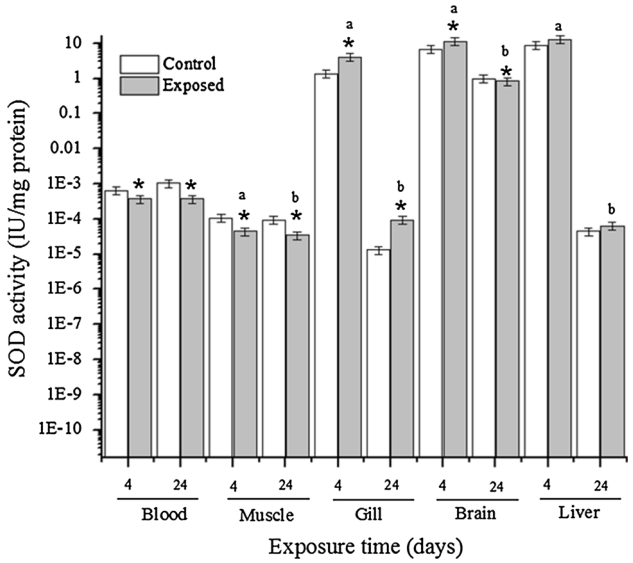

Fig. 4 Superoxide dismutase (SOD) activity in blood, muscle, gill, brain and liver of *C. carpio* exposed to DCF in short-term exposure (4 days) and long-term exposure (24 days). Values are the mean of three replicates ± SE. PT = protein. Significantly different from: *control group; *a* 4 days; *b* 24 days (ANOVA and Bonferroni's multiple comparisons, $P < 0.05$). $N = 216$ fish

24 days, a significant decreases in muscle, gills, brain and liver were observed (31.3, 4,300,438, 19,765,315.5 and 1,236.5 % respectively) (Table 3).

Catalase activity

Figure 5 shows CAT activity results. Significant increases with respect to the control group ($P < 0.05$) were found in

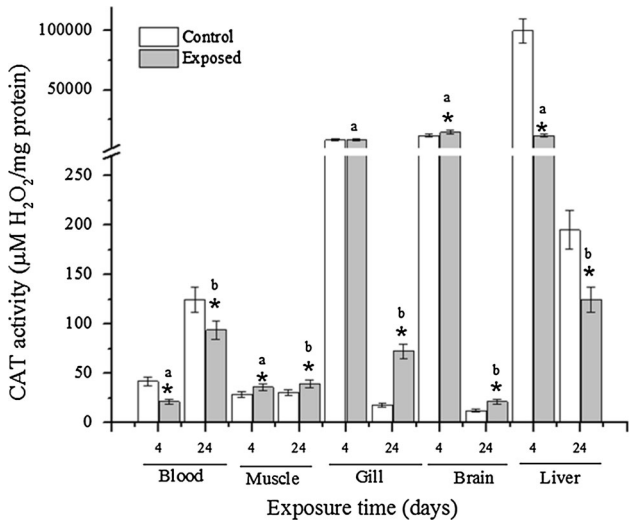

Fig. 5 Catalase (CAT) activity in blood, muscle, gill, brain and liver of *C. carpio* exposed to DCF in short-term exposure (4 days) and long-term exposure (24 days). Values are the mean of three replicates ± SE. PT = protein. Significantly different from: *control group; *a* 4 days; *b* 24 days (ANOVA and Bonferroni's multiple comparisons, $P < 0.05$). $N = 216$ fish

muscle, gills and brain (26, 2.2 and 24.9 % respectively) at 4 days, and 29.4, 312.9 and 42.5 % respectively at 24 days. At 4 days a significant decreases were observed in blood (49.1 %) and liver (88.3 %) and at 24 days of 75.3 and 36.1 %, respectively. Comparing CAT activity at 4 versus 24 days, a significant increases in blood and muscle of 341.8 and 9.4 % respectively were observed and a significant decreases in gills, brain and liver of 11,247.7, 69,417.6 and 9,241.8 % respectively were observed (Table 3).

GPx activity

Glutathione peroxidase activity results are shown in Fig. 6. Significant increases with respect to the control group ($P < 0.05$) were observed in blood (1.1 %) and liver (250 %) at 4 days, in blood, gills and liver (65.8, 60.4 y 250 % respectively) at 24 days. At 4 days a significant decreases were observed in gills (57.2 %) and brain (42.6 %); and at 24 days in muscle (59.4 %) and brain (48.6 %). Comparing GPx activity at 4 versus 24 days, a significant increases were observed in muscle, liver and gills of 41.3, 160.6 and 722 %, respectively. In contrast, a significant decreases of 7.7 and 37.9 % in blood and brain respectively, were observed (Table 3).

In Table 4, it shows the results of the Pearsońs correlation among DCF at 4 and 24 days and OS biomarkers in blood, muscle, gills, brain and liver of *C. carpio.* As shown, there are very close correlations between DCF and OS biomarkers.

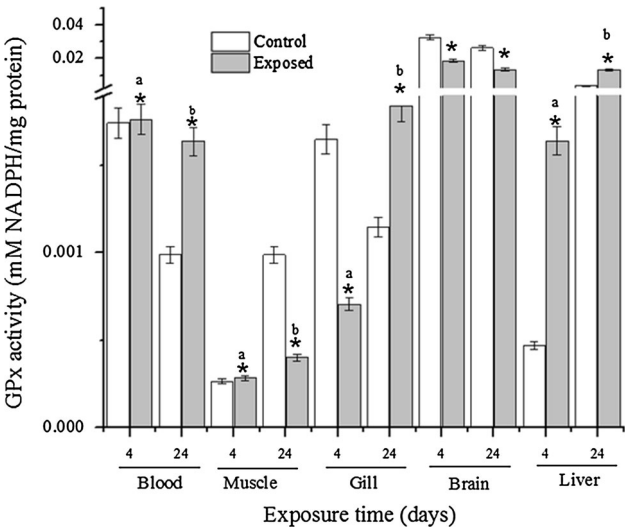

Fig. 6 Glutathione peroxidase (GP_X) activity in blood, muscle, gill, brain and liver of *C. carpio* exposed to DCF short-term exposure (4 days) and long-term exposure (24 days). Values are the mean of three replicates ± SE. PT = protein. Significantly different from: *control group; *a* 4 days; *b* 24 days (ANOVA and Bonferroni's multiple comparisons, $P < 0.05$). $N = 216$ fish

Discussion

Recent studies have reported NSAID-induced toxicity in aquatic organisms, since these organisms are more susceptible to toxic effects due to their continued exposure to wastewater discharges throughout the life cycle (Fent et al. 2006). The toxicity of NSAID is mediated by ROS formation as a result of biotransformation of these compounds through redox cycling (Abdollahi et al. 2004; Oviedo-Gómez et al. 2010; Gómez-Oliván et al. 2012, 2014; Islas-Flores et al. 2013, 2014).

Although most studies have shown that the DCF in concentrations ng/L to µg/L causes damage to aquatic organisms, in other studies biomarkers of damage (LPX and DNA damage) were significantly affected in marine mussel (*Mytilus spp*) by DCF exposure to 1,000 µg L^{-1} (1 mg L^{-1}) at 96 h (Schmidt et al. 2011). DCF concentrations in the order of mg L^{-1} may be relevant in saltwater bodies.

In the environmental waters, DCF has been shown to rapidly phototransform and to form several transformation products (Svanfelt 2013). Not only DCF itself but also these environmental transformation products may pose risk to aquatic organisms.

Diclofenac is photodegradable and biotransformed in: 5,4′-dihydroxy-diclofenac, 3-dihydroxy-diclofenac, 4′-dihydroxymethyl-diclofenac, 3′-hydroxymethyl-diclofenac, 4′-hydroxy-diclofenac and 5′-hydroxy-diclofenac (Deng et al. 2003). The latter two are oxidized to intermediates of benzoquinone imine, compounds that are highly toxic to aquatic organisms (Oviedo-Gómez et al. 2010).

The findings in this study show an increase of two biomarkers HPC and LPX in muscle, gills and liver at 4 and 24 days compared to control. The HPC also increased in acute and chronic exposure to DCF in brain. During LPX, polyunsaturated fatty acids with double bonds react with ROS, particularly the hydroxyl radical ($HO^{\cdot}$) and the reactive nitrogen species (RNS) peroxynitrite ($ONOO^{-}$), through a chain reaction mechanism. The final step in the LPX process is formation of lipid hydroxyperoxides that can readily break down into various chemical species such as lipid alkoxyl radicals, aldehydes, alkanes, lipid epoxides, and alcohols, most of which are toxic and mutagenic products (Porter et al. 1995). Studies by Islas-Flores et al. (2013) have shown that DCF-biotransformation starts at 12 h of exposure forming 4-hydroxy-DCF by reactions of Phase 1 (CYP). CYP is known to produce an oxygenated intermediate—the oxy-cytochrome P450 complex [P450 (Fe^{3+}) $O_2^{-\cdot}$]—during the biotransformation of NSAID (as DCF), with subsequent release of the superoxide anion by reaction decoupling (Doi et al. 2002). This ROS can produce increase of LPX and HPC.

In freshwater fish, the gills permit gas exchange, osmoregulation, ion exchange, acid–base regulation and excretion of nitrogenous waste (Evans et al. 2005). The gills are likewise known to carry out the oxidative metabolism of many toxic agents, thereby promoting production of the ROS responsible for HPC and LPX in the present study (Hoeger et al. 2005; Gomez et al. 2010; Islas-Flores et al. 2013) On the other hand, the liver is the main organ of NSAIDs biotransformation through cytochrome P450 (CYP). For this reason, in this organ the LPX was increased in our study.

Although most research has emphasised how lipid components are altered by oxidative deterioration in fish, proteins, including sarcoplasmic, myofibrillar and stromal proteins (forming the muscle) from fish, are also susceptible to oxidative damage. Oxidising lipids, metal ions and other prooxidants (as DCF), the agents that initiate oxidative damage, exist in animal muscle or are generated during meat processing (Xiong and Decker 1995; Li et al. 2013). Studies by Schmidt et al. (2011) and Gonzalez-Rey and Bebianno (2014) have shown that DCF induces estrogen and neurotoxic disturbances, and damage to tissue in *Mytilus* (spp.).

It is also well known that when NSAID (as DCF) are ingested they enters in contact with the vasculature where they acetylates the enzyme COX-2 present in endothelium or circulating leukocytes to produce 15-epi-lipoxin A4, which promotes nitric oxide (NO) synthesis mediated by endothelial (eNOS) and inducible (iNOS) nitric oxide synthase (Paul-Clark et al. 2004). When, the superoxide anion and NO bind they may form a reactive nitrogen specie (peroxynitrite) through a diffusion-limited reaction

Table 4 Pearsońs correlation among DCF (4 and 24 days) and oxidative stress biomarkers in blood, muscle, brain, gill and liver of *Cyprinus carpio*

Stress oxidative biomarker	Organ or tissue	DCF 4 days in carp 3,416.4 ± 80.3 μg L^{-1}	DCF 24 days in carp 2,044 ± 75.3 μg L^{-1}
CHP	Blood	−0.332	−0.007
	Muscle	−0.114	−0.206
	Brain	**−0.952**	−0.410
	Gill	−0.430	0.237
	Liver	−0.367	−0.367
LPX	Blood	0.116	−0.151
	Muscle	−0.162	−0.171
	Brain	−0.422	**−0.512**
	Gill	−0.500	**0.682**
	Liver	**0.998**	**−0.889**
CPC	Blood	−0.159	−0.115
	Muscle	−0.174	−0.057
	Brain	**0.771**	0.006
	Gill	−0.373	0.092
	Liver	**−0.848**	**−0.999**
SOD	Blood	0.169	−0.003
	Muscle	0.061	−0.199
	Brain	−0.386	0.401
	Gill	0.500	−0.497
	Liver	**−0.953**	−0.009
CAT	Blood	0.112	−0.002
	Muscle	−0.007	0.002
	Brain	**−0.850**	**0.653**
	Gill	−0.443	**0.653**
	Liver	**0.991**	**−0.938**
GP_X	Blood	−0.206	0.419
	Muscle	−0.007	0.100
	Brain	**−0.881**	−0.201
	Gill	**−1.000**	−0.229
	Liver	**−0.569**	**−0.834**

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

HPC hydroperoxide content, *LPX* lipid peroxidation level, *CPC* protein carbonyl content, *SOD* superoxide dismutase activity, *CAT* catalase activity, GP_X glutathione Peroxidase activity

(Huie and Padmaja 1993). 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).

Protein carbonyl content measured in this study was increased significantly at 4 and 24 days in gills, brain and liver. This result could be explained by the presence of NRS as peroxynitrite. ROS and RNS can remove protons from methylene groups in amino acids, leading to formation of carbonyls that tend to ligate protein amines and also induce damage to nucleophilic centers, sulfhydryl group oxidation, disulfide reduction, peptide fragmentation, modification of prosthetic groups, and protein nitration. These modifications lead to 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).

Superoxide dismutase 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) that is converted to hydrogen peroxide by SOD. Subsequently, H_2O_2 is sequestered and degraded to H_2O by CAT and GPx. In relation to the antioxidant enzymes, SOD activity in this study increased significantly in gills, brain and liver at 4 days with respect to controls ($P < 0.05$) and gills and liver at 24 days. These increases may be due to the fact that during the CYP-mediated biotransformation of DCF in carp, the superoxide anion radical O^{2*}, responsible for increased SOD activity, is released (Doi et al. 2002).

As regards CAT and GPx activity, the increased activity of these biomarkers found in this study may be due to a defense mechanism of the cells in order to offset the OS induced by increased H_2O_2 (Vlahogianni et al. 2007). Bagnyukova et al. (2006) state that LPX products are

apparently involved in the up-regulation of some antioxidant enzymes, including CAT and GPx. As stated above, in the present study an increase in SOD activity occurred in *C. carpio* exposed to DCF at 4 and 24 days, leading to increased hydrogen peroxide formation, which may act as a signal for CAT and GPx bioactivation in order to convert this highly toxic free radical to less toxic compounds. Similar responses have been found in other aquatic organisms exposed to other NSAIDs (Oviedo-Gómez et al. 2010; Gómez-Oliván et al. 2012, 2014; Islas-Flores et al. 2013).

The results of this study (Table 3) show that OS biomarkers were increased at 24 days of exposure in relation to 4 days in blood and liver. A possible answer to these findings might be found from bioaccumulation potential of DCF. The log K_{ow} of DCF is 4.4 (Cleuvers 2004) and the lipophilicity of this compound may favor bioaccumulation. In Table 1, we can see that DCF concentration in plasma is greater at 4 days than at 24. This could be because the DCF is biotransformed to hydroxylated metabolites. Islas-Flores et al. (2013) demonstrate that DCF concentrations in the water exposure system were decreasing over time and increasing in the carp. They also refer, that at 12 h, DCF biotransformation to 4-hydroxy-DCF in the common carp begins. This could explain the increase in OS biomarkers found in blood and liver.

On the other hand, Hoeger et al. (2008) demonstrated that DCF is not completely excreted through first pass metabolism in brown trout, but that a significant part of the applied DCF enters enterohepatic circulation. The resulting prolonged availability of DCF in the organism possible promotes accumulation of DCF.

Schwaiger et al. (2004) determined the bioconcentration of DCF in different organs of rainbow trout after exposure for 28 days. Their bioconcentration factors ranged from 69 in muscle to 2732 in liver at 1.0 μg L^{-1}. The liver may be exposed to increased local concentrations of DCF during detoxification process (Memmert et al. 2013).

After chronic exposure to DCF, several authors observed histopathological effects in trout species at relatively low concentrations around 1.0–5.0 μg L^{-1} (Schwaiger et al. 2004; Triebskorn et al. 2004; Hoeger et al. 2005). These findings are similar to those found in our study, except that biomarkers assessed were differents.

Furthermore, our results (Table 3) show that OS biomarkers were decreased at 24 days of exposure in relation to 4 days in gill, brain and muscle. These findings are consistent with chemical analysis by Schwaiger et al. (2004) who revealed a concentration-related accumulation of DCF were detected in the liver, followed by kidney, gills and muscle tissue in rainbow trout. BCFs ranged from 12 to 2732 in liver, from 5 to 971 in kidney, from 3 to 763 in gills and from 0.3 to 69 in the muscle, respectively, depending on applied DCF concentrations. This shows that bioconcentration is variable in each organ and these that are less able to bioconcentrate DCF are gills and muscle. These data consistent with those observed in our study.

Cyprinus carpio exposed to DCF were affected in OS status during the initial days of the study (at 4 days), exhibiting an increased response at 24 days in blood and liver. In contrast, a decrease was observed in muscle, gills and brain at 24 with respect to 4 days. This study confirms that, there is influence of exposure time (short and long-term) in OS biomarkers, but this influence depends on the organ or tissue evaluated.

Conclusions

In conclusion, DCF induces OS on blood, muscle, gills, brain and liver of *C. carpio* in short and long-term exposure (4 and 24 days, respectively). Oxidative stress is a reliable biomarker for evidencing acute and chronic exposure to concentrations of NSAIDs such as DCF.

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

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